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Identification of New *Cry* Genes in *Bacillus thuringiensis* Isolates by Multiplex PCR and RFLP

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Abstract: Proteins produced by the bacteria *Bacillus thuringiensis* are widely used for pest control in agriculture and reforestation. Those proteins are produced by genes known as *cry* genes. Some members of this class of genes present toxicity to only one insect order, whereas others present toxicity to more than one order. However, there still are a great number of important agricultural pests for which no toxin produced by *B. thuringiensis* is known. Moreover, frequent resistance to a given toxin raises the need for finding new and different *cry* toxin genes. Within the techniques used in the search for new *cry* genes, PCR has been distinguished by its detection level, facility, practicability, and quickness. The use of several primers in the same reaction increases the power of this technique and permits the identification of several genes simultaneously, thereby decreasing costs and optimizing time. However, DNA sequences of the same size may contain different base sequences and therefore belong to different gene families. Analysis based solely on amplicons can thus distinguish interesting sequences, but some of those identified may be false positives. Thus, the present work used multiplex PCR with RFLP in 41 *B. thuringiensis* isolates to analyze the presence of *cry* genes differing from those in standard strains. The results verified that 14 of the 41 studied isolates showed the same amplified DNA fragments for the *cry1* and *cry1Ac* genes as those exhibited by the standard strains. Nevertheless, after cleavage with restriction enzymes, three isolates showed sequences differing from the respective standard strains. Thus, we describe a valid method for identifying new *cry* genes from bacterial collections.

Key words: *Bacillus thuringiensis*, gene characterization, biological control, multiplex PCR, Cry toxins.

INTRODUCTION

Population growth (mainly in underdeveloped areas) and agricultural losses due to biotic and abiotic factors have spurred great efforts to increase food production. With regard to biotic factors, insect attacks are responsible for almost 15% of the world's agricultural losses [45]. Once agricultural insect pest control has been accomplished through the use of chemical insecticides (which are extremely toxic and exhibit a large range of action), severe consequences for men and environment will extend beyond that percentage. Further, because chemical insecticides are not selective, they also strike natural enemies of other pests and contaminate food, soil, and waters. The continuous use of such chemical products select for resistant insect populations, forcing agriculture to use larger doses of the products with every application [35]. Thus, any public politics proposing an increase of food production must consider the use of pesticides less aggressive toward the ecosystem. Biological control agents may contribute to both objectives of increasing food production and maintaining the tenable use of the planet.

Of the pathogenic insects used for biological control, the entomopathogenic bacteria *Bacillus thuringiensis* (*B. thuringiensis*) may be distinguished. It is responsible for almost 95% of the world's bioinsecticidal market [47] and represents about 2% of the world's insecticidal market [37].

Among the insect toxins produced by *B. thuringiensis*, the crystal proteins (Cry) are highly specific to several orders of insects like Lepidoptera, Coleoptera, and Diptera [47]. There are also reports of toxicity to nematodes [34], Hemiptera, Hymenoptera, Isoptera, Neuroptera, Orthoptera, Siphonaptera, Thysanoptera [6,37], Mallophaga, and Protozoa [16].

The great advantage of using Cry proteins in pest control is the fact that they are highly specific to certain species and do not affect other organisms (e.g.

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natural enemies or humans). However, the appearance of resistant individuals within their target populations may occur [12]. Therefore, due to the great potential for protein resistance and the large number of pests for which there is no known efficient Cry protein, several groups around the world are trying to identify new Cry genes that 1) provide efficient protection against other pests or 2) replace a Cry protein for which there is already a resistant population.

The proteins produced by *B. thuringiensis* may assume different forms, such as bi-pyramidal, cuboid, ramboid, ovoid, spherical, or even undefined [23]. These toxins are codified by *cry* genes; their toxicity is linked to the N-terminal region of the polypeptide chains, whereas the C-terminal portion is dispensable for toxicity [42] and may be involved in determining the structural form of the crystal [27]. Considering this fact, it is possible to suppose that small amino acid modifications on the N-terminal region of those proteins could change their specificity to a certain pest. Bravo *et al*., [8] demonstrated that a mutation in the N-terminal region of Cry1Ac protein affected the linkage and pore formation capacities of that protein in *Manduca sexta* membranes and decreased its insecticidal activity *in vivo*. Another example involves the Cry22 protein, which was initially identified as an active *B. thuringiensis* isolate against ants (Hymenoptera) [36]. Subsequently, other Cry22 proteins (Cry22Ab, Cry22Ba, and Cry22A) were characterized to show activity against certain Coleopterans. These proteins have the same molecular weight of roughly 75 to 86 kDa. However, Cry22A protein has four imperfect replicates of approximately 80 amino acids between the residues 261 and 575, whereas Cry22Ba essentially lacks the first of those replicates. A small variation in that region was thus able to alter protein specificity [30].

Since the cloning of the first *B. thuringiensis* gene coding for a protein toxic to insects in 1981 [43], several other genes have been identified and cloned by different methods. These methods include the use of antiserum [19,43], use of degenerated oligonucleotides complementary to the N-terminal region of previously-known *cry* genes sequences [13], use of complete or partial *cry* gene sequences as probes against genomic DNA of unknown isolates[2], use of the polymerase chain reaction (PCR) technique with specific primers for a certain *cry* gene [5,33,44,49,47,26,32,9], use of multiple primers in the same PCR reaction [17,38, 3,7], and use of the PCR technique followed by a DNA restriction fragment analysis (PCR-RFLP) [11,28,41,40,46,25].

Although methodologies based on antiserum have identified new genes, they have the disadvantage of requiring previous expression and purification of the protein. Further, the antibodies produced must be specific for the protein of interest, which is often quite difficult. The use of degenerated primers requires previous knowledge of the nucleotide sequences for the region of all searched genes. The probes also entail previous knowledge and isolation of a certain DNA sequence. In addition, all of these methods require large quantities of pure proteins or DNA and are extremely laborious; most of them are not suitable for a large-scale isolate analysis.

Although the PCR technique offers an efficient alternative in the search for new *B. thuringiensis cry* genes, its use on a large scale only became possible after the standardization of PCR with multiple primers via the multiplex PCR [48]. However, even reactions with several oligonucleotide primers in the same reaction do not guarantee accurate identification. Due to the similarity in their numbers of bases, amplified DNA segments ("amplicon") may represent distinctive (different) locos but occupy the same position on a gel (same size), [22,18].

Thus, multiplex PCR followed by an enzymatic cleavage of the "amplicon" (PCR-RFLP) provides a fast and accurate way to a large number of isolates for several different *cry* genes. RFLP permits the identification of differences of one base in the interior of the "amplicon". Because changes of few *cry* gene base pairs may alter specificity and/or recognition by insects, the use of this approach may be extremely useful for identifying isolates that contain variants of those genes. and, also, maintain as pests biological control, the use of new *cry* proteins.

Thus, this study aimed to determine the applicability of the use of multiplex PCR followed by RFLP analysis to identify strains of *B. thuringiensis* carrying *cry* genes variants on a high-throughput way.

MATERIALS AND METHODS

Using specific oligonucleotides, previously characterized for *cry* genes, total *B. thuringiensis* isolate DNA was amplified by multiplex PCR. The amplified products, with sizes of approximately 600 bp, were submitted to cleavage with different restriction enzymes. The isolates in the PCR product presenting a different restriction pattern from the standard isolate were then cloned into a specific vector, transformed in *E. coli* DH5α, and sequenced. Nucleotides sequences were submitted to Blastn and Blastx algorithm analysis [1] to determine their possible similarity to proteins already existing in Genbank.

2.1 – Bacterial Growing Conditions: Forty-one *B. thuringiensis* isolates and three standard strains (*B. thuringiensis* var. *kurstaki* HD1, *B. thuringiensis* var. *israelensis*, and *B. thuringiensis* var. *tenebrionis*)

belonging of the Bacterial Genetics and Applied Biotechnology Laboratory of the Department of Applied Biology of UNESP, Jaboticabal, SP, were cultivated in Petri dishes containing Agar Nutrient (3.0 g of meat extract; 5.0 g of peptone; 15.0 g of agar, 1 L of water, pH 6.8) for 12 h at 30ºC. The obtained colonies were inoculated in 50 mL of culture medium (Brain Heart Infusion – Biobras) and multiplied for 4.5 h at 30ºC under constant agitation of 200 rpm.

2.2. Extraction of Total DNA and PCR Reaction Conditions: The extractions of total DNA followed the methodology described by marmur[31], with slight modifications. Cell cultures from each isolate were centrifuged at 8,000g for 15 min at 4ºC. The bacterial pellet, obtained by centrifugation, had an average weight of 0.5 g and was resuspended in 6 mL of saline solution [0.5 M NaCl; 0.1 M EDTA, (pH 8.0)] with vortex agitation. Further, 0.6 mL of lysozyme was added and the suspension maintained for approximately 1 h in bath water with a temperature of 37ºC. Subsequently, 0.5 mL we added 25% SDS. Sodium acetate (2.5 M) was added to the mixture until a final concentration of 1M. Contaminating proteins were removed by chloroform/isoamly|alcoho| (24:1 v/v) extraction. The floating liquids were collected by centrifugation at 10,000g and dissolved in TE [10 mM Tris HCL (pH 7.4), 1 mM EDTA]. After adding RNase at a concentration of 50 µg/mL, the samples were incubated for 30 min at 37ºC. The suspension was then submitted to a chloroform/isoamly alcohol treatment, followed by the DNA reprecipitation with absolute ethanol and resuspension in TE. Finally, the solution was stored at -20ºC until the moment of its use.

2.3 – Analyses and PCR Reaction Conditions: With the objective of obtaining identical results for all standards in both amplifying situations and guaranteeing that the use of multiple oligonucleotides primers in the same reaction would not produce unspecified amplification of the standards, the PCR reactions were optimized for the use of only one pair of oligonucleotide primers (individual PCR) and the use of multiple oligonucleotide primers in the same reaction (multiplex PCR). In both situations, template mold DNA of *B. thuringiensis* standard strains (Table 1) was used as a positive control; water was used instead of the mold template DNA as a negative control.

The DNA samples were submitted to PCR in a final volume of 25 µL using an automatic thermocycler PTC 100 (MJ- Research). The sequence of the used oligonucleotide primers and their respective positive controls are listed in Table 1.

2.4. Standardization of the Individual Reactions: Initially, an individual amplifying reaction was performed with the DNA samples from each positive control. The reactions were conducted in final volume of 25 µL with the following components:

- 0.2 mM of dNTPs
- 0.4 pMoles of each primer
- 1 U of Taq DNA polymerase enzyme
- 1X of 10X PCR Buffer (Tris-HCL 200 mM pH 8.4 and KCL 500 mM)
- 2 mM of Mg^{2+} ($MgCl_2$ 50 mM)
- 30 to 500 ng of DNA template

The reactions were submitted to an amplifying process composed of a DNA denaturation period of 5 min at 94ºC followed by 35 amplifying cycles of 30 s at 94ºC, 30 s at 41.8ºC, and 1 min at 72ºC. At the end of the amplifying cycles, the samples were submitted to a final extension step of 7 min at 72ºC and maintained at 4ºC until they were removed from the thermocycler.

After confirmation, the amplified product from each standard sample was stocked to be used as a control during further electrophoresis.

2.5 - Standardization of Multiplex PCR Reactions: After the standardization of the individual PCRs, multiplex PCR was standardized with all *B. thuringiensis* isolates and standard strains studied in the present work. In this phase, each reaction was comprised of 2.5 µL of 10X PCF Buffer, 4 mM Mg^{2+}, 0.4 mM of each dNTP, 2 U Taq DNA polymerase enzyme, 10 pMoles of each of the ten primers, and approximately 100 ng of total DNA from the standard strain or isolate to be analyzed.

The reactions were submitted to an amplifying process composed of an initial denaturation cycle of 5 min at 94ºC and 35 amplification cycles of 30 s at 94ºC, 30 s at 49.1ºC, and 1 min at 72ºC. After the 35 amplifying cycles, the samples were submitted to a final extension step of 7 min at 72ºC and maintained at 4ºC until they were removed from the thermocycler.

2.6 - PCR-RFLP Analyses: The products of the multiplex PCR were submitted separately to cleavage with the following restriction enzymes: *Eco* RI, *Hind* III, *Hpa* II, and *Taq* I. These enzymes were randomly chosen from those in the laboratory with consideration of frequent recognition enzymes and low cost enzymes. The cleavage reactions were composed of 5 µL PCR product, 1 U of restriction enzyme, 2 µL of 10X PCF Buffer, and the volume completed to 20 µL with autoclaved bi-distillated water. The time and temperature conditions were those recommended by the restriction enzyme manufacturer (Invitrogen).

Sequentially, the reaction was analyzed with a 1% agarose gel, TAE buffer (Tri-Base 2 M, acetate 1 M, EDTA 0.1 M), and visualized by UV light. The image of gel was then saved in a computer.

Table 1: List of primers used in this present work

Genes	Primers	"Amplicon"	Standard strains
Gral-*cry*1[§]	5'CTGGATTTACAGGTGGGGATAT3'		
	5'TGAGTCGCTTCGCATATTTGACT3'	560 bp	*B. thuringiensis* var. *kurstaki* HD1
*cry*1 Ac[*]	5'ATCGCTCGTCTATCGGCATTG3'		
	5'AGCCAGCCCTCACGTTCTTC3'	400 bp	*B. thuringiensis* var. *kurstaki* HD1
cry 3[*]	5'CGTCCATCTCGTAAAGTCAAAG3'		
	5'ATGACAAGAAAGGGAGGAAG3'	200 bp	*B. thuringiensis* var. *tenebrionis*
*cry*11[*]	5'GGATGGATAGGAAACGGAAG3'		
	5'ATACTGCCGTCTGTTGCTTG3'	593 bp	*B. thuringiensis* var. *israelensis*
cyt 2[*]	5'CAAATGGTCTTCCTAATGCAG3'		
	5'TATGATTTGGACGATGTAAGC3'	498 bp	*B. thuringiensis* var. *israelensis*

bp – base pairs
"amplicon" – expected size of the amplified DNA fragment
§ - [7]
[*] - [21]

2.7 – Cloning and Polymorphic DNA Fragment Sequencing: After the separation of the fragments, the DNA of interest was purified from the gel using a Pure Link Quick Gel Extraction (Invitrogen) following the manufacturer's recommendations. The purified DNA sequences were individually cloned in the vector pGEM-T Easy (Promega) according to the manufacturer's indications, and the recombinant plasmids were transformed in *E. coli* DH5 α competent cells according to Sambrook[39]. For each transformation, 20 recombinant clones were selected. Each one of those clones was multiplied in medium with ampicillin, and plasmid DNA was extracted by the alkaline lysis method [39]. To confirm the expected size of the DNA fragments from cloning, a sample of each mixture was cleaved with the restriction enzyme *Eco* RI and submitted to electrophoresis with a standard fragment size (1Kb DNA Ladder -Invitrogen).

Among the clones with the expected DNA fragment sizes, four of each were selected and their plasmid DNA submitted to sequencing by an automatic sequencer ABI PRISM 3700 DNA Analyzer (Applied Biosystems) using Big Dye Terminator v3.1 kit (Applied Biosystems) according to the manufacture's suggestions. One universal oligonucleotide primer (T7 or SP6) was used.

To identify possible homologies with *cry* genes previously sequenced, the obtained chromatograms were analyzed with the Phred/phrap/consed programs [14, 15, 20] and the contig assembles were analyzed by the Blastx program [1].

RESULTS AND DISCUSSION

3 – Results: The use of multiplex PCR followed by RFLP for the identification of *B. thuringiensis* isolates *cry* gene variants on a large-scale offered a very powerful technique. It permitted the identification of differences between sequences with the same base pair size and allowed us to analyze a large number of samples in a short time.

3.1 – Standardization of PCR Reactions: Initial tests to determine the optimal individual and multiplex PCR conditions were performed.

For the individual reactions, a sash of annealing temperatures between primer and template DNA was initially tested using a gradient thermocycler (TGradient, Biometra). The results indicated a temperature of 41.8ºC as the optimal temperature. Maintaining constant all parameters in the reaction, except the DNA concentration, we showed that the ideal quantity of DNA in the reaction was of 100 ng.

Once the standardization of the reaction was complete, the DNA standard strain samples were amplified to obtain perfect amplifications. The lone exception to this was the *cry3* gene (Figure 1), which resulted in a smaller amplified DNA quantity that was nonetheless sufficient for analyses and visualization.

To standardize the multiplex PCR, the reaction components were varied. In this manner, the concentrations of reaction components like dNTPs, Mg^{2+}, 10X PCR Buffer, and DNA polymerase enzyme were varied (Table 2).

The tests permitted us to conclude that mixture T16 (Table 2) in multiplex PCR was satisfactory for DNA standard strain amplification. For those conditions, there is a greater requirement for magnesium, nucleotides, and DNA polymerase enzyme. After determining the ideal constitution of the reagents for the amplification reactions, we conducted a test to verify the optimal annealing temperature between the oligonucleotide primers and template DNA. In each

Table 2: Amount in microliters (µL) of each PCR component used in each test. T_0 to T_{18} = test zero to test eighteen. Test T_0 was considered the standard for comparison. Blank cells indicate maintenance of the same value used in the standard reaction. The stock concentration of each reagent is described in the material and methods section.

	T_0	T_1	T_2	T_3	T_4	T_5	T_6	T_7	T_8	T_9	T_{10}	T_{11}	T_{12}	T_{13}	T_{14}	T_{15}	T_{16}	T_{17}	T_{18}
H_2O	6.8	4.3	6.4	5.8	3.3	5.9	4.4	3.9	2.5	5.3	4.8	1.8	4.55	4.1	3.7	5.3	5.1	4.9	4.7
buffer 10x	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	4.0	1.25	2.5	2.5	2.5	2.5	2.5	2.5
Mg^{2+}	1.0	3.0	1.0	1.0	3.0	1.0	3.0	3.0	3.0	1.0	1.5	3.0	3.0	3.0	3.0	2.0	2.0	2.0	2.0
dNTPs	0.5	1.0	0.5	0.5	1.0	1.0	0.5	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Primers																			
Gral-*cry1*	1+1																		
cry1Ac	1+1																		
cry3	1+1																		
cry11	1+1																		
cyt2	1+1																		
Taq	0.2	0.2	0.6	1.2	1.2	0.6	0.6	0.6	2.0	1.2	1.2	1.2	1.2	04.	0.8	0.2	0.4	0.6	0.8
DNA	4.0																		

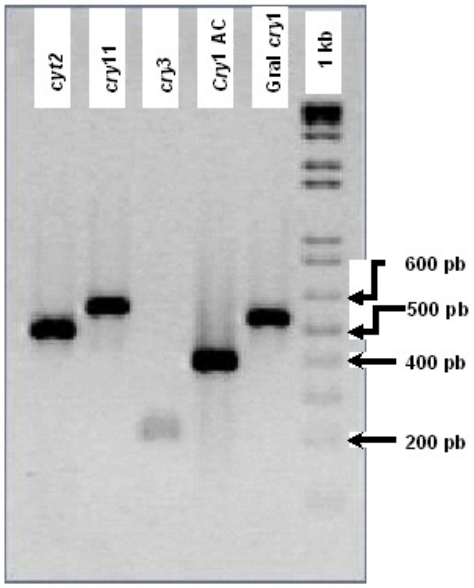

Fig. 1: Electrophoretic analysis of the PCR. The picture shows the results of amplification using 100 ng of template DNA and an annealing temperature of 41.8ºC. Genomic DNA of *B. thuringiensis* var. *kurstaki* HD1 (general gral *cry1* primer e *cry1Ac* genes), *B. thuringiensis* var. *tenebrionis* (*cry3*), and *B. thuringiensis* var. *israelensis* (*cry11* e *cyt2*) was used.

reaction, there were five PCR primers pairs. The gradient thermocycler tests revealed an ideal annealing temperature of 49.1ºC. This one is choosed because *cry3* oligonucleotides did not amplify at higher temperatures (Figure 2).

3.2 - Identification of *Cry* Genes by Multiplex PCR and RFLP: Analyses of 41 isolates via multiplex PCR using the previously-described standard conditions permitted us to identify 14 positive results for general gral-*cry1* primers (subgroups *cry1Ac* genes) (Figure 3, Table 3). The *cry* 1 gene was the most abundant.

The product of the multiplex PCR of 13 isolates and five standard strains was submitted to cleavage with four restriction enzymes: *Eco* RI, *Hin*d III, *Hpa* II, and *Taq* I. Additionally, amplified DNA from the isolates S_{497}, $S_{251,}$ and S_{1328} were also included in the cleavage due to the fact that they presented non-specific bands common to other isolates.

After those analyses, we verified that the isolates Br_{64}, $Br_{74,}$ and Br_{84} presented polymorphisms with respect to the standard strain *B. thuringiensis* var. *kurstaki* HD1. The isolate Br_{74} showed two distinctive bands, whereas the other two isolates showed only one polymorphic band (Figure 4).

3.3 – Cloning and Sequencing of DNA Polymorphic Fragments: After the cleavage of the multiplex PCR product with the *Taq* I enzyme and the separation of fragments in an agarose gel, the polymorphic bands at approximately 600 bp shown by the Br_{64}, $Br_{74,}$ and Br_{84} isolates (Figure 4 D) were individually removed from the gel. Those purified DNA sequences were cloned into the pGEM-T Easy vector and transformed into *E. coli*. Cleavage of the recombinant plasmids with the restriction enzyme *Eco* RI and subsequent electrophoresis confirmed the cloning of a fragment of the expected size.

The sequencing resulted in two contigs: one was composed of the amplified DNA sequences from the

Table 3: Isolates analyzed by the multiplex PCR technique and the respective genes identified. The "+" symbol indicates the presence and the "-" symbol indicates the absence of the gene in the isolate.

Isolates	*cry1*	*cry1Ac*	*cry3*	*cry11*	*cyt2*
1. Br_{13}	-	-	-	-	-
2. Br_{14}	-	-	-	-	-
3. Br_{19}	-	-	-	-	-
4. Br_{26}	-	-	-	-	-
5. Br_{32}	-	-	-	-	-
6. Br_{34}	-	-	-	-	-
7. Br_{44}	-	-	-	-	-
8. Br_{55}	-	+	-	-	-
9. Br_{64}	+	+	-	-	-
10.Br_{67}	-	-	-	-	-
11.Br_{74}	+	+	-	-	-
12.Br_{84}	+	+	-	-	-
13.Br_{85}	+	+	-	-	-
14.Br_{97}	-	-	-	-	-
15.Br_{98}	-	-	-	-	-
16.Br_{99}	-	-	-	-	-
17.Br_{100}	-	-	-	-	-
18.S_{75}	-	-	-	-	-
19.S_{165}	-	-	-	-	-
20.S_{170}	-	-	-	-	-
21.S_{204}	-	-	-	-	-
22.S_{248}	-	-	-	-	-
23.S_{350}	-	-	-	-	-
24.S_{443}	-	-	-	-	-
25.S_{479}	-	-	-	-	-
26.S_{497}	-	-	-	-	-
27.S_{251}	-	-	-	-	-
28.S_{646}	-	-	-	-	-
29.S_{1083}	-	-	-	-	-
30.S_{1328}	-	-	-	-	-
31.S_{1349}	-	-	-	-	-
32.MT_4	+	+	-	-	-
33.MT_5	+	+	-	-	-

Table 3: Continue

34.PA_1	+	+	-	-	-
35.SP_1	+	+	-	-	-
36.SP_2	+	+	-	-	-
37.SP_3	-	+	-	-	-
38.SP_4	+	+	-	-	-
39.SP_6	-	-	-	-	-
40.SP_{14}	+	+	-	-	-
41.SP_{15}	+	+	-	-	-

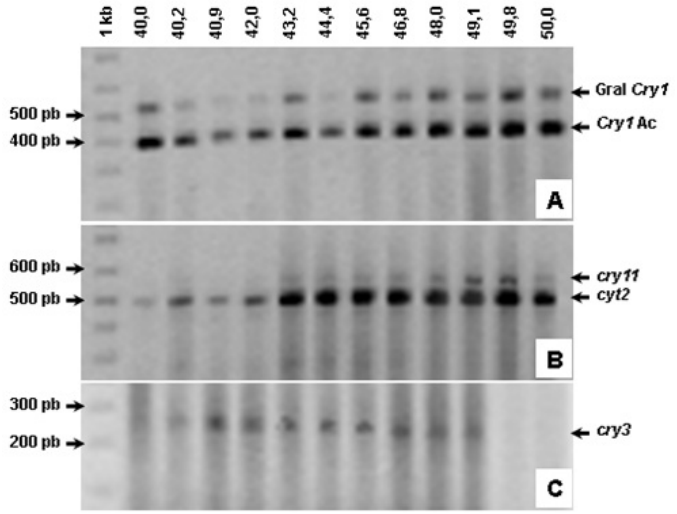

Fig. 2: Electrophoretic analysis of multiplex PCR in a temperature gradient varying from 40 to 50ºC (indicated on the upper part of the figure). "A" - *B. thuringiensis* var. *kurstaki* HD1 genomic DNA; "B" - *B. thuringiensis* var. *israelensis* genomic DNA; "C" - *B. thuringiensis* var. *tenebrionis* genomic DNA.

Br_{64} and Br_{84} isolates (Figure 5), and the other was formed by only the DNA sequences from the Br_{74} isolate (Figure 6). After using the phred/phrap/consed programs to analyze the results, we observed that the contig Br_{64}/Br_{84} contained 529 nucleotides and the contig Br_{74} contained 478 bases.

Comparison with other sequences, previously deposited in the National Center for Biotechnology Information (NCBI), GenBank, using the BLASTX program[1] allowed us to verify that the sequences of the Br_{64} and Br_{84} isolates were similar to that of the Cry1Ea protein (gb|ABX11258.1|;e-value=2e-92) on the first "hit". Although most proteins homologous to that sequence are active against insects of the Lepidoptera order, however, the second "hit" obtained from the BLASTX analysis showed similarity with the Cry 032 protein (gb|AAL50331|; e-value = 2e-92) active against nematodes. Multiple alignment with the predicted proteic sequence of those genes (Cry 032 and Cry1Ea) illustrated a difference of one amino acid between them and the contigs Br_{64}/Br_{84} (Figure 7). This finding likely indicates that the gene present in the Br_{64} and Br_{84} isolates is different from both used in the comparison.

On the other hand, the contig referent to the isolate Br_{74} was not similar to any other sequence currently registered in the GenBank.

4 – Discussion: Biological methods are still not frequently used for agricultural pest control, and they represent only 1% of the total of this activity. *B. thuringiensis* bacteria is responsible for 98% of the biological products used for pest control, and studies that increase this percentage are thus of extreme importance. Likewise, the results obtained in the present work may be useful to increase the participation of biological control in agriculture. Thus, the results achieved in this study may be useful to increase the participation of biological control in agriculture. We showed that multiplex PCR followed by RFLP is an extremely powerful method for the identification of *cry* gene variants in a rapid and efficient manner.

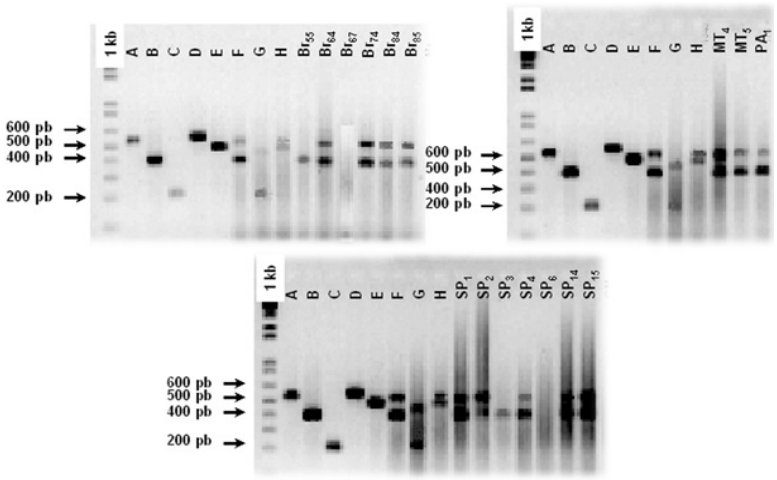

Fig. 3: Amplified profiles presented by *B. thuringiensis* isolates and standard strains. The isolates were submitted to multiplex PCR. The comparison patterns amplified by simple PCR are indicated by the letters "A" - *B. thuringiensis* var. *kurstaki* HD1 (Gral *cry1*), "B" - *B. thuringiensis* var. *kurstaki* HD1 (*cry1Ac*), "C" - *B. thuringiensis* var. *tenebrionis* (*cry3*), "D" - *B. thuringiensis* var. *israelensis* (*cry11*), and "E" - *B.thuringiensis* var. *israelensis* (*cyt2*). The letters "F" to "H" show the patterns obtained by multiplex PCR for *B. thuringiensis* var. *kurstaki* HD1, *B. thuringiensis* var. *tenebrionis,* and *B. thuringiensis* var. *israelensis*, strains respectively. 1 kb = molecular marker size of 1Kb DNA Ladder (Invitrogen).

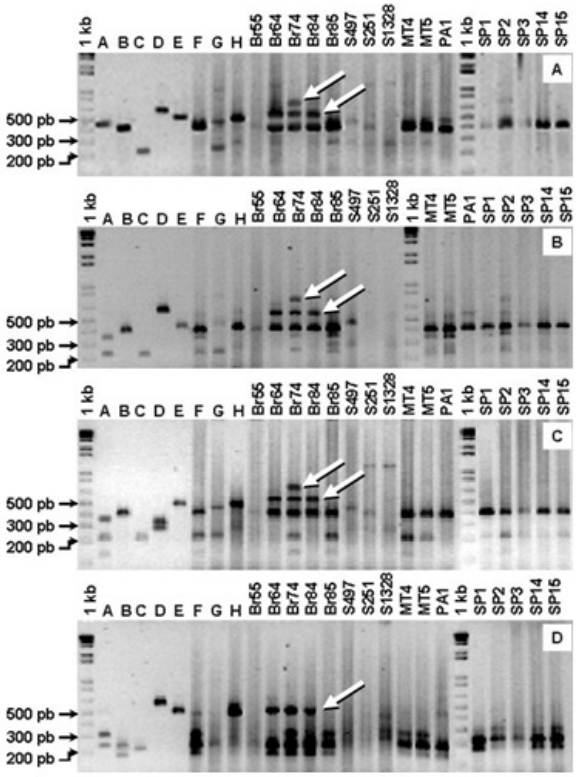

Fig. 4: DNA fragment restriction patterns presented by *B. thuringiensis* isolates after multiplex PCR products cleavage with the restriction enzymes *Eco* RI (gel A), *Hin*d III (gel B), *Hpa* II (gel C), and *Taq* I (gel D). From "A" to "E" are presented the individual PCR products with the oligonucleotides Gral *cry1*, *cry1Ac*, *cry3*, *cry11,* and *cyt2*, respectively. Panels "F" to "H" show the multiplex PCR products from all five primers pairs Gral *cry1, cry1Ac*, *cry3*, *cry11*, and *cyt2,* for *B. thuringiensis* var. *kurstaki* HD1 *B. thuringiensis* var. *tenebrionis,* and *B. thuringiensis* var. *israelensis,* respectively. 1Kb = molecular marker size 1kb DNA Ladder (Invitrogen). The arrows indicate the polymorphism sites.

```
GAAATACCATTGGTGAGTTTGTGTCTTTACAAGTCAATATTAACTCACCA
ATTACCCAAAGATACCGTTTAAGATTTCGTTATGCTTCCAGTAGGGATGC
ACGAATTACTGTAGCGATAGGAGGACAAATTAGAGTAGATATGACCCTTG
AAAAAACCATGGAAATTGGGGAGAGCTTAACATCTAGAACATTTAGCTAT
ACCAATTTTAGTAATCCTTTTTCATTTAGGGCTAATCCAGATATAATTAG
AATAGCTGAAGAACTTCCTATTCGTGGTGGTGAGCTTTATATAGATAAAA
TTGAACTTATTCTAGCAGATGCAACATTTGAAGAAGAATATGATTTGGAA
AGAGCACAGAAGGCGGTGAATGCCCTGTTTACTTCTACAAATCAACTAGG
GCTAAAAACAGATGTGACGGATTATCATATTGATCAAGTTTCCAATTTAG
TTGAGTGTTTATCGGATGAATTTTGTCTGGATGAAAAGAGAGAATTATCC
GAGAAAGTCAAATATGCGAAGCGACTCAA
```

Fig. 5: DNA nucleotide fragment sequence of 529 bp amplified from BR_{64} and BR_{84} isolates via multiplex PCR.

```
TAGCCAGCCCTCACGTTCTTCCTCCCTTTCTTGTCATAGCCAGCCCTCAC
GTTCTTCCTCCCTTTCTTGTCATAGCCAGCCCTCACGTTCTTCAATGCCG
ATAGACGAGCGATAGCCAGCCCTCACGTTCTTCAATGCCGATAGACGAGC
GATAGCCAGCCCTCACGTTCTTCCTGAGCCCTACAATGCCGATAGACGAG
CGATAGCCAGCCCTCACGTTCTTCAATGCCGATAGACGAGCGATAGCCAG
CCCTCACGTTCTTCAATGCCGATAGACGAGCGATAGCCAGCCCTCACGTT
CTTCCAATGCCGATAGACGAGCGATAGCCAGCCCTCACGTTCTTCCAATG
CCGATAGACGAGCGATAGCCAGCCCTCACGTTCTTCCCAATGCCGATAGA
CGAGCGATAGCCAGCCCTCACGTTCTTCCTGAGCCCTACAATGCCGATAG
ACGAGCGATAGCCAGCCCTCACGTTCTA
```

Fig. 6: DNA nucleotide fragment sequence of 478 bp amplified from the BR_{74} isolate via multiplex PCR.

```
Cry1Ea          VIKGPGFTGGDILRRNTIGEFVSLQVNINSPITQRYRLRFRYASSRDARITVAIGGQIRV 540
Cry032          VIKGPGFTGGDILRRNTIGEFVSLQVNINSPITQRYRLRFRYASSRDARITVAIGGQIRV 540
BR64_BR84       ---------------NTIGEFVSLQVNINSPITQRYRLRFRYASSRDARITVAIGGQIRV 45
                               ****************************************

Cry1Ea          DMTLEKTMEIGESLTSRTFSYTNFSNPFSFRANPDIIRIAEELPIRGGELYIDKIELILA 600
Cry032          DMTLEKTMEIGESLTSRTFSYTNFSNPFSFRANPDIIRIAEELPIRGGELYIDKIELILA 600
BR64_BR84       DMTLEKTMEIGESLTSRTFSYTNFSNPFSFRANPDIIRIAEELPIRGGELYIDKIELILA 105
                ************************************************************

Cry1Ea          DATFEEEYDLERAQKAVNALFTSTNQLGLKTDVTDYHIDQVSNLVECLSDEFCLDEKREL 660
Cry032          DATFEEEYDLERAQKAVNALFTSTNQLGLKTDVTDYHIDQVSNLVECLSDEFCLDEKREL 660
BR64_BR84       DATFEEEYDLERAQKAVNALFTSTNQLGLKTDVTDYHIDQVSNLVECLSDEFCLDEKREL 165
                ************************************************************

Cry1Ea          SEKVKHAKRLSDERNLLQDPNFRGINRQPDRGWRGSTDITIQGGDDVFKENYVTLPGTFD 720
Cry032          SEKVKHAKRLSDERNLLQDPNFRGINRQPDRGWRGSTDITIQGGDDVFKENYVTLPGTFD 720
BR64_BR84       SEKVKYAKRL---------------------------------------------------- 175
                *****:****
```

Fig. 7: Multiple alignment between the predicted amino acid sequences for the contig Br_{64}/Br_{84}, Cry 032, and Cry1Ea genes.

If we had used just individual PCR, it would have been necessary to perform 42 reactions for each pair of oligonucleotides - a total of 210 reactions for the five oligonucleotides used in this study. With the use of multiplex PCR, however, only 49 reactions were necessary (less than 25% of that needed for individual PCRs) for the same quantity of isolates and primers. Furthermore, this reduction also decreased the number of enzymatic cleavage reactions (RFLPs). If the RFLP technique had not been used, none of the isolates would have shown polymorphisms relative to the standard since the amplifications resulted in sequences of similar sizes. The isolates showed more than one *cry* gene, because they also showed the same cleavage pattern of the standard strain in addition to the polymorphism after the enzymatic cleavage. Also, note that the isolates had more than one gene cry because, after the cleavage by the standard strain This fact also suggests the possibility that the polymorphic sequence is part of a different *cry* gene.

In all of the isolates studied, the *cry1* gene was the most abundant. These results are compatible with previously published data [33,7,4,10]. Previous authors demonstrated that the genes coding Lepidoptera toxic proteins are normally the most plentiful in the collections of this type of bacteria. On the other hand, no genes coding for Coleoptera or Diptera Cry proteins (e.g. *cry3*, *cry11,* or *cyt2*) were found, suggesting that these proteins are not abundant in the group samples studied. These results also agree with those from published literature. Ben-dov *et al*., [4] also failed to find *cry11* and *cry3* genes in a collection of *B. thuringiensis* strains collected in Israel, Kazakhstan, and Uzbekistan soils.

Because that small mutations in the toxin's recognition regions may drastically alter its specificity

for insect targets [24], these isolates reveal new candidate genes different from the standards strain. These genes that may be efficient for use against other pests or substitute for a gene that is no longer effective due to resistance of its insect target population.

Due to its extreme importance to both the environment and men, the search for new *B. thuringiensis cry* genes is an enviable fact. The search for new cry genes in *B. thuringiensis* is highly coveted because of its importance to the environment and humans. Therefore, the present results show that the use of multiplex PCR along with PCR-RFLP facilitates analyses of several *B. thuringiensis* isolates in a rapid and accurate manner.

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