



# Genetic and toxigenic diversity of *Bacillus cereus* group isolated from powdered foods

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Revised: 13 July 2020 / Accepted: 6 August 2020 / Published online: 18 August 2020  
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**Abstract** *Bacillus cereus* is a human pathogenic bacterium that produces emetic and diarrheal foodborne diseases. This study evaluated the genetic and toxigenic diversity in *B. cereus* group isolates from powdered foods collected in public educational institutions, bakeries and powdered food companies located in Medellín, Colombia. *B. cereus* was detected in 35 of 305 (11%) powdered food samples and 52 *B. cereus* were isolated. The presence of ten toxin genes, *hblCDAB*, *nheABC*, *cytK2*, *entFM* and *cesB*, was evaluated in the isolates by multiplex PCR. The *nheABC* operon was found in all isolates (100%), *hblCDAB* in 22 (42%), *hblCDA* in 8 (15%) and *hblCD* in 3 (6%); the *cytK2* gene was detected in 32 isolates (62%) and *entFM* in 32 (62%). Notably, the *cesB* gene was not detected. According to the presence of toxin genes, fifteen profiles were identified. The predominant toxigenic profile contained all toxin genes but *cesB*. A large genetic diversity was observed by GTG<sub>5</sub> fingerprinting with 46 isolates grouped in seven clusters and the remaining six clustering individually. There was no relationship between toxigenic profiles and genetic clusters, but some genetic clusters seemed to be related to particular powdered food types. In general, the

results evidenced high genetic and enterotoxigenic diversity among the *B. cereus* group isolates.

**Keywords** Toxin genes · Infant formula · Powdered milk · Wheat flour · Corn starch · GTG<sub>5</sub> fingerprinting

## Introduction

The *Bacillus cereus* group or *Bacillus cereus sensu lato* (*s.l.*) comprises nine species characterized by a high degree of genetic similarity, *B. cereus sensu stricto* (*s.s.*), *Bacillus thuringiensis*, *Bacillus mycoides*, *Bacillus pseudomycooides*, *Bacillus anthracis*, *Bacillus weihenstephanensis*, *Bacillus cytotoxicus* and *Bacillus toyonensis* (Jimenez et al. 2013), and the recently identified *B. wiedmannii* (Liu et al. 2017). These Gram-positive, spore forming, human pathogens are naturally found in soil. Therefore, soil contaminated food products carrying these bacteria constitute risk factors for foodborne infection or intoxication, mostly because their spores can survive cooking or pasteurization processes (De Jonghe et al. 2008).

*Bacillus cereus* causes two types of foodborne diseases, an emetic syndrome and a diarrheal syndrome (Stenfors-Arnesen et al. 2008). The emetic syndrome is caused by emetic toxin, cereulide, produced by a nonribosomal peptide synthetase encoded by the polycistronically transcribed cereulide synthetase (*ces*) gene cluster, located on a megaplasmid (Rajkovic et al. 2008). The diarrheal syndrome is caused by one or a combination of three enterotoxin types, hemolysin BL (HBL) encoded by the *hblCDA* or *hblCDAB* operons, non-hemolytic enterotoxin (NHE) encoded by the *nheABC* operon (Granum et al. 1999), and the cytotoxin K encoded by the *cytK* gene, a single protein similar to the  $\beta$ -toxin of *Clostridium perfringens* (Logan

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2012). Two variants have been described to *cytK* gene, *cytK1* gene encoding the more toxic variant of the CytK toxin while *cytK2* gene, is the most common variant and least toxic (Stenfors-Arnesen et al. 2008). HBL and NHE toxins are both tripartite proteins. HBL contains two lytic components L2 and L1, and a binding component B, encoded by *hblC*, *hblD* and *hblA*, respectively (Ryan et al. 1997). In addition, an open reading frame, *hblB*, according to sequence analysis, was generated by duplication of *hblA* gene and is considered a pseudogene (Stenfors-Arnesen et al. 2008). NHE also contains a cytolytic protein NheA and two binding components NheB and NheC, encoded by *nheA*, *nheB* and *nheC*, respectively (Fagerlund et al. 2008). In addition, most *B. cereus* strains are potential producers of enterotoxin FM (EntFM), codified by the *entFM* gene (Stenfors-Arnesen et al. 2008).

Studies in distinct geographic locations showed high incidences of enterotoxin genes in *B. cereus s.l.* strains isolated from foods, as marketed food products, rice and spices, respectively (Fogele et al. 2018; Kim et al. 2014; Samapundo et al. 2011). A few studies have been performed in Colombia to identify toxin genes of *B. cereus s.l.* in DNA extracted directly from powdered foods, and the results showed that the enterotoxin genes were by far, more frequent than the emetic gene (Sánchez et al. 2014a, b). Knowledge about the genetic and toxigenic diversity of the *B. cereus s.l.* isolates contributes important epidemiological information, such as the differentiation of their clinical potential, finding of contamination sources, tracking isolates along the food chain and define strains distribution within foods (De Jonghe et al. 2008). Furthermore, *B. cereus* toxin genes have been detected in Colombia where it causes one third of the total foodborne illnesses (INS 2011). Therefore, the aim of this study was to evaluate the genetic and toxigenic diversity of *B. cereus s.l.* isolated from infant formula, flours and powdered milk in Medellín, Colombia.

## Materials and methods

### Food samples and *B. cereus s.l.* isolation

The samples evaluated were powdered food products collected in six public educational institutions, seven bakeries and two powdered food companies located in Medellín, Colombia (designated as A to O). They included, infant formula (n = 75), powdered milk (n = 75), wheat flour (n = 79) and corn starch (n = 76). *B. cereus* isolation from powdered foods was performed according to ISO 7932:2004, for the enumeration of presumptive *B. cereus* cells (ISO 2004). Briefly, 25 g of sample were dissolved on 225 ml of peptone water and serial dilutions were

performed ( $10^{-1}$ – $10^{-3}$ ); a 100 µl sample from each dilution was spread on mannitol egg yolk polymyxin agar (MYP) (Merck Millipore, Darmstadt, Germany) using a sterile triangular cell spreader, and performed in triplicates in order to reduce experimental error, followed by incubation at 37 °C for at least 48 h. The colonies obtained were counted in plates that showed growth of 30–300 CFUs. Presumptive colonies were analyzed using biochemical tests, according to standard protocols that included catalase, motility, Voges-Proskauer reaction, glucose, xylose and arabinose utilization; starch, casein and gelatin hydrolysis; β-hemolytic activity on blood agar plates. Positive colonies to catalase, motility, Voges-Proskauer reaction, glucose utilization, β-hemolytic activity and, starch, casein and gelatin hydrolysis were considered *B. cereus s.l.* The isolates were preserved in cryotubes containing Tryptic Soy Broth and 15% (v/v) glycerol, and stored at – 80 °C. Emetic *B. cereus* NVH 1257 and F4810/72 and enterotoxigenic *B. cereus* ATCC 14579 and ATCC 10987 served as reference strains for phenotypic and molecular assays, and were kindly provided by Dr. Niels Hendriksen and Dr. Per E. Granum. All strains were kept as glycerol stocks at – 80 °C until required.

### DNA extraction and toxin gene determination among *B. cereus s.l.* isolates

Bacteria strains were cultured in Luria–Bertani (LB) medium (Difco, Detroit, Mich., USA) at 37 °C overnight for DNA extraction (D'Alessandro et al. 2007). DNA concentration and purity were checked in a NanoDrop 2000 UV/VIS spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA). The DNA was stored at – 20 °C for the following experiments. The *B. cereus* isolated from powdered foods were screened for the presence of the genes that encode the toxins cereulide (*cesB*), non-hemolytic enterotoxin (*nheA*, *nheB*, and *nheC*), hemolytic BL (*hblC*, *hblD*, *hblA*, and *hblB*), enterotoxin FM (*entFM*), and cytotoxin K (*cytK2*) by three multiplex PCR assays according to previously described methodology (Sánchez et al. 2020).

### *Bacillus cereus s.l.* (GTG)<sub>5</sub> fingerprinting

The *B. cereus* group isolates from powdered foods were analyzed to establish their genetic diversity according to amplification of repetitive element palindromic based-polymerase chain reaction (rep-PCR) using the single (GTG)<sub>5</sub> primer (De Jonghe et al. 2008). Briefly, a total reaction volume of 20 µl containing 0.6 µM (GTG)<sub>5</sub> primer, 200 µM dNTPs mix, 3.5 mM MgCl<sub>2</sub>, 1.6 U of *Taq* DNA polymerase (Thermo Fisher Scientific) and 50 ng of DNA template. PCR amplifications were performed under

the following conditions, an initial denaturation of 5 min at 94 °C, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 45 °C for 1 min and elongation at 65 °C for 8 min, and a final extension at 65 °C for 16 min. Amplification was carried out in a DNA thermocycler MyCycler (Bio-Rad, California, USA). PCR products were electrophoresed in a 1.5% (w/v) agarose gel (Amresco, Ohio, USA) with ethidium bromide for 2.5 h at a constant voltage of 120 V in 1X TBE buffer (0.1 M Tris base, 0.09 M boric acid, 0.01 M EDTA, pH 8.4), at 4 °C. The gene ruler 1 Kb DNA ladder (Thermo Fisher Scientific) was used as a size standard. The rep-PCR profiles were visualized under ultraviolet light, followed by digital capture with UVP Gel Doc system (UVP, California, USA). The resulting fingerprints were analyzed using the BioNumerics 6.6 software package (Applied Maths Inc. St Martens, Belgium), with 1% optimization and 1% position tolerance. Genetic similarity among digitized profiles was calculated using the Pearson correlation coefficient and the Unweighted Pair Group Method with Arithmetic average (UPGMA) was used to obtain a dendrogram of the profiles, using a 94% similarity value for cluster analysis.

## Results and discussion

### Incidence of *B. cereus* s.l. in powdered foods

The *B. cereus* count was below  $10^3$  cfu/g or ml in all powdered food samples, therefore, these foods are considered safe for human consume. *B. cereus* were detected in 35 of 305 (11%) powdered food samples and 52 *B. cereus* group isolates were recovered from these samples. The *B. cereus* incidence in powdered foods was similar, in powdered milk was 13%, followed by corn starch (12%), infant formula (11%) and wheat flour (10%). Although, infant formula only presented an 11% incidence, the highest number of *B. cereus* isolates (19) was recovered from this powdered food (Table 1).

The incidence of *B. cereus* s.l. in food products greatly vary; compared to those reports, the *B. cereus* incidences in powdered foods found in this study, are lower. For

example, higher incidence was found in a Korean study that reported incidence of 78.3% *B. cereus* in rice samples (Kim et al. 2014). Variable *B. cereus* s.l. incidences were reported in a study carried out in food products marketed in Belgium (5% to 100%), such as rice, sauces, lasagna and cooked pasta (Samapundo et al. 2011). In Scotland, *B. cereus* s.l. were reported varying within 20–88% in soil, feces, raw and processed vegetables (Altayar and Sutherland 2006). In studies accomplished in Korea, Iran and New Zealand, *B. cereus* was identified in 40% of dried foods as raw rice (Jang et al. 2006); infant foods (Rahimi et al. 2013) and dehydrated potato (King et al. 2007), respectively. The results of the above studies are higher to the ones of the present work in which a low *B. cereus* incidence was found in powdered milk, corn starch, infant formula and wheat flour samples. A possible reason for this low incidence of *B. cereus* s.l. in these powdered foods is the low humidity present in these samples that prevents microbial growth and only sporulating bacteria can survive in these food types.

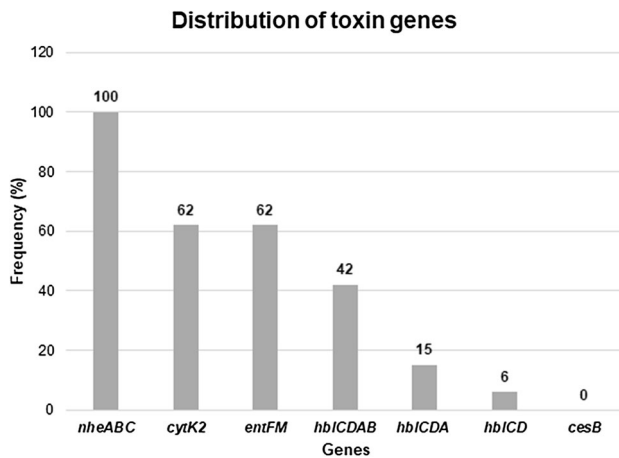
### Distribution of toxin gene profiles among *B. cereus* s.l. isolates

A total of 52 *B. cereus* s.l. were isolated from powdered foods. The distribution of toxin genes was as follows, the three genes of the *nhe* operon were detected in the 52 *B. cereus* s.l. (100%). All four genes of the *hbl* operon were found in 22 isolates (42%). The *hbl* operon missing the *hblB* gene in eight isolates (15%); notably, three isolates lacked the *hblAB* genes, indicating that these strains would not produce a functional hemolysin BL. The *cytK2* and *entFM* genes were found in 32 isolates (62%) (Fig. 1). None of the 52 *B. cereus* group isolates contained the *cesB* gene.

These results showed that the enterotoxin genes were widely distributed among the *B. cereus* s.l. isolated from powdered foods. The *nhe* operon was highly conserved, with 100% of *B. cereus* harboring all three genes (*nheABC*) that encode the non-hemolytic enterotoxin. In general, studies have reported that *B. cereus* strains isolates from foods and soil carried all three genes of the *nhe* operon

**Table 1** Incidence of *B. cereus* s.l. in powdered food samples

Product	No. of analyzed samples	No. of contaminated samples with <i>B. cereus</i> group (%)	No. of <i>B. cereus</i> isolates
Infant formula	75	8 (11)	19
Powdered milk	75	10 (13)	12
Wheat flour	79	8 (10)	9
Corn starch	76	9 (12)	12
Total	305	35 (11)	52



**Fig. 1** Distribution of toxin genes among *B. cereus s.l.* isolates from powdered foods

(Chaves et al. 2011; Kim et al. 2014; Ngamwongsatit et al. 2008). All this information and evidence from bioinformatic analysis suggest that the *nhe* operon is highly conserved, because of the vertical inheritance, which is possibly caused by a second, unknown but fitness relevant function of *nhe* operon (Böhm et al. 2015).

Regarding the *B. cereus s.l.* isolates that carried the *hblCDAB* operon, a wide genetic diversity was observed. The complete operon (*hblCDA*) was detected in 30 *B. cereus* isolates (57%), from which 22 isolates (42%) harbored the *hblB* pseudogene. Three *B. cereus s.l.* isolates (6%) carried only the *hblCD* genes. Similar *hbl* operon frequencies have been previously reported, describing that *hblCDA* operon is generally present in 45–65% of *B. cereus* strains (Thaenthanee et al. 2005). Studies carried out in Denmark and Brazil reported that 50–70% of *B. cereus* isolates harbored *hblCDA* operon (Chaves et al. 2011; Hansen and Hendriksen 2001). Higher frequencies (84–100%) of *B. cereus* strains carrying the *hbl* genes were reported in studies accomplished in Belgium (Samapundo et al. 2011) and Korea (Kim et al. 2014). Additionally, it has been found that 43% of *B. cereus* environmental/food strains carried *hbl* operon, as isolated strains in this study, while that a higher proportion of clinical strains (81%) harbored the *hbl* genes (Ghelardi et al. 2007). Previous studies have reported the lack of one or two components of the *hbl* operon (Kim et al. 2009; Wehrle et al. 2009). Similarly, in some isolates of the present study, the *hblAB* genes were absent; this polymorphisms of the *hbl* operon is suggestive of an inactive hemolysin BL in these isolates. Polymorphism of enterotoxin genes has been observed in other food-related *B. cereus* isolates (Chaves et al. 2011; Guinebretière et al. 2002). Further studies will be necessary to evaluate which factor(s) is/are responsible for the observed lack of *hbl* operon components.

The *cytK2* gene was found in 62% of *B. cereus s.l.* isolates. These results and those of other studies reporting frequencies varying within 45.4%–88.81% (Chaves et al. 2011; Guinebretière et al. 2002; Ngamwongsatit et al. 2008; Samapundo et al. 2011) indicate that *cytK2* gene is widely spread among the *B. cereus s.l.* strains. Similar to *cytK2* gene, *entFM* gene was found in 32 isolates (62%), and results obtained previously in other studies suggest that *entFM* gene frequency and distribution among *B. cereus s.l.* strains varies greatly (45 to 100%) (Carter et al. 2018; Kim et al. 2009; Osman et al. 2018; Rahimi et al. 2013).

On the other side, none of the 52 *B. cereus s.l.* isolates carried the *cesB* gene required for the production of emetic toxin cereulide. *B. cereus* emetic strains are not frequently found (Altayar and Sutherland 2006), as shown in other studies in which the *ces* gene is not detected; for example in *B. cereus* strains from Brazil (Chaves et al. 2011). However, in Argentina a highly cytotoxic *B. cereus* strain isolated from cooked chicken harbored the *ces* gene (López et al. 2015), and in Korea was also reported *B. cereus* emetic strain isolated from rice (Kim et al. 2014). Notably, emetic strains have been isolated mainly from rice dishes, rice paddy fields and its processing environment in Asian countries, which suggests an association between *B. cereus* emetic strains and rice (Logan 2012).

According to the presence or absence of the toxin genes, *hblCDAB*, *nheABC*, *cytK2*, *entFM* and *cesB*, in 52 *B. cereus s.l.* isolates from powdered foods, fifteen different toxin gene profiles were identified (Table 2). Regarding the toxin gene profiles, 22% of the isolates (11 strains) had the profile with all genes for hemolysin BL, non-hemolytic enterotoxin, cytotoxin K and enterotoxin FM, and 18% of them had the *hblB* pseudogen (profile I), while in the rest, 4% (2 *B. cereus*) it was absent (profile II). This result is agreement to results obtained in other studies, which toxin gene profiles that included all enterotoxin genes were the most frequent profiles (Chaves et al. 2011; Kim et al. 2009; Ngamwongsatit et al. 2008; Samapundo et al. 2011). These results mean that only diarrheal syndromes can be caused by *B. cereus s.l.* isolated here, but more studies are needed to know if these strains can generate all toxins simultaneously to cause food poisoning.

### (GTG)<sub>5</sub> fingerprinting

Evaluation of the genetic diversity of *B. cereus s.l.* isolated from powdered foods by (GTG)<sub>5</sub> genotyping showed that 46 isolates grouped into seven clusters and six clustered individually (Fig. 2). Interestingly, a relationship was found between particular clusters and specific powdered foods; for example, cluster 1 only contained isolates from infant formula, cluster 3 from corn starch, cluster 5 from wheat flour and cluster 7 from powdered milk. Regarding

**Table 2** Toxin gene profiles observed in 52 *B. cereus s.l.* isolates from powdered foods

Profile	Hemolysin BL complex				Non-hemolytic enterotoxin complex			Cytotoxin K <i>cytK2</i>	Cereulide <i>cesB</i>	Enterotoxin FM <i>entFM</i>	Total (%) <i>n</i> = 52
	<i>hblC</i>	<i>hblD</i>	<i>hblA</i>	<i>hblB</i>	<i>nheA</i>	<i>nheB</i>	<i>nheC</i>				
I	+	+	+	+	+	+	+	+	–	+	9 (18)
II	+	+	+	–	+	+	+	+	–	+	2 (4)
III	+	+	–	–	+	+	+	+	–	+	1 (2)
IV	+	+	+	+	+	+	+	+	–	–	7 (13)
V	+	+	+	–	+	+	+	+	–	–	2 (4)
VI	+	+	–	–	+	+	+	+	–	–	1 (2)
VII	+	+	+	+	+	+	+	–	–	+	5 (9)
VIII	+	+	+	–	+	+	+	–	–	+	2 (4)
IX	+	+	–	–	+	+	+	–	–	+	1 (2)
X	–	–	–	–	+	+	+	+	–	+	7 (13)
XI	+	+	+	+	+	+	+	–	–	–	1 (2)
XII	+	+	+	–	+	+	+	–	–	–	2 (4)
XIII	–	–	–	–	+	+	+	+	–	–	3 (6)
XIV	–	–	–	–	+	+	+	–	–	+	5 (9)
XV	–	–	–	–	+	+	+	–	–	–	4 (8)

+ = PCR product of the expected size was formed (gene is present)

– = no PCR product was formed (gene is absent)

toxigenic profiles and genetic clusters, there were predominant toxigenic profiles in some clusters as clusters 2 (profile X) and 3 (profile I); however, the various toxigenic profiles were distributed among the 52 *B. cereus* group isolates. This observation indicates that the toxigenic profile is not related to the genetic background, as previously suggested (Chaves et al. 2011; Lee et al. 2012).

No association was found between cluster and place of powdered food collection, but there was a relationship between cluster and powdered food type. Notably, cluster 3 was formed by eight *B. cereus* isolates from corn starch exclusively, however five isolates carried all enterotoxin genes (profile I), and were isolated from a special corn starch used to cook Colombian buñuelos, which could indicate a contamination by a particular strain in this type of corn starch. Additionally, in cluster 2 the majority of *B. cereus s.l.* harbored the toxigenic profile X and were isolated from infant formula in different institutions, this may indicate a systematic contamination due to this particular powdered food is manufactured by the same factory.

In other studies, using (GTG)<sub>5</sub> PCR fingerprinting, a relationship was observed between clusters and sample source. In South Africa, from 49 *B. cereus* strains obtained from extended shelf life, raw and pasteurized milk and filler nozzles, three groups comprised isolates exclusively from filler nozzles while other three groups were isolates from filler nozzles, raw and pasteurized milk (Mudagza and Buys 2017). In other study in Belgium, 61 of 80 *B. cereus*

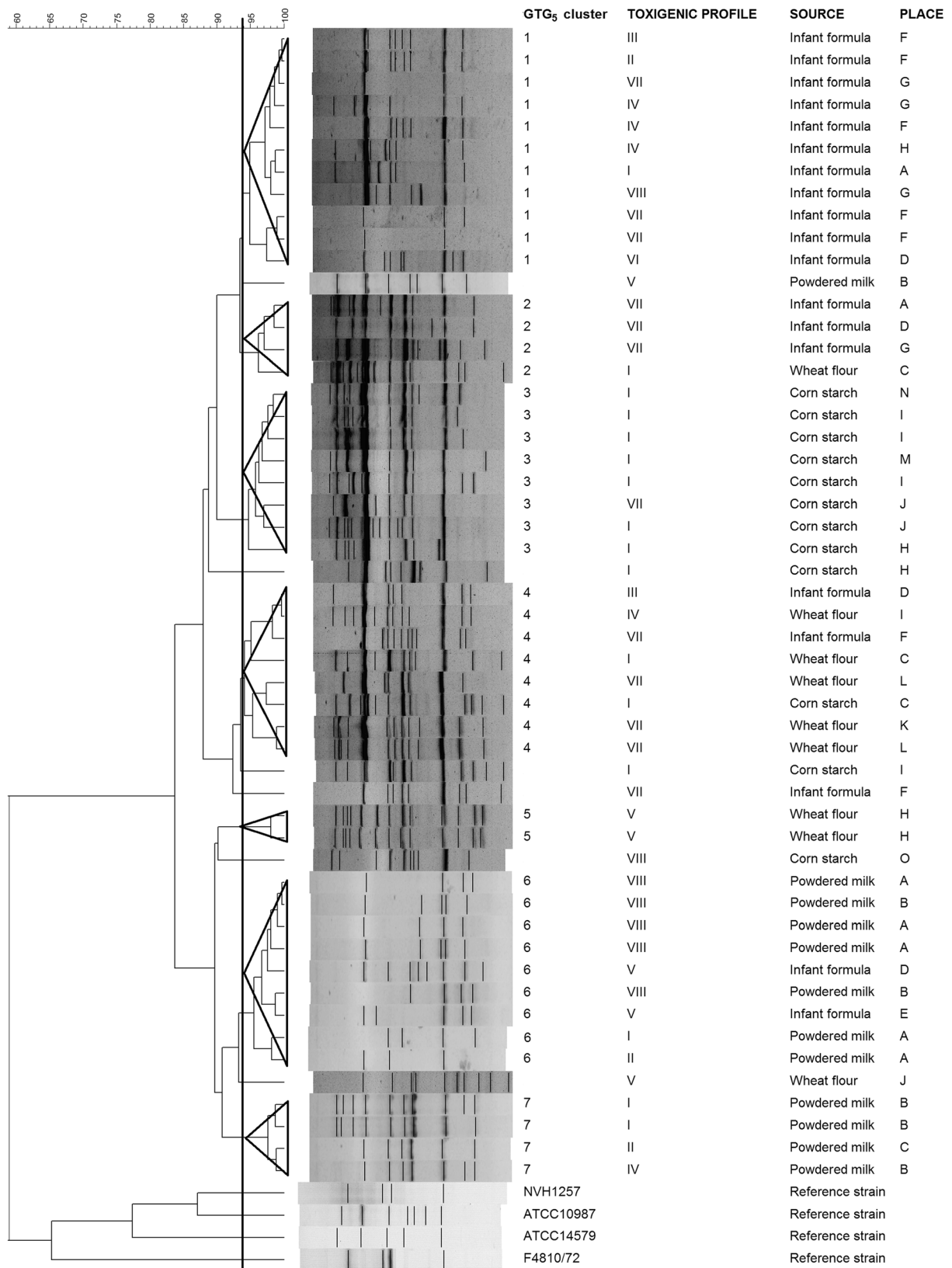
group isolated from food products marketed formed 15 distinct clusters and the remaining 19 were each clustered separately, the cluster 2 harbored mainly *B. cereus* group isolates from lasagna and bolognese sauce, and cluster 6, 7 and 11, isolates from raw basmati rice, lasagna and béchamel sauce, respectively (Samapundo et al. 2011).

In general, the (GTG)<sub>5</sub> fingerprint results revealed a high diversity among the 52 *B. cereus* group isolates obtained from infant formula, powdered milk, wheat flour and corn starch. The large genetic diversity obtained is similar to the high genetic diversity detected in Brazil among 97 foodborne *B. cereus s.s.* strains collected during three decades, which were grouped in 15 clusters (Chaves et al. 2011). Although a high genetic diversity was detected in *B. cereus s.l.* isolates from powdered foods, it was achieved to identify some contamination sources related to food type or to the producing food factory. This information contributes to the assessment of food safety/risk to aid decision-making in public health in Colombia.

## Conclusion

The results indicate that *B. cereus s.l.* isolates obtained from powdered foods show a potential risk to cause diarrheal-type food poisoning as most of them contained all enterotoxin genes. According to *B. cereus s.l.* genetic diversity, no correlation was found between the genetic





**Fig. 2** Genetic diversity of the *B. cereus* s.l. isolates from powdered foods. Dendrogram from (GTG)<sub>5</sub> fingerprints using Pearson correlation coefficient (94% indicated by bold vertical line) and UPGMA

background and toxigenic profiles. However, the results suggest an association between *B. cereus s.l.* genetic background and food type, indicating that (GTG)<sub>5</sub> fingerprinting is useful to identify contamination source in foods. Data on the incidence, toxigenic profiles and genetic diversity for *B. cereus s.l.* isolated from powdered foods constitute useful information that provide the bases for the assessment of the potential risk of this pathogen to cause food poisoning; it also contributes to a better understanding of the epidemiology of *B. cereus s.l.* in powdered food products.

**Acknowledgements** This research was funded by the Department of Science, Technology and Innovation -Colciencias- (Grant No. 386) and Committee for Research Development -CODI- at the University of Antioquia, Colombia.

#### Compliance with ethical standards

**Conflict of interest** The authors declare no competing financial interest.

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