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Genetic diversity, antimicrobial resistance and toxigenic profiles of *Bacillus cereus* isolated from food in Brazil over three decades

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ABSTRACT

Bacillus cereus is an ever-present problem. It is widely distributed in several environments such as soil and plants and is commonly isolated from food and additives. In this study we analyzed 97 foodborne *B. cereus sensu stricto* strains isolated in Brazil in the 1980's, 1990's and 2000's in order to investigate the genetic diversity (assessed by Rep-PCR), antimicrobial resistance and toxigenic profiles (presence of *hblA*, *hblC* and *hblD*; *nheA*, *nheB* and *nheC* as well as *cytK*, *ces* and *entFM* genes) of such strains. The majority of the strains (79, 81.4%) were β-hemolytic. The NHE complex was found in 82 strains (84.5%) and HBL complex was found in 61 (62.9%) strains. All strains were negative to *ces*. The *cytK-2* gene was found in 44 (45.4%) strains. The predominant toxigenic pattern was type I (32, 33%) which included strains positive for all toxin genes but *ces*. Computer assisted cluster analysis of Rep-PCR profiles showed a high genetic diversity. Seven major clusters comprising two or more strains were found and cluster 1 was predominant (ten strains, nine of them showing 100% similarity). This cluster included strains isolated in the 1980's and the 1990's. Cluster analysis of Rep-PCR profiles based on decade of isolation, source, hemolytic pattern, toxigenic and antibiotic resistance patterns revealed a similar clustering pattern as found in the analysis including all strains. The inability to observe a predominant band pattern when Rep-PCR cluster analysis was based on decade of isolation suggests that this diversity has been maintained over time. All strains were susceptible to gentamicin. We detected resistance to tetracycline (11 strains showing intermediate resistance and nine completely resistant strains), clindamycin (ten intermediate strains) and vancomycin (one strain). Clindamycin resistance showed statistical association with strains isolated in 2000's. The predominant resistance pattern was type A (72, 72.2%) which included strains susceptible to all drugs tested. Our results suggest that the majority of the strains present in several types of food in Brazil pose a potential risk to cause food poisoning due to the high prevalence of toxin genes found in these strains. However, additional studies involving cytotoxicity tests and affiliation of these strains to phylogenetic groups based on molecular data would be useful to better evaluate this potential and could provide a more accurate indication of the risk.

1. Introduction

Bacillus cereus is an ever-present problem due to its ubiquitous occurrence, its nutritional versatility and the ability to form endospores and to grow over a broad temperature range (Setlow and Setlow, 1994). It is widely distributed in several environments such as soil and plants and is commonly isolated from food and additives (Abriouel et al., 2007; Kim et al., 2009).

Many *B. cereus* strains cause food poisoning and other infections. Two main types of food poisoning have been described: emetic and diarrheal.

The emetic type is caused by a small cyclic heat-stable peptide, cereulide, and is characterized by the occurrence of nausea and vomiting within up to six hours after ingestion (Agata et al., 1995; Ehling-Schulz et al., 2004; Rajkovic et al., 2008). The operon responsible for cereulide production is situated on a large plasmid that seems to be subjected to lateral transfer (Ehling-Schulz et al., 2006). It has been hypothesized that cereulide-producing strains represent a recently emerged clone in the *B. cereus* population that are progressively diversifying (Ehling-Schulz et al., 2005; Vassileva et al., 2007).

The diarrheal type is attributed to enterotoxins, a group of proteins including two heat-labile toxins, the hemolysin BL (HBL, encoded by *hblA*, *hblC* and *hblD*) (Beecher et al., 1995) and non hemolytic enterotoxin (NHE, encoded by *nheA*, *nheB*, and *nheC*) (Granum et al., 1999), and the cytotoxin K (encoded by *cytK*), a single protein similar to the β-toxin of *Clostridium perfringens* (Lund et al., 2000). The syndrome is characterized by the occurrence of abdominal pain and

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watery diarrhea eight to 16 h after ingestion (Granum and Lund, 1997).

An additional enterotoxin, enterotoxin FM (EntFM) (Asano et al., 1997) has been reported and was found to be cytotoxic to Vero cells (Boonchai et al., 2008).

There are only few studies addressing the characterization of *B. cereus* strains isolated in Brazil (Aires et al., 2009; Aragon-Alegro et al., 2008) in contrast with several studies involving strains isolated in temperate climates (Ankolekar et al., 2009; Godic and Seme, 2009; McIntyre et al., 2008; Turner et al., 2006; Wijnands et al., 2006; Zhou et al., 2008). In addition, studies involving *B. cereus* Brazilian strains include only strains recently isolated; characteristics of strains isolated in past decades remain largely unknown.

The aim of this study was to investigate the genetic diversity, antimicrobial resistance and toxigenic profiles of *B. cereus* strains isolated from food and spices in Brazil in the 1980's, 1990's and 2000's decades.

2. Materials and methods

2.1. Bacterial strains

Ninety-seven *Bacillus cereus sensu stricto* strains isolated from food and spices collected in the southwest region of Brazil were analyzed. None of the strains, to the best of our knowledge, was involved in food poisoning cases. All strains were isolated in food surveys. Fifty-seven strains were isolated in the 1980's, 20 in the 1990's and 20 in the 2000's. Sources of isolation are shown in Table 1. All strains were randomly selected from Coleção de Culturas do Gênero *Bacillus* e Gêneros Correlatos – CCGB, Instituto Oswaldo Cruz – FIOCRUZ, where they were kept lyophilized, and grown in Nutrient Broth or Nutrient Agar at 33 °C ± 1 °C for 18 h. *B. cereus* strains were identified as previously described (Rhodehamel and Harmon, 2001). Growth at 7 °C was investigated on bovine blood agar plates as previously described (Stenfors Arnesen et al., 2007) in order to exclude *Bacillus weihenstephanensis*. None of the strains presented visible growth within two weeks and were all recorded as negative.

Table 1
Source of isolation of *Bacillus cereus* strains.

Source	Number of stains according to the decade of isolation			Total
	1980's	1990's	2000's	
Infant milk based formula	2	7	2	11
Milk	6	2	1	9
Cumin	7	–	1	8
Chocolate pudding	3	5	–	8
Black pepper	1	4	2	7
Ready to eat dishes[a]	7	–	–	7
Beef	4	–	1	5
Corn[b]	3	–	2	5
Yogurt	5	–	–	5
Rice cream	4	–	–	4
Cassava flour	–	–	4	4
Wheat	2	1	0	3
Oregano	–	–	3	3
Lettuce	3	–	–	3
Chocolate powder	3	–	–	3
Other[c]	7	1	4	12
Total	57	20	20	97

[a] Includes: beef jerk/pumpkin—3, chicken pie—2, and steak—2.
[b] Includes corn flour and corn flakes.
[c] Includes: powder soup, ice cream, ham, spinach , sausage, meat/soy sauce, paprika, nutmeg, dry parsley, mixed spice, curry, and pudding powder—1.

2.2. Determination of lecithinase and hemolytic activities

The hemolytic activity was determined at 33 °C on blood agar plates as described by Pruss et al. (1999). With respect to the hemolytic activity the strains were classified as α (partial), β (total), D (discontinuous β-hemolysis) or non hemolytic.

Evaluation of lecithinase activity was performed as previously described (Vasconcelos and Rabinovitch, 1994).

2.3. Detection of enterotoxin genes

Genomic DNA of *B. cereus* strains was extracted by thermal lysis according to the methodology described by Nunes et al. (1999). Briefly, an aliquot of 1 mL of the culture grown overnight at 37 °C in 5 mL of BHI was centrifuged for 4 min at 1000 rpm. The supernatant was discarded, the pellet was washed in 1 mL of TE buffer (10 mM Tris, 1 mM EDTA, pH 7.8) and the cells were resuspended in 100 μL of TE buffer. The suspension was placed in a 100 °C water bath for 10 min. and centrifuged for 30 s at 10,000 rpm at 4 °C. The supernatant was collected and used as template. Genes belonging to the HBL complex (*hblA*, *hblC* and *hblD*) and the NHE complex (*nheA*, *nheB* and *nheC*), the cereulide peptide synthetase gene *ces* and *entFM* were amplified according to the methodology described by Yang et al. (2005). The *cytK* gene was amplified as described by Guinebretiere et al. (2002) and Yang et al. (2005).

2.4. Bacillus Rep-PCR

The repetitive element sequence polymorphism-PCR (Rep-PCR fingerprinting) was used for chromosomal comparisons of *B. cereus* strains. The primers and the conditions for amplification were those described by Reyes-Ramirez and Ibarra (2005). Briefly, amplifications were performed with a GeneAmp System 9700 (Applied Biosystems, Foster City, CA, USA) in a total volume of 25 μL containing: 300 ng of each primer, 200 μM of dNTP, 5 mM of $MgCl_2$, 2.5 U of *Taq* Polymerase (Invitrogen, São Paulo, Brazil) and 1 μL of template DNA. To visualize band patterns, amplicons were electrophoresed in 1.2% agarose gels in Tris–borate EDTA buffer (Tris–borate 89 mM; EDTA 2 mM pH 8.0) at 2 V/cm for four hours. Amplicons were visualized under ultraviolet light, after treatment of the gels with 0.5 μg/mL Ethidium Bromide solution for 15 min. As a reference, 100 bp DNA molecular size marker (Invitrogen, São Paulo, Brazil) was used. Banding patterns were analyzed by visual inspection and by computer-assisted analysis with GelCompar II (version 1.5) software (Applied Maths, Kortrijk, Belgium) using 0.40% optimization and 1% position tolerance. The similarity of banding patterns was assessed using the Dice index and the unweighted pair group method with arithmetic average (UPGMA). For cluster analysis 80% of similarity was used (Abriouel et al., 2007; Manzano et al., 2009).

2.5. Antimicrobial susceptibility test

Minimal inhibitory concentrations (MIC) of vancomycin, gentamicin, tetracycline and clindamycin were determined for all strains according to the standard CLSI methodology (CLSI, 2006, 2007, 2009).

2.6. Statistical analysis

Statistical analysis was performed to evaluate associations between antimicrobial resistance profiles and decade of isolation, clusters by Rep-PCR and source of isolation. Association between toxigenic and antibiotic resistance profiles and decade of isolation and source of isolation were evaluated as well. Bivariate analysis was performed by x^2 test or Fisher's exact test with Epi Info (version 3.5.1).

3. Results

3.1. Lecithinase and hemolytic activities

The ability to degrade lecithin was widespread among foodborne *B. cereus* strains. Only seven strains lacked lecithinase activity. These strains were restricted to the 1980's (four strains) and the 1990's (three strains) and were isolated from five different kinds of food (wheat flower, black pepper, infant milk based formula, ice cream and ready-to-eat dishes).

The majority (79, 81.4%) of the strains were β-hemolytic. Ten strains presented a discontinuous pattern of β-hemolysis forming a ring at a distance of a few millimeters from the colony. As with the lecithinase negative strains, ᴅ-hemolytic strains were restricted to the 1980's (eight strains) and the 1990's (two strains). Only one strain was non-hemolytic. Seven strains were α-hemolytic and these strains were restricted to the 1980's.

3.2. PCR amplification of toxin genes and identification of toxigenic profiles

All PCR reactions generated fragments of the expected size. *entFM* was the most common gene and was detected in 94 strains (96.9%). The NHE complex was found in 82 strains (84.5%) positive for the three genes (*nheA*, *nheB* and *nheC*). Only three strains were negative for all three genes and 12 (12.4%) showed amplification products for one or two genes. HBL complex (*hblA*, *hblC* and *hblD*) was found in 61 (62.9%) strains. Thirty-five strains were negative for all three genes and one was negative only for *hblD*. All strains were negative for *ces*. The *cytK* gene was found in 44 (45.4%) strains in the PCR amplification performed according to Guinebretiere et al. (2002). When these same strains were submitted to the amplification protocol described by Yang et al. (2005) in order to discriminate between *cytK-1* and *cytK-2*, they were all positive for *cytK-2* gene.

Nineteen different toxigenic patterns were identified based on the amplification products generated by PCR with 14 of them being unique. The predominant pattern was type I (32, 33%) which included strains positive for all toxin genes but *ces*, followed by type II (24, 24.5%) which did not contain *cytK* and *ces*, type III (18, 18.6%) which contained only the NHE complex and *entFM* type IV (7, 7.2%) which did not contain HBL complex or *ces* genes and type V (2, 2.1%) which included strains negative for all toxin genes. Statistical associations between the predominant toxigenic patterns identified and decade of isolation or source were not observed.

3.3. Rep-PCR typing and computer assisted cluster analysis

Amplification of genomic DNA of *B. cereus* strains by Rep-PCR generated fragments ranging from 100 to 4000 bp. Altogether, 69 band profiles were observed with 53 represented by only one strain. This result indicates a high degree of genetic diversity among these strains. The computer assisted cluster analysis of Rep-PCR profiles using 80% similarity reveled seven main clusters: cluster 1 was the predominant (ten strains, nine of them showing 100% similarity) followed by cluster 2 (five strains with two pairs of strains with 100% similarity) and 3 (four strains presenting 100% similarity). Clusters 4, 5 and 6 comprised three strains each with 80% similarity. Eight additional clusters with two strains each were observed. Examples of the Rep-PCR profiles are shown in Fig. 1. The major Rep-PCR cluster, cluster 1, included strains isolated in the 1980's and the 1990's from different types of food (beef, chocolate pudding, infant milk based formula and spices). Among the strains belonging to Rep-PCR cluster 1, half exhibited toxigenic pattern II and the other half exhibited toxigenic pattern III. In addition, nine in ten strains were included in antibiotic resistance profile A. However, statistical analysis revealed that these associations were not significant (p values of 0.17 and 0.18 respectively).

Computer assisted cluster analysis of Rep-PCR profiles based on decade of isolation, source, hemolytic activity, toxigenic and antibiotic resistance patterns revealed a similar clustering pattern as that obtained by the analysis including all 97 strains.

3.4. Antimicrobial susceptibility

All strains tested were fully susceptible to gentamicin, with MICs ranging from 0.25 μg/mL to 2 μg/mL. Ten strains presented intermediate resistance to clindamycin with MICs ranging from 1 μg/mL to 2 μg/mL, whereas susceptible strains had MICs ranging from 0.25 μg/mL to 0.5 μg/mL. Resistance to tetracycline was predominant, with 11 strains showing intermediate resistance (MIC 8 μg/mL) and nine showing complete resistance (MICs ranging from 16 μg/mL to 128 μg/mL). Only one strain presented resistance to vancomycin with a MIC of 8 μg/mL. This strain was susceptible only to gentamicin and showed the highest MIC to tetracycline.

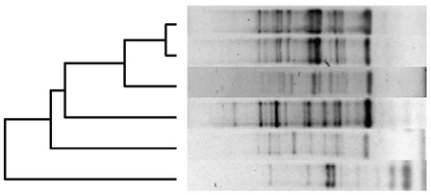
Intermediate resistance to clindamycin was statistically associated with strains isolated in 2000's ($p = 0.00048$, Fisher 2-tailed).

Antimicrobial resistance patterns were identified based on MICs results (Table 2). The predominant pattern was type A (72, 72.2%) which included strains susceptible to all drugs tested, followed by type B (10, 10.3%) comprising strains susceptible to vancomycin, gentamicin and clindamycin and intermediate to tetracycline; type C (5, 5.1%) with strains susceptible to vancomycin, gentamicin and tetracycline and intermediate to clindamycin and type D (5, 5.1%) with strains susceptible to vancomycin, gentamicin and clindamycin and resistant to tetracycline. Antimicrobial resistance patterns found were not associated with decade of isolation or type of food.

4. Discussion

In the present study, 97 *B. cereus sensu stricto* strains isolated during 1980's, 1990's and 2000's from different types of food were fingerprinted by Rep-PCR and evaluated for toxigenic potential and antimicrobial resistance.

Rep-PCR has been used in other studies to characterize *Bacillus* strains (Manzano et al., 2009; Reyes-Ramirez and Ibarra, 2005). In the present study we used primers specifically designed for Rep sequences found in the *Bacillus cereus* group. The cluster analysis of fingerprints generated by Rep-PCR revealed a high genetic diversity among the *B. cereus* strains, with



Source	Decade of isolation	Cluster
cumin	1980	1
chocolate pudding	1990	1
milk	1980	3
wheat flour	2000	U
chicken pie	1980	5
pudding powder	1980	4

Fig. 1. Genotypic profiles of representative *Bacillus cereus* isolates belonging to major Rep-PCR clusters. Dendrogram from computer-assisted analysis of Rep-PCR profiles obtained for *B. cereus* isolates using Dice index and the unweighted pair group method with arithmetic average (UPGMA). U, Unique pattern.

Table 2
Antimicrobial susceptibility patterns of *Bacillus cereus* strains as determined by minimal inhibitory concentration test.

Antimicrobial susceptibility pattern (n/%)	Antimicrobial agent			
	Van	Cli	Gen	Tet
A (72, 72.2)	S	S	S	S
B (10, 10.3)	S	S	S	I
C (5, 5.1)	S	I	S	S
D (5, 5.1)	S	S	S	R
E (3, 3.1)	S	I	S	S
F (1, 1)	S	I	S	I
G (1, 1)	R	I	S	R

S, susceptible; R, resistant; I, intermediate; Van, vancomycin; Cli, clindamycin; Gen, gentamicin; Tet, tetracycline.

only few clusters at 80% of similarity. This result is in agreement with others (Abriouel et al., 2007; Bartoszewicz et al., 2008; Cherif et al., 2007; Manzano et al., 2009). We could not observe predominant band patterns when the Rep-PCR cluster analysis was based on decade of isolation, suggesting that this diversity has been maintained over time. In a study using PFGE, the same genetic diversity among *B. cereus* strains was observed by others (Park et al., 2009). These results show that this diversity can be observed regardless of the technique applied.

We observed that strains isolated from the same type of food or carrying the same toxigenic profile or antimicrobial resistance pattern presented distinct Rep-PCR patterns. This suggests that the presence of toxin encoding genes or antimicrobial resistance determinants is not related to genetic background as, for instance, in species like *Staphylococcus aureus* (Vivoni et al., 2006).

Enterotoxin encoding genes were widely spread among the strains analyzed. According to current reports, the enterotoxins HBL and NHE are considered to be the major virulence factor of *B. cereus* related to food poisoning (Stenfors Arnesen et al., 2008).

Several studies reported *cytK* on being abundant among foodborne *B. cereus* (Brillard and Lereclus, 2004; Guinebretiere et al., 2002; Rosenquist et al., 2005). In the present study, the strains positive for *cytK* harbored the variant *cytK-2*. Although CytK-2 is not as toxic as *CytK*–1, its potential to cause disease cannot be underestimated (Fagerlund et al., 2004). The occurrence of *cytK-1* is rare and strains harboring this gene belong to a distant phylogenetic group comprising *Bacillus cytotoxicus* strains (Guinebretiere et al., 2010).

The most common toxigenic profile, type I, included strains possessing all known genes associated with enterotoxin production but *ces*. This result is in agreement with another study involving strains from Korea (Kim et al., 2009). In a study including strains isolated from food in Brazil, Aragon-Alegro et al. (2008) found that more than half of the strains contained NHE and HBL complexes and *cytK-2*. It shows that the occurrence of these genes in Brazilian *B. cereus* strains seems to be very common. In contrast with the 62.8% of strains found positive for *hbl* genes in this study, strains isolated from fermented food in Africa showed a very low frequency of these genes, only one in 26 strains (Abriouel et al., 2007).

Other publications report a lack of PCR amplification of one or two components of HBL or NHE complexes (Abriouel et al., 2007; Ankolekar et al., 2009; Kim et al., 2009; Wehrle et al., 2009). In the present study we observed the same problem for some strains. Since NHE and HBL are tripartite toxins, in both cases the three components are necessary to generate the active toxin (Beecher and MacMillan, 1991; Lindback et al., 2004). Thus, our results suggest the presence of non-functional combination or the occurrence of toxin-encoding gene polymorphism (Guinebretiere et al., 2002). Further studies are necessary to evaluate which factor is responsible for the lack of amplification observed for some components.

In this study, the *entFM* was found to be the most prevalent enterotoxin gene in foodborne *B. cereus* isolates. The FM-1 enterotoxin isolated from *B. cereus* has been shown to be cytotoxic to Vero cells, and the degree of its cytotoxicity depends on the bacterial strain (Boonchai et al., 2008; Hsieh et al., 1999). Tran et al. (2010) demonstrated that this toxin has cell wall peptidase activity implicated in adhesion, biofilm formation, and virulence.

Among the strains analyzed, hemolysis was a widely spread characteristic with 91.7% of the strains presenting β-hemolysis. Abriouel et al. (2007) reported that 100% of the strains isolated in Africa from fermented food were hemolytic. That percentage is slightly different from what we found. Curiously, 32 strains that were negative for the HBL encoding genes exhibited β-hemolysis, suggesting the production of other products with hemolytic activity such as sphingomyelinase (Oda et al., 2010).

MICs of vancomycin, gentamicin, tetracycline and clindamycin were determined for all strains in order to evaluate the antimicrobial resistance. Our results suggest that antimicrobial resistance is not a common feature among Brazilian *B. cereus* isolated from food. Resistance to at least one drug was found in 27.8% of the strains, most of them being resistant to tetracycline. Among the studies published in the literature that evaluate antimicrobial resistance in foodborne *B. cereus*, our work is in good agreement with Godic and Seme (2009) and Rosenquist et al. (2005) which determined the antimicrobial susceptibility by Etest and broth dilution test, respectively.

The broth dilution test methodology was chosen following the CLSI guidelines which reports that, at this time, recommendations for disk diffusion of *Bacillus* spp. cannot be made due to the limited data related to disk diffusion test for this genus (CLSI, 2006). However, the methodology chosen generated some difficulty in comparing our results with those described in the literature since most of the authors use only the disk diffusion technique (Ankolekar et al., 2009; Park et al., 2009; Roy et al., 2007). In addition, it led us to question the reliability of these data generated by disk diffusion technique since, up to now; the break points published by the CLSI for this technique do not include interpretative standards for the classification of *Bacillus* spp. strains as resistant, intermediate or susceptible.

Interestingly, intermediate resistance to clindamycin was statistically associated with strains isolated more recently (in the 2000's). Further studies involving contemporary strains would be useful to evaluate whether this is an emerging resistance pattern in Brazilian foodborne *B. cereus* strains.

To our knowledge, this is the first report presenting molecular and toxigenic characterization and antimicrobial resistance profiles of foodborne *B. cereus* strains isolated in Brazil that covers a period of three decades. We acknowledge that the analysis of strains known to be involved in cases of food poisoning would bring valuable information about Brazilian *B. cereus* strains. However, such strains are extremely difficult to recover since food poisoning cases in Brazil are not monitored or reported.

Since the strains analyzed in the present study came exclusively from food surveys, our results suggest that the majority of the strains present in several types of food in Brazil pose a potential risk to cause food poisoning due to the high prevalence of toxin genes found in these strains. Cytotoxicity tests and affiliation to phylogenetic groups based on molecular data from fluorescent amplified fragment length polymorphism patterns in association with ribosomal gene sequences have been recently proposed as methodologies to provide a more accurate indication of the risk of *B. cereus* strains (Guinebretiere et al., 2010). The application of these methodologies to the strains analyzed in this work could generate additional data and contribute to better evaluate risk potential of such strains.

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