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PCR Detection of *cry* Genes in Local *Bacillus Thuringiensis* Isolates

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Abstract: Twelve local *B. thuringiensis* strains are used in the study, which previously isolated from the soil of seven governorates and characterized by their high toxicity against 2nd instars larvae of cotton leafworm (*Spodoptera littoralis*). The PCR amplification analysis of the 12 Bt isolates revealed the presence of amplified fragments characteristic of five different *cry* genes. Results showed the expected sizes of PCR products of *cry1Aa, cry1Ac, cry1I, cry2* and *cry3* genes ranging from 2595 to 2985, 209 to 447, 275 to 463, 2028 to 2471 and 1933 to 2130 bp, respectively. Out of the six *cry* genes investigated, only *cry1Ab* gene was not found in any of the 12 Bt isolates. Three different Bt. isolates; As-5, As-6 and Ts-7 showed *cry1Ac* gene only with PCR product size of 238 bp. While, five of the 12 Bt isolates were harboring a combination of different *cry* genes, such as Gh-3 harbored lepidoptera-active *cry1Aa* and *cry1Ac*, lepidoptera-diptera active *cry2* gene and coleoptera-active *cry3* gene. Two other Bt isolates; Sn-4 and Ts-1 harbored *cry1Ac* and *cry2*, while As-2 is containing *cry1Aa* and *cry1Ac*. Gh-5 was harbored *cry1Aa* and *cry1I*. These profiles of *cry* genes indicate that Gh-3 isolate was active against lepidopteran, dipteran and coleopteran insects. Four Bt isolates; Qa-6, Qa-7, Fa-8 and Is-1 did not react with any of the six PCR specific *cry* gene primers. These isolates may have had some other *cry* genes not identified by the six specific primers used. consequently combined strains are good candidates in the search for biocontrol agents with a wider spectrum of action against many different insects. On the other hand, *cry1* genes were the most frequently found among the 12 local Bt isolates and the most common profile of *cry1* genes contained *cry1Ac* and the second abundant genes was *cry1Aa* and *cry2*.

Key words: local Bt isolates, lepidopteran, dipteran and coleopteran *cry* genes, PCR.

INTRODUCTION

Crystal proteins from the gram-positive spore forming bacterium *Bacillus thuringiensis* are toxic to a wide variety of insects that are economically important as pests. over 100 *cry* gene sequences encoding the *B. thuringiensis* endotoxin have been isolated, characterized and classified in 22 groups and different subgroups with regard to their amino acid similarity (Crickmore *et al.,* 1998 and Schnepf *et al.,* 1998). The proteins toxic for lepidopteran insects belong to the Cry1, Cry9, and Cry2 groups; toxins active against coleopteran insects are the Cry3, Cry7, and Cry8 proteins as well as the Cry1B and Cry1I proteins, which have dual activity. The Cry5, Cry12, Cry13, and Cry14 proteins are nematocidal, and the Cry2, Cry4, Cry10, Cry11, Cry16, Cry17, Cry19, and Cyt proteins are toxic for diptera insects. The characterizations for most of the *B. thuringiensis* collections were based on bioassays against different insect larvae without identification of the *cry* genes present in the *B. thuringiensis* strains.

PCR-based techniques have been proposed to identify different *cry* genes in *B. thuringiensis* strains (Porcar and Juarez-Perez, 2003). For example, multiplex PCR method was established to rapidly identify and classify *B. thuringiensis* strains containing *cry* genes toxic to species of lepidoptera, coleoptera and diptera (Ben-Dov *et al.,* 1997). A two-step PCR strategy allows the ability for further clone and sequence genes for which no specific primers are available, whereas a variable region exists between two conserved regions (Juarez-Perez *et al.,* 1997). Forty three *B. thuringiensis* isolates from Brazil and 3 from Argentina were screened using the PCR for various

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coleoptera-specific *cry* genes (Marquez *et al.,* 2000). Moreover, the analysis of 496 Bt strains collected from Mexico was based on multiplex PCR with novel general and specific primers to detect different *cry* genes (Bravo *et al.,* 1998). Six Bt isolates obtained from soil in Nigeria, confirmed to be toxic to mosquito larvae were differentiated using a PCR-based technique (Ogunjimi *et al.,* 2000). A pair of highly degenerated primers was used by Shevelev *et al.* (1998) to carry out a single step PCR-detection of any known and probably unknown *cry* genes of *cry1*, *cry4* and *cry9* encoding for 130 kD protein d-endotoxin in the natural *B. thuringiensis* strains. Thirty different PCR profiles were found in the strains with *cry1* genes when they were analyzed with specific primers from *cry1A* to *cry1F* (Uribe *et al.,* 2003). To verify the d-endotoxin gene types of K1, Li *et al.* (2002) performed PCR analysis with specific *cry* gene primers; they proved that K1 contained a new *cry* gene in addition to *cry1Aa*, *cry1Ab*, *cry1Ac*, *cry1E* and *cry2* genes. With PCR products as probes, Kuo *et al.* (2000) have cloned two new *cry*-type genes from *B. thuringiensis* subsp. wuhanensis. These two clones were named *cry1Gb1* toxic to *Plutella xylostella* larvae and *cry1Bd1* toxic to *Pieris rapae* larvae. Two genes encoding the 32 and 40 kDs polypeptides of *B. thuringiensis* crystals were cloned, expressed in an acrystalliferous *B. thuringiensis* strain and sequenced (Kim *et al.*, 2003). A new *cry1Ca9* gene encoding a protein of 1189 amino acid residues was cloned and sequenced from a *B. thuringiensis* isolate native to Taiwan (Kao *et al.,* 2003). The PCR method is highly efficient for identification the *cry* genes present in *B. thuringiensis* strains. However, the *cry* gene list is increasing, and novel PCR primers are needed in order to identify some of the recently described *cry* genes.

The aim of the present study is to detect and isolate different *cry* genes toxic towards the larvae of members of the orders lepidopteran, dipteran and coleopteran in some local *B. thuringiensis* isolates based on PCR method with specific primers, as well as predicates their insecticidal activity.

MATERIALS AND METHODS

Bacterial Strains:

Twelve local *B. thuringiensis* isolate strains were used in the study (Table 1). They were previously isolated from the soil of seven governorates and characterized by their high toxicity against 2^{nd} instars larvae of cotton leafworm (*Spodoptera littoralis*) identify and classify *B. thuringiensis* strains containing *cry* genes toxic to species of lepidoptera, coleoptera and diptera.

DNA Extraction From Bacillus Thuringiensis:

The cultures of 12 *B. thuringiensis* isolates were incubated overnight at 30°C in LB broth medium with shaking. Five milliliters of LB medium was inoculated with 0.1 ml of the overnight culture and incubated at 30°C for 3 hrs with vigorous shaking. DNA was extracted and was used as a template for PCR according to Carozzi *et al.* (1991).

PCR Amplification and Primers Designed of Cry Genes:

Amplification was carried out in a DNA thermocycler (biometra, biomedizinische analytik gmbh) for 30 reaction cycles each. PCR reactions were carried out in 25 µl containing 50 ng of template DNA was mixed with reaction buffer, 150 mM deoxynucleoside triphosphate, 0.5 mM (each) primer, and 0.5 U of *Taq* DNA polymerase (Promega). Template DNA was denatured for 1 min at 94°C and annealed to primers for 1 min at 54°C, and

Table 1: *B. thuringiensis* strains with their names that isolated from different governorates.

Code Number	Local Bt isolates
1	Gharbeya-3
2	Gharbeya-5
3	Aswan-2
4	Aswan-5
5	Aswan-6
6	Toshka-1
7	Toshka-7
8	Qalubeya-6
9	Qalubeya-7
10	Sinai-4
11	Ismaileya-1
12	El-Fayoum-8

Table 2: Characteristics of specific primers for six *cry* genes *cry1Aa*, *cry1Ab*, *cry1Ac*, *cry1I*, *cry2* and *cry3* genes.

Primer pair[a]	Sequences of primers	*cry* genes recognized	Position[b]	Product size (bp)
1 (d)	5′ ATTATCATATTGATCAAGTTC	*cry1Aa*	2595-2590	390
(r)	5′ CATAAGGAACCCGTACCTGG		2965-2985	
2 (d)	5′ GGACCAGGATTTACAGGAGG	*cry1Ab*	1474-1495	1111
(r)	5′ GTTCTCCTACTAATGGTTTCC		2564-2585	
3 (d)	5′ CTCAATGGGACGCATTTCTT	*cry1Ac*	209-229	238
(r)	5′ CGGTTGTAAGGGCACTGTTC		427-447	
4 (d)	5′ AGCTATGGCCTAAGGGGAAA	*cry1I*	275-296	189
(r)	5′ TTCCAACCCAACTTTCAAGC		442-463	
5 (d)	5′ CAAAAGAATGGATGAGAGTG	*cry2*	2028-2049	443
(r)	5′ CTTTATTTGCACAGGCACG		2451-2471	
6 (d)	5′ CCTGGCTATGTGGATTTCGT	*cry3*	1933-1954	197
(r)	5′ CGAATGGCTTGGTTATGGAA		2109-2130	

[a] (d and r) refereed to direct and reverse primers, respectively.

[b] Starting from the first base of the sequence (of respective *cry* gene) in the GenBank database.

extensions of PCR products were achieved at 72°C for 1 min and an extra step of extension at 72°C for 5 min according to Mahadi *et al.* (1998). PCR amplification products were separated using agarose gel electrophoresis in 1.2% TAE buffer and stained with 0.2 mg/ml ethidium bromide according to Sambrook *et al.* (1989). PCR products were visualized under UV transilluminator and the sizes of the fragments were estimated based on a DNA ladder of 100 to 1500 base pairs. The direct and reverse primers for the six *cry* genes were designed from the GenBank database according to Pinto and Fiuza (2003). Whereas, four accessions; AF288683, Q269474, AY993931 and *cry1Aa13*-AF510713 contained different *cry1Aa* gene sequences were alignment from the very similar sequences within consensus. Two accessions; DQ092482 and AY847289 for *cry1Ab* gene, three accessions; AY225453, AY122057 and U89872 for *cry1Ac* gene, five accessions for *cry2* genes; *cry2*-AF200816, *cry2Aa*-AJ488143, *cry2Aa5*-AJ132464, *cry2Aa6*-AJ132465 and *cry2Ad*-AJ132463. The two single accessions *cry1I*-AY959880 and *cry3*-AY572010 for *cry1I* and *cry3* genes, respectively. The sequences of the designed primers are presented in Table (2).

RESULTS AND DISCUSSIONS

Twelve local Bt. isolate strains that used in the study were previously characterized by their high mortality percentages against larvae of cotton leafworm (Aly *et al.,* 2003).

Identification of Cry Genes in B. Thuringiensis Local Isolates:

The PCR amplification analysis of the 12 Bt isolates revealed the presence of amplified fragments characteristic of six different *cry* genes. Results showed the expected sizes of PCR products of *cry1Aa, cry1Ac, cry1I, cry2* and *cry3* genes, ranging from 2595 to 2985, 209 to 447, 275 to 463, 2028 to 2471 and 1933 to 2130 bp, respectively (Fig. 1 and Table 2). Out of the six *cry* genes investigated, only *cry1Ab* gene was not found in any of the 12 Bt isolates.

Three different Bt isolates; As-5, As-6 and Ts-7 showed *cry1Ac* gene only (Table 3) with PCR product size of 238 bp (Table 2). While, five of the 12 Bt isolates were harboring a combination of different *cry* genes, such as Gh-3 harbored lepidoptera-active *cry1Aa* and *cry1Ac*, lepidoptera-diptera active *cry2* gene and coleoptera-active *cry3* gene. Two other Bt isolates; Sn-4 and Ts-1 harbored *cry1Ac* and *cry2*, while As-2 is containing *cry1Aa* and *cry1Ac*. Gh-5 was harbored *cry1Aa* and *cry1I* (Fig. 1 and Table 3). These strains are good candidates in the search for biocontrol agents with a wider spectrum of action. The profiles of *cry* genes indicated that Gh-3 isolate was active against lepidopteran, dipteran and coleopteran insects. Four Bt isolates; Qa-6, Qa-7, Fa-8 and Is-1 did not react with any of the six PCR specific primer and gave negative results by PCR. These isolates may have had some other *cry* genes not identified by the six specific primers used (Table 3).

The three isolates; Gh-3, Sn-4 and Ts-1 which harboring the lepidoptera-diptera active *cry2* gene could be used for wide spectrum of diptera insects**.** The Cry2, a diptera- and lepidoptera-specific protein and Cry3, a coleoptera-specific protein were reported (Hofte and Whiteley, 1989 and Head, 2005). The presence of different *cry* genes in the same *B. thuringiensis* strain has been reported, for example Aronson (1994) and Ben-Dov *et al.* (1997) reported the presence of *cry1* genes and *cry3*, *cry8*, or *cry7* genes in the same *B. thuringiensis* strain. While,

Table 3: PCR-detection of different *cry* genes in the local Bt isolates.

Local Bt isolates		*cry* genes with PCR product size (bp)						
		Lepidoptera-active genes				Lepidoptera-diptera active *cry2* gene	Coleoptera-active *cry3* gene	
		cry1Ac	*cry1Aa*	*cry1I*	*cry1Ab*	443 bp	197 bp	**
		238 bp	390 bp	189 bp	1111 bp			
1	Gh-3	•	•			•	•	67%
2	Sn-4	•				•		33
3	Ts-1	•				•		33
4	Gh-5		•	•				33
5	As-2	•	•					33
6	As-5	•						17
7	As-6	•						17
8	Ts-7	•						17
9	Qa-6							0
10	Qa-7							0
11	Fa-8							0
12	Is-1							0
*0		58%	25	8	0	25	8	

• The presence of each *cry* gene.

* Percentage of each *cry* gene in all isolates.

** Percentage of all *cry* genes in each isolate.

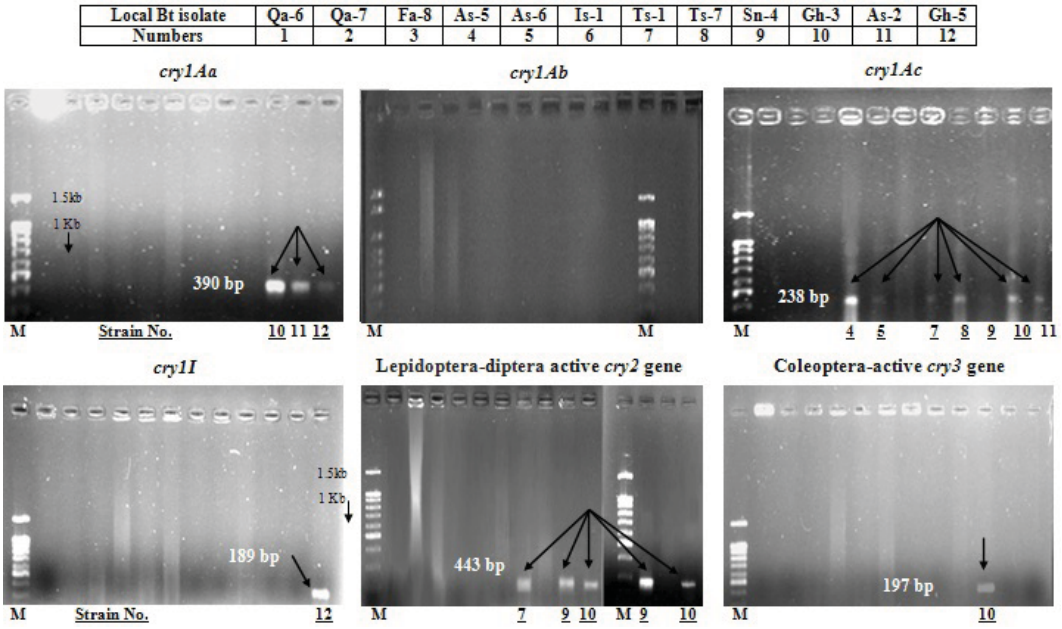

Fig. 1: PCR amplification products from local Bt isolates. M= DNA 1kb ladder separated by agarose electrophoresis.

Bravo *et al.* (1998) observed lepidopteran-active *cry1* genes and coleopteran-active *cry3A*, *cry3Ba* and *cry7A* genes in the same strain. They suggested that such *B. thuringiensis* strains which harbored more than one *cry* gene, have a high frequency of genetic information exchange.

The *cry1* genes were the most frequently found among the 12 local Bt isolates and the most common profile of *cry1* genes contained *cry1Ac* and the second abundant genes was *cry1Aa* and *cry2* (Table 3). Ben-Dov *et al.* (1997) found that strains containing *cry1* genes were the most abundant; however, strains harboring *cry4* genes were the second most abundant, while strains with *cry3* genes were absent. While, Chak *et al.* (1994) reported that *cry1A* genes were the most abundant, followed by the *cry1C* and *cry1D* genes; only four strains harbored *cry4*

genes, and no strains harbored *cry3*, *cry1B*, *cry1E*, or *cry1F* genes. Song *et al.* (2003) showed that *cry1I*-type genes appeared in 95 of 115 *B. thuringiensis* isolates and 7 of 13 standard strains. A novel *cry1I*-type gene was found in one standard strain and six isolates.

The identification of known *cry* genes in the *B. thuringiensis* strains is important, since the specificity of action is known for many of the Cry toxins. This fact allows the possibility of selecting native strains that could be used in the control of some targets and of selecting strains with the highest activity.

The PCR screening is a rapid method for detecting and differentiating *B. thuringiensis* field strains by their PCR product profiles and for predicting their insecticidal activities in order to direct them for subsequent toxicity assays against lepidoptera, coleoptera and diptera.

The eight Bt isolates displaying new profiles that containing combinations of *cry* genes should be characterized and further developed for integration with other toxicity assays against coleoptera, diptera and other lepidoptera insects. Moreover, such Bt isolates point strongly as good gene sources for developing transgenic plants resistant to insect pests. This was agreed with Ben-Dov *et al.* (1997) who mentioned that a strain may contain a novel gene with sequences annealing to the primers for known genes but different sequences in other regions defining a new insecticidal activity. This limitation can be resolved by a set of specific primers through the sequence of a particular gene (Kalman *et al.*, 1993).

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