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Genotypic analysis of *Bacillus thuringiensis* serovars by RAPD-PCR

Análise genotípica de sorovares de *Bacillus thuringiensis* através do RAPD-PCR

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Abstract

In this study, genomic DNA fingerprints of 40 *Bacillus thuringiensis* serovars representing different serotypes were obtained by Randomic Amplification of Polymorphic DNA (RAPD-PCR) using three different randomic primers. The electrophoretic analysis of RAPD band profiles showed the presence of polymorphism among the serovars and serotypes. Between the primers used in this study, primer 2 was the most discriminatory and, therefore, was used to build the dendrogram. The similarity analysis of the electrophoretic profiles showed low level of similarity (16%) among the different *B. thuringiensis* serovars. The serovars were divided into two main groups, and these indicated a high level of genetic divergence both intra and inter-group. Results suggested that genomic fingerprints obtained by RAPD can be used for the genotypic characterization and identification of *B. thuringiensis* serovars as a complement to flagellar serology.

Key words: *Bacillus thuringiensis*, RAPD-PCR, genetic similarity, flagellar serology.

Resumo

O *fingerprintings* do DNA genômico baseado na amplificação aleatória de DNA polimórfico (RAPD-PCR) de 40 sorovares de *Bacillus thuringiensis* representando diferentes sorotipos foi realizado usando três iniciadores randômicos. A análise eletroforética dos perfis de RAPD revelou a incidência de polimorfismo entre os sorovares e sorotipos. Entre os iniciadores utilizados, o iniciador nº 2 foi o mais discriminatório e, portanto, foi usado para construir um dendrograma. A análise da similaridade entre os perfis eletroforéticos demonstrou baixo nível de afinidade genética (16%) entre os diferentes sorovares de *B. thuringiensis*, agrupando esses sorovares em dois grupos principais com alta divergência genética intra e intergrupo. Logo, o RAPD baseado no *fingerprintings* genômico de *B. thuringiensis* pode ser usado na caracterização genotípica e identificação de sorovares de *B. thuringiensis* como um complemento à sorologia flagelar.

Palavras-chave: *Bacillus thuringiensis*, RAPD-PCR, afinidade genética, sorologia flagelar.

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Introduction

Bacillus thuringiensis (Bt) is defined as mesophilic, facultative anaerobic or aerobic, Gram-positive, spore-forming bacilli that are able to synthesize parasporal crystalline inclusions during the sporulation (Schnepf *et al.,* 1998). The introduction of serotypes based on flagellar antigens (H-antigen) serotyping along with biochemistry characters helped the description of new *Bt* serotypes (Bonnefoi and De Barjac, 1963; De Barjac and Franchon, 1990).

The commercial interest in biological control of insects stimulated intensive screening programs to search for new strains in different countries, allowing the discovery of new serovars presenting different spectrums of entomopathogenic activity (Kawalek *et al.,* 1995; Rabinovitch *et al.,* 1995; Seleena *et al.,* 1995; Orduz *et al.,* 1996; Ferrandis *et al.,* 1999). In the 1990's, 82 different flagellar serovars, 69 serotypes and 13 sub-antigens groups of *Bt* were identified. Flagellar serotyping is a useful methodology for the classification of *Bt*, but it presents some limitations such as autoagglutinating strains and non-motile strains (Lecadet *et al.,* 1999).

In addition, the identification and characterization of *Bt* strains have been accomplished through molecular techniques such as colony DNA hybridization, PCR analysis using conserved primers for ribosomal DNA sequences, RFLP analysis and selective amplification of genomic fragments by AFLP-PCR. These techniques can collaborate to group new strains and help the identification of those that can not be serotyped by the flagellar antigen technique (Bourque *et al.,* 1993; Hansen *et al.,* 1998; Pattanayak *et al.,* 2000; Joung and Côté, 2001).

Genomic DNA fingerprinting using random amplified polymorphic DNA PCR (RAPD-PCR) has been applied in studies to analyze inter or intra-specific differences in genomic DNA and it has been demonstrated to be useful to discriminate related organisms (Welsh and McClelland, 1990; Williams *et al.,* 1990). The analysis of RAPD profiles has shown that *Bt* serovars can be distinguished and identified based on their fingerprints. Even strains belonging to the same serotype can be distinguished among themselves or from other serotypes (Brousseau *et al.,* 1993; Hansen *et al.,* 1998; Pattanayak *et al.,* 2001; Rivera and Priest, 2003).

Moreover, RAPD-PCR has been commonly used in typing pathogenic strains of *B. sphaericus* (Woodburn *et al.,* 1995), to discriminate differences between *Bt* serovars (Brousseau *et al.,* 1993), to identify *Brevibacillus laterosporus* strains (Oliveira *et al.,* 2004), to distinguish among *Bt* serotypes (Pattanayak *et al.,* 2001) and for molecular characterization of *Bt* serovars (Rivera and Priest, 2003; Chaves *et al.,* 2008).

The objective of this study was to evaluate the genetic similarity among *Bt* strains of different serovars by applying RAPD-PCR. By analyzing RAPD profiles, we attempted to establish genetic relatedness among *Bt* serovars and serotypes.

Material and methods

Bacterial strains

Forty-one strains representing 40 serovars and 30 serotypes of *Bt* are shown in Table 1. The strains analyzed in this study were obtained from International Entomopathogenic *Bacillus* Centre (IEBC) Collection at Institute Pasteur, Paris, France, and were held at Coleção de Culturas do Gênero *Bacillus* e Gêneros Correlatos – CCGB, except strains LFB-FIOCRUZ 855, 869, 891 that were isolated by the Laboratório de Fisiologia Bacteriana team and serotyped at Institute Pasteur.

All strains were grown in Nutrient Broth or Nutrient Agar OXOID (Meat extract-3g/L; Meat peptone-5g/L; Agar- 15g/L pH 6.8 $\pm$ 2) at 33ºC $\pm$ 1ºC for 18h as needed. The strains were lyophilized and stored at the CCGB at Laboratório de Fisiologia Bacteriana, Instituto Oswaldo Cruz/FIOCRUZ.

DNA extraction and RAPD-PCR

The DNA of the *Bt* strains was extracted according to the methodology described by Harwood and Cutting (1990) and Santos (2000). After extraction, DNA concentration was determined according to Sambrook *et al.* (1989). For the amplification assays the kit "Ready to Go RAPD Analysis" (Amersham Pharmacia Biotech) was used.

The methodology, as well as the cycles for RAPD-PCR, followed the manufacturer recommendations. The primers used are part of the "Ready to Go RAPD Analysis" kit. Each primer is an arbitrary sequence of 10 base pairs in the concentration of 2,5 nmol (Table 2). Amplifications were carried out in a total volume of 25 µL containing: 18 µL of distilled water, 5µL of primer (25pmol) and 2 µL of template DNA. *E. coli* BL21 strain was used as positive control for the kit. In addition, a negative control without DNA was performed. The amplification was carried out with 45 cycles at 95ºC for 1 min, 36ºC for 1 min and 72ºC for 2 min for denaturation, annealing and primer extension, respectively. All samples for RAPD-PCR were submitted to pre-amplification for 5 min at 95ºC and post-amplification for 7 min at 72ºC. For analysis of the amplification products, 5 µL of amplicons were electrophoresed in 1.5% agarose gels in Tris-borate EDTA (TBE 1X) (Tris-borate 89mM; EDTA 2mM pH 8.0) buffer at 75V for 3 h. The visualization of amplified products was performed under ultraviolet light, after treatment for 15 min with 0.5 µg/mL Ethidium Bromide solution. As a reference, 100bp DNA molecular weight (Amersham Pharmacia Biotech) was used.

Table 1. Assignment of *Bacillus thuringiensis* strains used in the study.

LFB FIOCRUZ[a]	Serovar	Flagellar serotype	Country of origen[b]
674	*thuringiensis*	H 1	Canada
683	*finitimus*	H 2	USA
474	*kurstaki*	H 3a3b3c	Chade
678	*alesti*	H 3a3c	France
472	*sotto*	H 4a4b	Pakistan
716	*sotto* biotype *dendrolimus*	H 4a4b	France
1260	*kenya*	H 4a4c	Kenya
675	*galleriae*	H 5a5b	URSS
677	*canadensis*	H 5a5c	Canada
682	*entomocidus*	H 6	Canada
891	*aizawai*	H 7	Brasil
476	*morrisoni*	H 8a8b	ND
849	*morrisoni*	H 8a8b	USA
717	*ostriniae*	H 8a8c	China
725	*nigeriensis*	H 8b8d	Czechoslovakia
562	*tolworthi*	H 9	England
731	*darmstadiensis*	H 10a10b	Germany
1316	*londrina*	H 10a10c	Brazil
715	*toumanoffi*	H 11a11b	Germany
727	*kyushuensis*	H 11a11c	Japan
724	*thompsoni*	H 12	USA
563	*pakistani*	H 13	Pakistan
584	*israelensis*	H 14	Israel
723	*dakota*	H 15	USA
718	*indiana*	H 16	USA
721	*tohokuensis*	H 17	Japan
713	*kumamotoensis*	H 18a18b	Japan
722	*yunnanensis*	H 20a20b	China
726	*pondicheriensis*	H 20a20c	India
729	*colmeri*	H 21	USA
719	*shandongiensis*	H 22	China
730	*japonensis*	H 23	Japan
720	*neoleonensis*	H 24a24b	Mexico
728	*coreanensis*	H 25	South Korea
1261	*mexicanensis*	H 27	Mexico
1317	*monterrey*	H 28a28b	Mexico
1163	*jegathesan*	H 28a28c	Malaysia
1164	*medellin*	H 30	Colombia
1318	*malaysiensis*	H 36	Malaysia
855	*oswaldocruzi*	H 38	Brazil
869	*brasiliensis*	H 39	Brazil

Notes: (a) Register of the lineages in the Coleção de Culturas do Gênero *Bacillus* (CCGB). (b) ND, not determined.

Table 2. Amplification products of the *Bacillus thuringiensis* serovars used random decamer primers.

Primer	Sequence (5' – 3')	Number of fragments generated	Size range (bp)
1	GGTGCGGGAA	21	200-1600
2	GTTTCGCTCC	28	250-2300
3	AACGCGCAAC	24	100-1600

Numerical analysis

Results of RAPD-PCR were transformed in binary matrixes and evaluated using the NTSYS-pc (2.1 version) numerical analysis program. Profile analysis was performed based on the calculation of Dice Coefficient, used to establish the similarity matrix. This matrix was transformed into a dendrogram using the unweighted pair group method (UPGMA), according to Rohlf (2000). A co-phenetic matrix was constructed using the similarity matrix to test the validity of clusters generated. Correlation (Mantel *t* test) between cophenetic matrix and similarity was determined using MXCOMP module (Sneath and Sokal, 1973).

Results and discussion

In this study 41 *Bt* strains representing 40 serovars and 30 serotypes were characterized by RAPD-PCR using three different randomic primers. All primers used allowed the amplification of multiple fragments of polymorphic DNA for all *Bt* serovars tested. These primers generated a total of 73 fragments ranging from 100bp to 2300bp for the 41 *Bt* strains were analyzed, and an average of 24 fragments per primer (Table 2). Amplification reactions for each primer were performed twice and the results were reproducible. Among the primers used, primer 2 was the most discriminatory since it generated 28 polymorphic fragments (Figure 1, C and D). *Bt* ser. *kumamotoensis* and *Bt* ser. *sotto* biotype *dendrolimus* presented the same band profile with

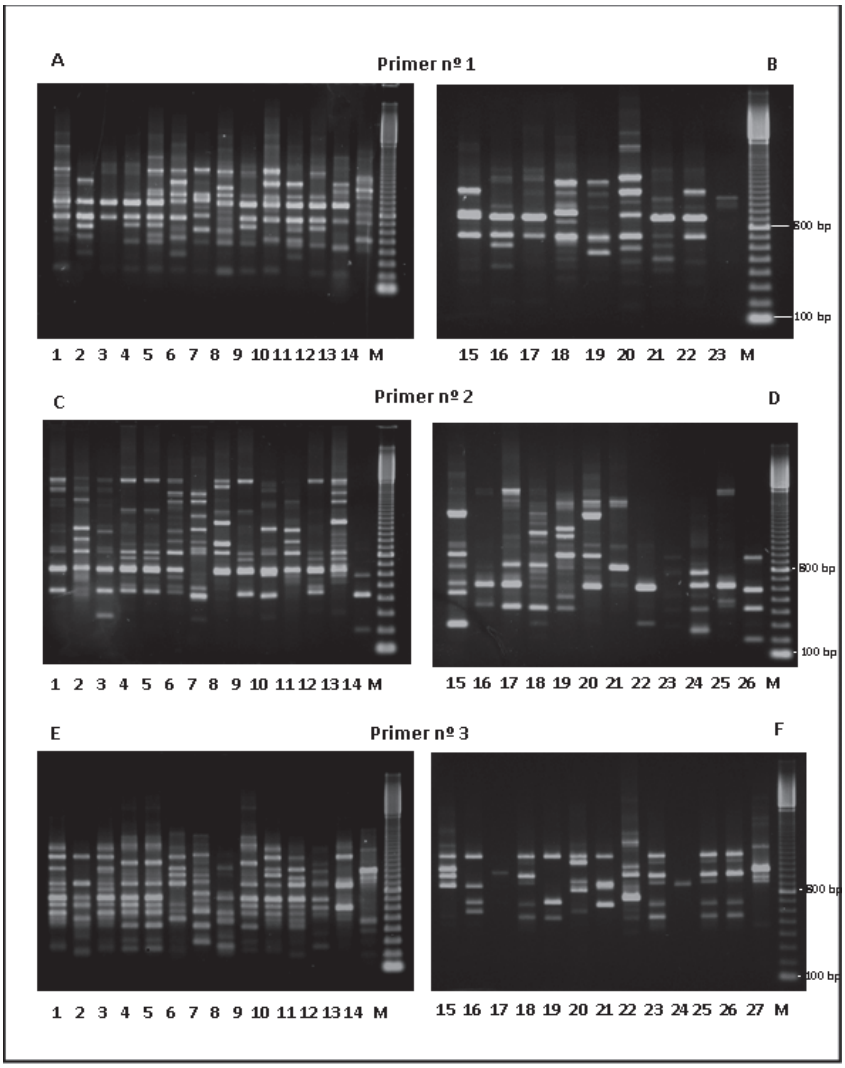

Figure 1. Examples of RAPD profiles of the *Bacillus thuringiensis* serovars generated by primers 1, 2 and 3. **Primer nº 1, 2, 3 - (A), (C)** and **(E)** 1, *israelensis*; 2, *kurstaki*; 3, *morrisoni*; 4, *kumamotoensis*; 5, *sotto* biotipo *dendrolimus*; 6, *indiana*; 7, *neoleonensis*; 8, *tohokuensis*; 9, *yunnanensis*; 10, *dakota*; 11, *thompsoni*; 12, *nigeriensis*; 13, *colmeri*; 14, *E. coli* BL21; **M**, molecular weight – 100 bp ladder, the 800 bp and 100 bp bands of the ladder are indicated; **Primer nº 1, 2, 3 - (B)** and **(D)** 15, *japonensis*; 16, *darmstadiensis*; 17, *morrisoni*; 18; *oswaldocruzi*; 19, *brasiliensis*; 20, *aizawai*; 21, *jegathesan*; 22, *medellín*; 23, *finitimus*; 24, *toumanoffi*; 25, *ostriniae*; 26, *E. coli* BL21; **M**, molecular weight (same as above). **(F)** 15, *japonensis*; 16, *darmstadiensis*; 17, *morrisoni*; 18; *oswaldocruzi*; 19, *aizawai*; 20, *jegathesan*; 21, *medellín*; 22, *finitimus*; 23, *toumanoffi*; 24, *ostriniae*; 25, *E. coli* BL21; **M**, molecular weight (same as above).

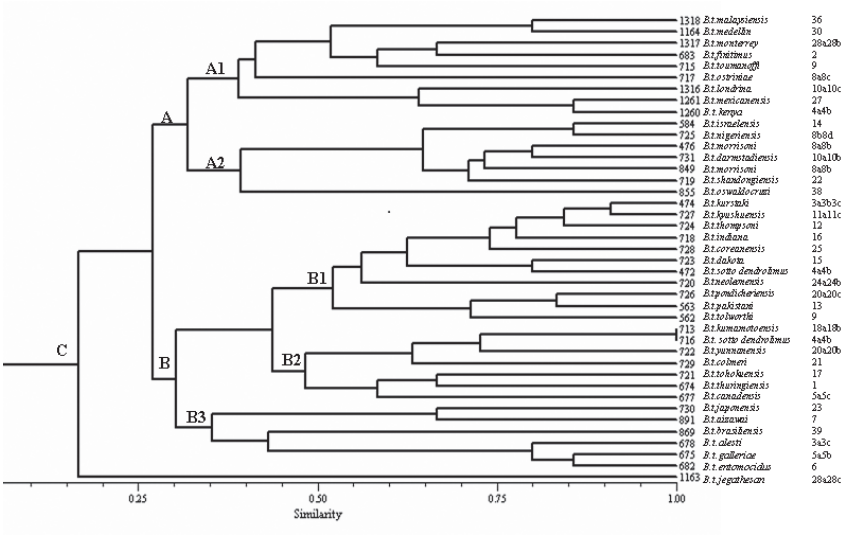

Figure 2. Dendrogram based on the RAPD profiles of the 41 *Bacillus thuringiensis* strains generated by primer 2 using the Dice Coefficient and UPGMA cluster analysis.

100% identity, when amplified with primers 2 and 3 (Figure 1, C4 and C5; E4 and E5) but were discriminated when primer 1 was used for amplification (Figure 1, A4 and A5).

RAPD-PCR band profiles of 41 *Bt* strains presented similarity in terms of co-migration of some DNA fragments which reflects the fact that the strains belong to the same species. The variation in PCR products generated by RAPD reflects the variation on internal sequences of amplified fragments of genomic DNA of the different serovars (Welsh and McClelland, 1990; Rivera and Priest, 2003). The RAPD analysis allowed the differentiation of the *Bt* serovars and, moreover, it revealed some genetic similarity among serovars and serotypes.

The analysis of band profiles generated by primer 2 showed low genetic similarity (16%) among *Bt* serovars, including these serovars into two major clusters, A and B (Figure 2). Cluster A was divided in two subclusters, A1 and A2, which were then subdivided in smaller clusters, some with only one serovar as in the case of the serovar *oswaldocruzi*. In the cluster A, the higher similarity (85%) was seen between serovars *mexicanensis* (serotype 27), *kenyae* (serotype 4a4c), *israelensis* (serotype 14) and *nigeriensis* (serotype 8b8d) which presented related RAPD profiles. These serovars synthesize proteins that demonstrate biological activity for both Diptera and Lepidoptera.

It has been reported that *Bt* serovars can be differentiated and identified based on the genomic fingerprints obtained by RAPD. Favorable results obtained with the application of RAPD were reported by Brousseau *et al.* (1993). The authors described the discrimination of *Bt* serotypes by RAPD and, additionally, the discrimination of *Bt* serovar *kurstaki* from different commercial products. The application of RAPD-PCR allows the discrimination of *Bt* lineages, even among strains belonging to the same serotype (Hansen *et al.,* 1998).

In subcluster A2, the strains LFB-FIOCRUZ 476 and 849, which belong to the same serotype (8a8b), were discriminated by using primer 2 (Figure 1, C3 and D17).

Cluster B was divided in three subclusters (B1, B2 and B3). The highest similarity (92%) was detected between the serovars *kurstaki* (serotype 3a3b3c) and *kyushuensis* (serotype 11a11c), both producers of glycoproteins with mosquitocidal activity. The serovar *kyushuensis* synthesizes the toxin Cyt2Aa that shows toxicity to Diptera while some strains of serovar *kurstaki* synthesize a dual-specificity crystal protein, Cry2A. This protein (Cry2A) has been reported as responsible for Diptera toxicity presented by these strains (Yamamoto and McLaughlin, 1981). Furthermore, serovars *galleriae* (serotype 5a5b) and *entomocidus* (serotype 6) included in subcluster B3 presented high similarity (around 85%). These serovars, however, synthesize toxins with different insecticidal activities.

Serovar *jegathesan* (serotype 28a28c) formed a group of only one member that presented 16% of similarity with the other groups and a low level of similarity to the other serovars. This serovar synthesize a polypeptidic complex of six proteins Cry and one protein Cyt, many of them presenting homology with the proteins produced by serovar *israelensis* (Seleena *et al.,* 1995). Notwithstanding, the fragments amplified by primer 2 showed an expressive divergence between this serovar and the serovar *israelensis*.

The coefficient of co-phenetic correlation in the dendrogram was relatively high (0.70) indicating the validity of the clusters generated in the present study (Sneath and Sokal, 1973).

The low level of similarity among some *Bt* serovars may be associated to the high levels of genetic diversity within this species. This diversity can be demonstrated by the diversity of agglutination reactions of flagellar antigens and by the presence of different proteins which present insecticidal activity against insects of specific Orders (Schnepf *et al.,* 1998; Pattanayak *et al.,* 2000).

The genetic divergence of different *Bt* serovars is probably due to the preferential location of the toxin genes in plasmids, usually conjugative and frequently associated to mobile elements, which determine the large diversity of these genes. Therefore, the occurrence of lineages presenting

a combination of genes that generates different toxicity profiles can be observed (Rosso *et al.,* 2000).

These facts are supported by the polymorphism of RAPD profiles and the low level of similarity among some *Bt* serovars. By establishing a genetic correlation among the serovars and the serotypes, Pattanayak *et al.* (2001) were able to distinguish *Bt* serotypes by RAPD-PCR.

Malkawi *et al.* (1999) demonstrated the genetic polymorphism among *Bt* isolates from different regions of Jordan by applying RAPD-PCR. According to these authors, RAPD markers common to some isolates could be used to develop specific DNA probes that can help in the identification of new *Bt* strains. Additionally, this methodology was applied by Rivera and Priest (2003) to analyze 126 *Bt* strains belonging to 56 serovars isolated from different countries. The strains analyzed were grouped into 58 genomic types according to the respective RAPD profiles. Although the profiles reflected the heterogeneity of the species, strains belonging to certain serovars, such as *israelensis*, were genomic homogeneous and represented clonal groups. However, other serovars, such as *aizawai*, were different and, for these serotypes, RAPD profiles were not correlated to the serotype. Chaves *et al.* (2008) reported that RAPD-PCR was useful in the identification of autoagglutinating *Bt* strains that could not be typed by the classic flagellar serotyping technique.

Genomic fingerprints generated by RAPD-PCR have been useful for the discrimination of related organisms based on intra and inter-specific differences of the genomic DNA which are reveled by the profiles of amplified products (Welsh and McClelland, 1990; Williams *et al.,* 1990). Stephan *et al.* (1994) and Stephan (1996) used this technique to differentiate *B. cereus*, *B. lentus* and *B. licheniformis* strains. Ronimus *et al.* (1997) were able to discriminate strains of thermophilic and mesophylic *Bacillus* by applying RADP-PCR. Hendriksen and Hansen (2006) demonstrated that *Bt* serovar *kurstaki* HD1 can be distinguished from other *Bt* strains by the analysis of the RAPD pattern generated by only one primer.

The genetic polymorphism reveled by the RAPD markers is useful for the classification of microorganisms. By comparing the profiles generated for different lineages or species it is possible to detect differential fragments that can be specific for the groups or species of interest but absent on related species (Rosato *et al.,* 2002). Levy *et al.* (2005) applied RAPD-PCR to identify genetic markers in *B. anthracis* strains.

The application of RAPD allowed Oliveira *et al.* (2004) to identify a molecular marker in *Brevibacillus laterosporus* lineages that has not been detected in other genetic related species belonging to the genus *Brevibacillus*. That marker can be useful for the isolation and identification of new bacterial lineages of this specie. Daffonchio *et al.* (1999), using this technique, identified a specific marker of *B. anthracis* which allowed the differentiation of this species from *B. cereus*, *B. thuringiensis* and *B. mycoides*. Zahner *et al.* (1999) were able to identify a molecular marker for mosquitocidal *B. laterosporus* strains using RAPD.

Based on the results obtained in this study, we suggest that the RAPD-PCR represents an important tool for the detection of polymorphism among *Bt* serovars. The identification of molecular markers common to certain species can be applied for the development of DNA probes specific for *Bt*, as well as other species belonging to the genus *Bacillus* and correlated genus.

The analysis of the profiles generated for *Bt* serovars suggests that RAPD-PCR technique can provide an accurate way of evaluating the genetic similarity among the serovars. Therefore, genomic fingerprints of *Bt* obtained by RAPD-PCR can be used for the genotypic characterization and identification of *Bt* serovars as a complement, in addition to flagellar serology.

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