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Molecular Characterisation of Soil-Dwelling *Bacillus thuringiensis* using Transcriptional Regulator, *XRE* Gene and the Crystal Protein, *cry2* gene

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Abstract
Bacillus thuringiensis (Bt) is the organism that is used most frequently in biological pest management, which is distinguished by the capacity to possess crystalline inclusions throughout the sporulation phase. There is an increasing need to use biological control in controlling plant pathogens due to the inherent advantages. However, the detection of Bt has become more time consuming and cumbersome due to the numerous available crystal genes. The goal of the study was to isolate strains of *Bacillus thuringiensis* from the soil, characterise the isolates using the transcriptional regulator, XRE gene and the crystal proteins cry2gene and compare the efficiency of these two biomarkers in identifying Bt species. Five different *Bacillus thuringiensis* strains were isolated from soil samples in Zaria, Nigeria. Polymerase chain reaction was used to detect the existence of the cry2 and XRE genes. Four (80%) of the five isolates harboured the XRE genes, while none (0%) harboured the cry2 genes. This observation is a likely indication that the XRE gene is a reliable biomarker in the identification of Bt isolates from environmental samples. In order to ensure speed and reproducibility in the detection of Bt from environmental samples, molecular techniques targeting the XREgene are recommended.
Keywords: *Bacillus thuringiensis*; transcriptional regulator, XRE; crystal protein, cry2

INTRODUCTION

Bacillus thuringiensis (Bt) is an aerobic, Gram-positive rod-shaped soil bacterium found in diverse ecosystems around the world, including soil, water, warehouses particulates, woody and coniferous tree leaves, dead insects and insectivorous animals (Dharmender *et al.*, 2008; Roh *et al.*, 2017). It is well documented that during the sporulation process, it has the capacity to produce crystal inclusions (cry toxins) containing insecticidal proteins known as δ-endotoxin (Schünemann *et al.*, 2014). These endotoxins are specific to their targets; for instance, bipyramidal and cuboidal inclusions encoded by *cry1*, *cry2*, *cry7*, *cry8*, *cry9*, *cry15*, *cry22* and *cry51*genes are effective towards *Lepidoptera* and *Coleoptera* insect pests (Frankenhuyzen, 2009; Jain *et al.*, 2017). The spherical, composite, flat and other crystals inclusions are toxic to *Diptera*, *Hemiptera*, *Hymenoptera*, *Hemiptera*, *Siphonoptera* insects (Frankenhuyzen, 2009).

There are currently 78 known *cry* genes families with 823 distinct *cry* genes and 3 cyt families with 40 distinct cyt genes (Crickmore, 2020). Hence, the characterisation of *Bacillus thuringiensis* from environmental samples becomes even more time consuming and cumbersome using the currently available *cry* genes as biomarkers as cry protein products vary with the different categories of *cry* genes. Using universal primers, the 16S rRNA gene sequences showed high genetic relatedness between *Bacillus thuringiensis* and *Bacillus cereus* (Helgason, 2000; Rasko *et al.*, 2005; Bartoszewicz and Marjanska, 2017). Furthermore, the characterization of Bt isolates with specific crystal toxins, becomes difficult when one considers that the genes encoding these various toxins are mostly borne on plasmids, which can be temporarily or permanently transferred to each other or to *Bacillus cereus*.(Rolle *et al.*, 2005; Fiuza, 2015).To circumvent these limitations, the *XRE* gene (that regulates the most common type of crystal protein production) was targeted to detect *Bacillus thuringiensis* (Wei *et al.*, 2019).Wei *et al.* (2019)developed a real-time PCR method to explain the detection of *Bacillus thuringiensis* targeting the transcriptional regulator, *XRE* gene, in spiked food samples from South Korea. However, to the best of our knowledge, there has been no published work on the detection of Bt isolated from environmental samples targeting the *XRE* genes. The *cry2* gene is one of the most common crystal genes used in characterizing *Bacillus thuringiensis*.

The transcriptional regulator, *XRE* gene has not been the focus of studies based on the identification of *Bacillus thuringiensis* in Nigeria.

There is increased interest globally in the control of plant pathogens using biological agents such as *Bacillus thuringiensis*, molecular methods are, hence, important in the comprehensive identification of these agents. Nigeria is in dire need of more studies in this area of research as culture dependent techniques alone do not highlight key markers of this biocontrol agent (*Bacillus thuringiensis*). Therefore, in view of the highlighted concerns above, the current study was conducted to isolate *Bacillus thuringiensis* strains from various soil types in Zaria, Nigeria, and to compare the effectiveness of the *XRE* gene with that of the established crystal protein, *cry2* gene in detecting the putative *Bacillus thuringiensis* isolates using PCR.

MATERIALS AND METHODS

Collection of Soil Samples

Soil samples were collected from fields of three sites in Zango, Zaria namely, Tomato farmland, Cow rangeland and Refuse dump site. Soil sample of 10g was collected from the surface to a depth of 5 to 10cm in each of ten spots within each site. The ten soil samples from each site were bulked and thoroughly mixed to obtain representative composite soil samples(Stefani *et al*., 2015).The soil samples from the three sites were stored in polythene bags and brought to the Environmental Research Laboratory, Department of Microbiology, Ahmadu Bello University, Zaria for isolation of *Bacillus thuringiensis* from the samples

Isolation of Bacillus thuringiensis

Bacillus thuringiensis were isolated using the sodium acetate selection method outlined by Travers *et al*. (1987) with some modifications. Ten grams soil was dissolved into 90mL of distilled water and incubated for 45 mins at 28ºC on a rotatory shaker (B. Bran scientific & instrument company, England) at 250 rpm. Thereafter, 2 mL of the broth culture was added to 20 mL of sterile Luria Bertani (LB) (Tryptone 10g/L, yeast extract 5g/L, NaCl 5g/L) broth buffered with 0.25 M sodium acetate (pH 6.8) in 50 mL Erlenmeyer flask and incubated for 4 hours at 28ºC on a rotatory shaker at 250 rpm. After incubation, 5 mL aliquots from each culture were placed in hot water bath operating at 80ºC for 3 minutes. Thereafter, 0.1 mL was spread on LB agar (Tryptone 10g/L, yeast extract 5g/L, NaCl 5g/L and Agar 15g/L) and incubated at 28ºC for 24 hr.

Purification and Preservation of Typical Bacillus thuringiensis Isolates

Colonies having morphology typical of *Bacillus thuringiensis* (cream coloured, dry surface with entire margin) were selected and purified by subculturing on T3 agar (Tryptone 3g/L, yeast extract 1.5g/L, Peptone 2g/L, Sodium phosphate 0.05M, $MnCl_2$ 0.005g/L and Agar 15g/L) to obtain pure cultures and then preserved at 4ºC for further studies.

Biochemical Characterisation of Isolates

The characteristic cultural and biochemical properties of the suspected *Bacillus thuringiensis* strains were determined following Gram staining and spore staining procedures (Bergey, 2004; Willey, 2008).Biochemical tests conducted include: motility, casein hydrolysis, oxidase, citrate utilization, Methyl red-Voges Proskaeur, catalase, and arginine hydrolysis.

Detection of cry2 gene and transcriptional regulator (XRE) gene

Qiagen DNA easy extraction kit (Jiangsu Mole Bioscience Co., Ltd, China) was used to extract DNA based on the manufacturer's instructions. The PCR conditions for the amplification of the *cry2* and the *XRE* genes were performed as described by Ben-Dov *et al*. (1997) and Wei *et al*., (2019) respectively.

Polymerase chain reactions were carried out in 25 µL reaction mixture containing 8 µL template DNA, 150 mM dNTPs, 20 pM of each of the four primers (Table 1) and 0.5U of *Taq* DNA polymerase. The *XRE* gene was amplified in a DNA thermocycler using the following program: 1 initial denaturing cycle at 94ºC for 3 minutes, 35 cycles containing: denaturing at 94ºC for 30 seconds, annealing at 49ºC for 30 seconds and extension for 30 seconds at 72ºC and then the reaction being terminated by a final extension for 10 minutes at 72ºC. The amplification of the *cry2* gene was carried out with the program: 1 initial denaturing cycle at 94ºC for 3 minutes, 35 cycles containing: denaturing at 94ºC for 30 seconds, annealing at 55ºC for 30 seconds and extension for 1 minute at 72ºC, thereafter, a final extension for 10 minutes at 72ºC to complete the reaction.

The *XRE* gene and the *cry2*gene bands were visualized using agarose gel electrophoresis. An aliquot (15 µL) of the amplification products was loaded onto 1.5% agarose gel and runat 100volts for 1 hour in TAE buffer (40mM Tris-Acetate, 1 mM EDTA). The gels were stained with ethidium bromide and a molecular weight marker of 100 bp was used to document them.

Table 1. Primer sets used in the amplification of the *cry2* and the *XRE* genes

Genes	Primer Sequence (5'-3')	Product size (bp)	Reference
cry2	F GTTATTCTTAATGCAGATGAATGGG	689	Ben-Dov *et al*. (1997)
	R CGGATAAAATAATCTGGGAAATAGT		
XRE	F AAGATATTGCAAGCGGTAAGAT	246	Wei *et al*. (2019)
	R GTTTTGTTTCAGCATTCCAGTAA		

RESULTS

The colonial morphology, as well as the microscopic and biochemical characteristics of the isolates showing the expected characteristics of *Bacillus thuringiensis* are as shown in Table 2. Colonies that appeared creamy white, circular, dry, with flat elevation and wavy margin on LB agar were regarded putatively as*Bacillus thuringiensis*.

Table 3 shows the frequency and percentage of isolates with the desired microscopic and biochemical characterization. The occurrence of *Bacillus thuringiensis* isolates from various soil types namely, agricultural farmlands, refuse dump site and cow rangeland, are as shown in Table 4.

The five isolates identified using cultural, microscopic and biochemical characterizations were tested for the presence of the *cry2* andthe *XRE* genesby PCR. The amplicons of the *XRE* gene (246 bp) were detected in four of the strains of *Bacillus thuringiensis* (Plate I). In all of the strains, the *cry2* gene (689 bp) was not amplified.

Table 2. Biochemical characterization of isolates *Bacillus thuringiensis*

Isolate code	C1	C2	C6	R3	R4
*Colonial morphology	+	+	+	+	+
Gram reaction/Shape of cells	+/R	+/R	+/R	+/R	+/R
Chains of cells	+	+	+	+	+
Endospore stain	+	+	+	+	+
Motility	+	+	+	+	+
Catalase	+	+	+	+	+
Oxidase	+	+	+	+	+
Methyl Red	-	-	-	-	-
Voges Proskauer	+	+	+	+	+
Casein hydrolysis	+	+	+	+	+
Arginine hydrolysis	-	-	-	-	-
Utilization of citrate	+	+	+	+	+
Remark	Bt	Bt	Bt	Bt	Bt

C1, C2and C6 = Isolates from Cow rangeland
R3 and R4 = Isolates from Refuse dump site
***** = colonies that appear creamy white, circular, dry, flat elevation and with wavy margin on LB agar
+ = positive reaction. - = negative reaction.
R = rod.
Bt = *Bacillus thuringiensis*.

Table 3. Frequency and percentage of isolates with the desired microscopic and biochemical characteristics

Test	Result expected for *Bacillus thuringiensis*	Number of isolates tested	Number with the desired result (%)
Gram reaction	Gram positive	16	13 (81)
Shape of cells	Rod shaped	16	12 (75)
Chains of cells	Rods in chain	16	11 (69)
Endospore stain	Green spores with pink vegetative tissue	11	8 (73)
Motility	Motile organism	8	8 (100)
Catalase	Positive reaction (presence of bubbles)	5	5 (100)
Oxidase	Positive (purple colour)	5	5 (100)
Methyl Red	Negative (No change in colour)	5	5 (100)
Voges Proskauer	Positive reaction (Red colour)	5	5 (100)
Casein hydrolysis	Positive (production of halo zone around the colony)	5	5 (100)
Arginine hydrolysis	Negative (No clear zone around the colony)	5	5 (100)
Utilization of citrate	Positive (colour change from green to blue)	5	5 (100)

Table 4. Frequency of distribution of *Bacillus thuringiensis* isolates in various soil types after biochemical characterization

Sources of soil samples	*Number of Bacillus like isolates	Number of Bt isolates	[a]Bt index	Frequency of distribution of Bt (%)
Agricultural farmland	5	0	0.00	(0)
Cow rangeland	6	3	0.50	(50)
Refuse dump site	5	2	0.40	(40)
Total	16	5	0.31	(31)

***** Creamy white, circular, dry, flat elevation, with wavy margin

[a] Bt Index: Divide the number of *Bacillus thuringiensis* isolates by the total number of *Bacillus*-like colonies.

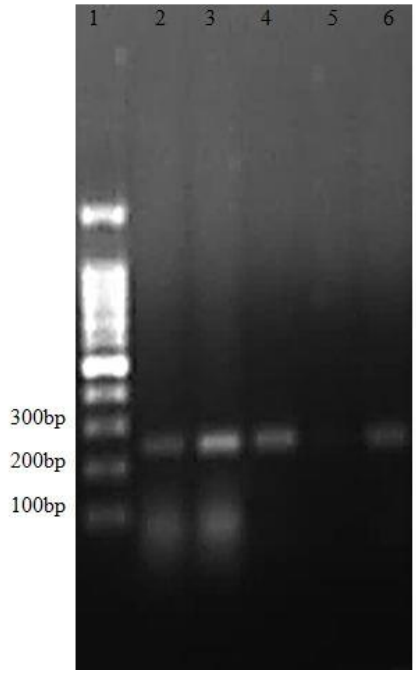

Plate I. Amplification of *XRE* gene (246 bp) in *Bacillus thuringiensis* isolated from soil samples

Key: Lane 1: DNA ladder (100bp), Lanes 2 - 4: Bt isolated from Cow range (C1, C2, C6, respectively), Lanes 5 and 6: Bt isolated from Refuse dump (R3 and R4, respectively)

DISCUSSION

An isolation procedure similar to the one used in this study was also utilised by Bello and Hussaini, (2017) and Adeyemo *et al*. (2018) and numerous *Bacillus thuringiensis* strains were isolated from different soil samples in Zaria and Okitipupa respectively.

The suspected *Bacillus thuringiensis* isolates were subjected to biochemical characterization and the observations made were in agreement to those reported by Kaur *et al*. (2002) and Eswarapriya *et al*. (2010)who reported that in addition to producing parasporal crystal bodies, the strains of *Bacillus thuringiensis* also have positive reactions for catalase production, citrate utilization, casein and starch hydrolysis.

The estimated value of successful *Bacillus thuringiensis* isolation (Bt index) varies with types of soil, with 0.31 being the average Bt index observed in this present study. The average Bt index varies among soil samples across the globe, as previously reported (Vilas-Boas and Lemos, 2004; Lone *et al*., 2016; Lone *et al*., 2017). The possible cause of the variation in Bt index may be a consequence of the difference in topography, nutrient availability, humidity, isolation source, geography and the interaction between bacterium and pests which could significantly affect their populations in different habitats.

The higher frequency of *Bacillus thuringiensis* isolated from cow rangeland relative to refuse dump site and agricultural farmland could be attributed to the fact that this soil type likely has higher organic matter content which favours the growth and proliferation of *Bacillus thuringiensis* which naturally occur on saprophytic plants and nourishes on dead organic matter. This finding corroborates the study of Bello and Hussaini, (2017) where higher occurrence of *Bacillus thuringiensis* was observed in Cow rangeland soil type.

Owing to the speed and reproducibility of the PCR-based approach, it has been widely used since its introduction by Carozzi *et al*. (1991) for the detection of established and new*cry* genes in strains of *Bacillus thuringiensis*. For the five strains isolated in this study, the *cry2* gene was not amplified, implying that the isolates have not acquired the plasmids harbouring the *cry2* gene. It also indicates that the isolates may have other kinds of *cry* genes that were not targeted throughout the study; as the *B. thuringiensis* strain has been reported to harbour one or even more crystal toxin genes (Crickmore *et al*., 2011). The primary cause for toxin genes diversity could be attributed to the fact they are mostly expressed on plasmids which are easily transferred partially or completely among *B. thuringiensis* strains (Fiuza, 2015; Liu *et al*., 2015). This agrees with the reports of Bello and Hussaini (2017) and Jain *et al*. (2017) which indicate that a large number of isolates do not carry the *cry2* genes. However, the predominance of the *cry2* gene has also been reported (Liang *et al*., 2011; Lone *et al*., 2017; Wei *et al*., 2019).The amplification of the *XRE* gene in this study revealed that out of five strains of putative strains of *B. thuringiensis*, the gene was detected in four (80%) when end-point PCR was used which only detects the gene and does not quantify it; while Wei *et al*. (2019)utilised a real-time PCR which quantifies the number of genes targeted in the amplification of the *XRE* gene in the detection of *Bacillus thuringiensis* with a specificity of 94% achieved. According to report by Wei *et al*.(2019), the *XRE* gene is more dominant than the *cry2* gene when compared in strains of *Bacillus thuringiensis*. The primary reason for the efficiency of *XRE* gene over *cry2* gene could be due to the fact that the former is a transcriptional regulator, itregulates the main type of crystal protein production (Wei *et al*., 2019) and there are currently 78 distinct *cry* genes families available as biomarkers since several translated cry protein product vary with the different categories of *cry* genes.

CONCLUSION

Strains of *Bacillus thuringiensis* were isolated from various soil types in Zaria, Nigeria. The *XRE* gene was found to be a more reliable biomarker than the *cry2*gene in the molecular detection of indigenous strains of *Bacillus thuringiensis* in the present study. The molecular detection of functional and regulatory genes is a reliable method of studying bacteria with biotechnological applications such as biocontrol.

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Author contributions

AEO, MDA and AHI designed the experiments. AEO conducted the experiments. AEO wrote the manuscript, and MDA and AHI reviewed the manuscript.

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