



## Detection of Multiple *Cry* Genes in *Bacillus thuringiensis* Isolated from Different Soil Types in Zaria

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### Authors' contributions

This work was carried out in collaboration between both authors. Author AB designed the study, performed the experiment, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author IMH managed the analyses of the study and managed the literature searches. Both authors read and approved the final manuscript.

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### ABSTRACT

**Aims:** To isolate and characterize *Bacillus thuringiensis* from different soil types within Zaria and screen them for the possession of *cry* genes.

**Methodology:** Soil samples were collected from different sites in Zaria. A total of twelve *Bacillus thuringiensis* strains were isolated from the different soil types. This isolates screen for the possession of *cry1*, *cry2*, *cry3* and *cry4* genes using Polymerase Chain Reaction.

**Results:** Eight of the 12 isolates showed the presence of at least one *cry* gene, while the remaining four showed none. *Cry1* was the most frequently detected gene (58.88%), followed by *cry2* (16.67%), while *cry3* and *cry4* had the least occurrence (8.33%). Isolate L3 had *cry1* and *cry2*, L2 had *cry1* and *cry3* while isolate L1 had *cry2* and *cry4*.

**Conclusion:** *Bacillus thuringiensis* isolates from different soil types in Zaria harbor possess *cry* genes and as such are potential biocontrol agents. The presence of multiple *cry* gene in one isolate is of entomological importance as it could have broad spectrum of toxicity against different pests.

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## 1. INTRODUCTION

The control of insect pest using bacteria started from the agricultural sector and has now been extended to the public health. *Bacillus thuringiensis* and *B. sphaericus* are considered pathogenic against vectors of human diseases, such as mosquitoes and *Simulium* species. Some of the diseases caused by these vectors are malaria, yellow fever, filariasis, onchocerciasis and leishmaniasis; which are known to affect between 500 million to a billion people each year [1]

Malaria is considered the most challenging among the diseases because of the morbidity-mortality ratio. Globally, 300-500 million cases and infections, and over one million deaths are reported annually; 90 percent of these occur in tropical Africa. Nigeria is known for its high prevalence of malaria. Malaria accounts for over 45 percent of all out-patient cases in Nigeria [2].

The use of chemical insecticides as prophylaxis measure in applications such as coils, sprays, insecticide-treated nets (ITNs) have posed threat to human health and the ecosystem. An important alternative measure to chemical insecticides is biological control measure which involves the regulation of pest population using natural control agents such as predators, nematodes and microbial insecticides [3].

It is the use of one biological organism to control another; releasing beneficial bacteria, fungi or arthropods to limit pest infestation. The safety offered by microbial insecticides is their greatest strength. Bacteria and fungi have been shown to kill mosquitoes to varying degrees. *Bacillus thuringiensis israeliensis* (BTI) and *B. sphaericus* are being used in worldwide field test designed to control mosquitoes' population [2].

The situation demands the safer pesticides and biopesticides are the most desired alternatives. Bacteria, especially *B. thuringiensis* and *B. sphaericus* are the most potent and successful group of organisms for effective control of insect pests and vectors of diseases [4].

*B. thuringiensis* accounts for about 5-8% of *Bacillus* spp. population in the environment. *B. thuringiensis* is able to produce an intracellular protein crystal during sporulation which is toxic to mosquitoes. The protein crystals so produced are solubilized in the mid-gut of the insect to form

proteins called delta-endotoxins that are toxic in low concentrations to insects that have specific receptors located in the mid-gut epithelium. Although *B. thuringiensis* has no adverse effects on variety of vertebrates and invertebrates animals, however under certain conditions, it was reported to be pathogenic to earthworm *Lumbricus terrestris*. Isolation of *Bacillus thuringiensis* from soil used to be cumbersome however the development of better isolation techniques and media has enhanced research on *B. thuringiensis* [5].

*B. thuringiensis* strains can carry one or more crystal toxin genes, and therefore, strains of the organism may synthesize one or more crystal protein. Transfer of plasmids among *B. thuringiensis* strains is the main mechanism for generating diversity in toxin genes [6].

The insecticidal spectrum of each crystal protein is unique. As such, Cry proteins have been classified based of their host specificity and their amino acid compositions. The different forms of crystal proteins are 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, Cry11 is both active against Lepidopterans and Coleopterans, whereas Cry1B shows toxicity against Lepidoptera, Coleoptera, and Diptera [7].

More than 3000 species of insect included in 16 orders have been found to be susceptible to different crystal proteins. Insecticidal crystal proteins are toxic to insects within the orders Lepidoptera, Diptera, Coleoptera, Hymenoptera, Homoptera, Orthoptera, Mallophaga as well as non-insect organisms such as nematodes, mites, protozoa, and plathelminthes [7]. *Bacillus thuringiensis* produces crystal inclusion bodies comprising two non-related families of delta-endotoxins which belong to Cry (Cry4Aa, Cry4Ba, Cry10Aa and Cry11Aa) and Cyt (Cyt1Aa1, Cyt1Ca and Cyt2Ba). These toxins have been characterized as pore forming toxins (PFT). The absence of resistance development

to *B. thuringiensis israelensis* based products is primarily due to the synergistic combinations of Cry and Cyt proteins in its crystal. In fact, Cyt1Aa protein has been reported to delay resistance development to Cry4Aa, Cry4Ba and Cry11Aa in *Culex* larvae. Unlike Cry toxins structure, cytolytic protein Cyt1A is a single a/b domain comprise two outer layers of  $\alpha$ -helix hairpins wrapped around a sheet. It does not require specific receptors in the target midgut cells. Indeed, they interact directly with non-saturated membrane lipids, get inside, and then, form pores. Interestingly, several reports show a synergism between Cyt1Aa and *B. thuringiensis* dipteran specific toxins (such as Cry4Aa, Cry4Ba, Cry11Aa and Cry2Aa). In fact, Cyt1Aa has been reported to synergize with Cry11Aa against *Aedes aegypti* larvae by acting as a functional receptor for Cry11Aa providing an efficient formation of oligomer pre-pore structure. Cry4Ba is the most active *B. thuringiensis israelensis* toxin against *A. aegypti*. Cry4Ba structure reveals a wedge-shaped form with three distinct domains common for the majority of Cry toxins. Domain I consists of the N-terminal seven-helix bundle involved in the membrane insertion, toxin oligomerization and ion-leakage pore formation. However, both domains II and III are responsible for receptor recognition [7].

All Cyt toxins react directly with phospholipids without the need for a membrane protein receptor. Spores are known to synergize the insecticidal activity of crystals when tested against insects. This may be related to the invasion of haemocoel through the ulcerated midgut, and the subsequent development of septicemia. The efficiency and potency of Cry toxins in insects control could be increased by the addition of enzyme chitinase in *B. thuringiensis* preparations. The chitinase acts on the peritrophic membrane which is composed of a network of chitin and proteins. The identification of Bt Cry genes by PCR has proven to be a very useful method for strain characterization and selection. Studies have found a correspondence between toxicity of bt isolates and the PCR amplification of the cry gene hence PCR is a useful tool for the prediction *B. thuringiensis* insecticidal activity [8].

## 2. MATERIALS AND METHODS

### 2.1 Isolation Procedure

*Bacillus thuringiensis* strains were isolated from soil samples from different soil types namely cow

range land, refuse dump site and agricultural soil. The soil samples were stored at 4°C until analyzed. For each sample, 0.5 g of soil was added in 10 mL of LB medium (composition per 1L: Tryptone 10 g/L, yeast extract 5 g/L, NaCl 5 g/L) to which 0.25 M sodium acetate was added and incubated in shaking incubator at 30°C and 250 rpm for 4 hours. From each sample 2 mL was taken and heat shocked in a water bath at 80°C for 20 minutes. Serial dilutions of treated samples were prepared using 1 mL of the treated sample and then 0.1 mL was spread on T3 agar (composition per 1L: Tryptone 3 g/L, Yeast extract 1.5 g/L, Tryptose 2 g/L, MnCl<sub>2</sub> 0.005 g/L, Sodium phosphate 0.05 M and Agar 15 g/L) and incubated for 2 days at 26°C. Colonies with *B. thuringiensis* like morphology (entire margin, off white color, dry and rich growth of colony) were picked up at random and purified by streaking them LB agar plates (Tryptone 3 g/L, Yeast extract 1.5 g/L, Tryptose 2 g/L, MnCl<sub>2</sub> 0.005 g/L, NaH<sub>2</sub>PO<sub>4</sub> 6.9 g/L, Na<sub>2</sub>HPO<sub>4</sub> 8.9 g/L and Agar15 g/L) [9].

### 2.2 Characterization of *Bacillus thuringiensis*

The isolates were characterized using Gram reaction and endospore staining, motility, growth above 45°C, catalase activity, Voges Proskauer test and Microgen™ Bacillus-ID. The isolates were conserved / stored on nutrient agar slant and then stored at 4°C.

### 2.3 Molecular Detection of Cry Genes in the *Bacillus thuringiensis* Strains Isolated

#### 2.3.1 DNA extraction

PCR was done using crude DNA. Overnight culture of *B. thuringiensis* LB broth was transferred into Eppendorf tubes and centrifuged at 1000 rpm for one minute. The supernatant was discarded and the pellet was resuspended in 50  $\mu$ L of lysis buffer, vortex mixed and incubated at 37°C overnight. The resulting cell lysate was briefly centrifuged for 10 seconds at 10,000 rpm and then supernatant was used for the Polymerase Chain Reaction [10].

#### 2.3.2 Detection of cry genes by PCR

PCR analysis was carried out to identify *cry1*, *cry2*, *cry3* and *cry4* genes in the 12 isolates as described by [10]. The reaction was done in 20  $\mu$ L reaction mixture containing 5  $\mu$ L template

**Table 1. Primer sets used in the PCR assay**

<b>Cry genes</b>	<b>Primer Sequence (5'-3')</b>	<b>product size (bp)</b>
<i>Cry1</i>	F TTG TGA CAC TTCTGC TTC CCA TT R CATGATTCATGCGGCAGATAAAC	270
<i>Cry2</i>	F GTTATTCTTAATGCAGATGAATGGG R CGGATAAAATAATCTGGGAAATAGT	689
<i>Cry3</i>	F CAT CTG TTG TTTCTG GAG GCA AT R CGT TAT CGC AGAGAG ATG ACA TTA AC	590
<i>Cry4</i>	F GCATATGATGTAGCGAAACAAGCC R GCGTGACATACCCATTTCCAGGTCC	440

F = Forward primer, R = Reverse primer

DNA, 150 mM dNTPs, 20 pM of each of the 4 primers (Table 1) and 0.5 U of *Taq* DNA polymerase. Amplification was carried out in a DNA thermocycler with the program: one denaturing cycle at 94°C for 4 minutes, 35 cycles containing: 94°C for 45 seconds, annealing at 48-55°C for 45 seconds and 1 minute at 72°C and then the reaction being terminated by a for 4 minutes one at 72°C. The *cry* gene bands were visualized through agarose gel electrophoresis. The gels were stained with ethidium bromide and documented with a 100 bp molecular weight marker.

### 3. RESULTS AND DISCUSSION

An alternative approach for pest control is the use of microorganisms as biocontrol agents. The microbial pesticides are essentially nontoxic to humans as such there are no concerns for human health effects with *Bacillus thuringiensis*. Researchers have also shown that microbial larvicides do not pose risk to wildlife, non-target species or the environment and retain a good activity in polluted water [10,11].

The search for alternative biocontrol agents such as *Bacillus thuringiensis*-based biopesticides is increasingly attracting interest. This bacterium produces parasporal crystalline inclusions (Cry proteins) which are toxic to many important agricultural pests. The Cry proteins are encoded by *cry* genes and so far, many *cry* genes have been identified in different *B. thuringiensis* strain collections [10,11].

Twelve strains of *B. thuringiensis* were isolated from different soil sample following isolation using colonial morphology on specific media, microscopic and biochemical characterization. The identity of the isolates were further confirmed using Microgen™ Bacillus-ID system kit.

Agarose gel electrophoresis result of the PCR amplification product (amplicon) showed that the expected fragments of 270 bp, 690 bp, 590 bp, and 440 bp corresponding to *cry1*, *cry2*, *cry3*, and *cry4* genes. The most frequent *cry* gene identified was *cry1* (found in 7 isolates out of 12 (58.88%)) followed by *cry2* (found in 2 isolates out of 12 (16.67 %)) while *cry3* and *cry4* had same frequency (found in 1 isolate out of 12(8.88%).

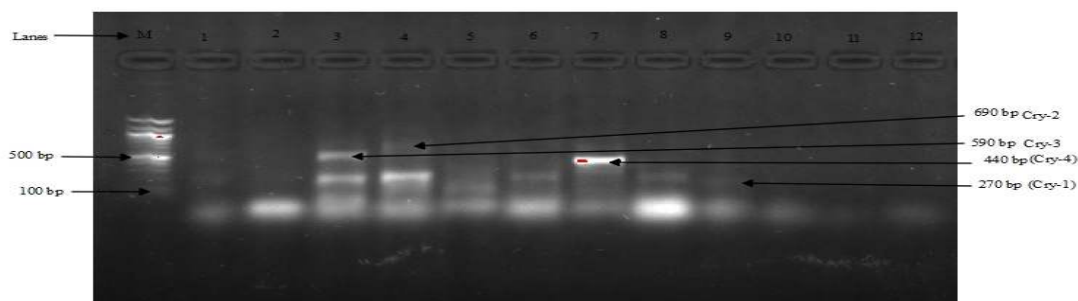
A high frequency of *cry1* observed in this study is in agreement with the work of [10,11,12,13,14]. The observed high frequency of incidence of *cry1* genes might be because *cry1* gene-containing strains of *Bacillus thuringiensis* are possibly more abundant in nature.

Cry1 and Cry2 proteins are reported to show strongest toxicity to *Lepidopterans*. Proteins belonging to the class Cry4 are specifically toxic to Dipterans, while Cry3 proteins show insecticidal activity against Coleopterans. So also, Cry2 and Cry4 are toxic to dipteran insects [15,16].

Some Cry proteins on the other hand display toxicity to more than one insect order. For example, Cry1I is active against both *Lepidopterans* and *Coleopterans*, whereas Cry1B shows toxicity against *Lepidoptera*, *Coleoptera*, and *Diptera* [17].

Eight of the isolates (L3, N3, L2, D2, L7, L1, L6, and A1) were found to harbour at least 1 of the genes, three of the isolates (L3, L2 and L1) harbour 2 different genes, while four isolates were found not to harbour any of the genes checked. The presence of different *cry* genes in the same *B. thuringiensis* strain as observed in this study was also reported by [10].

Presence of multiple *cry* genes in one strain might be as a result of genetic information



**Fig. 1. Agarose gel electrophoresis of PCR products obtained with universal primers for *cry1*, *cry2*, *cry3* and *cry4* genes in a number of *B. thuringiensis* strains. Lane numbers correspond to the number of isolates. M: Marker (100 bp ladder)**

exchange between different strains. This is of great advantage in biocontrol measure as such isolates could show simultaneous toxicities towards different insect families and will be good candidates in the search for biocontrol agents covering a wider spectrum of action.

**Table 2. Distribution of the *Cry* genes among the 12 *Bacillus thuringiensis* isolates from different soil sites**

Isolates	<i>Cry 1</i>	<i>Cry 2</i>	<i>Cry 3</i>	<i>Cry4</i>
L3	+	+	-	-
N3	+	-	-	-
L2	+	-	+	-
D2	+	-	-	-
N2	-	-	-	-
L7	+	+	-	-
L1	-	-	-	+
L6	+	-	-	-
A1	+	-	-	-
C2	-	-	-	-
D1	-	-	-	-
E2	-	-	-	-

Key: + mean the *cry* gene is present - mean the *cry* gene is absent

Despite the fact that chemical insecticides provide many benefits to food production and human health and have proven to be very effective at increasing agriculture and forestry productivities, it also pose some hazards, such as contamination of water and food sources, poisoning of non-target fauna and flora, concentration in the food chain and selection of insect pest populations resistant to the chemical insecticides [17].

#### 4. CONCLUSION

Prolonged use and exposure to the modern synthetic insecticides has been associated with

cancer, liver damage, immune-toxicity, birth defects and reproductive problems in humans and other animals.

The PCR results show that some of the isolates of *Bacillus thuringiensis* strains from different soil types within Zaria harbour *cry* genes which implies that the isolates could be good candidate for the control of wide variety of pests: Lepidopterans, Coleopterans and Dipterans.

The isolates, especially those possessing the *cry* genes can be used for future research regarding assessment of their toxicity against mosquito larvae and other pest.

This research will benefit the community by using the isolates as biocontrol agent in the control of mosquitoes, which are important vectors in the transmission of diseases and in the control of butterfly larvae, which are agricultural pests.

#### COMPETING INTERESTS

Authors have declared that no competing interests exist.

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