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For: Journal of Clinical Microbiology

Running title: *Food poisoning power and B. cereus phylogeny*

Keywords: food poisoning power, cytotoxicity, Toxins, *Bacillus cereus* Group, phylogenetic groups, food safety

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ABSTRACT

Cytotoxic activity levels of culture filtrates and toxin distribution varied according to the phylogenetic group (I-VII) inside the *B. cereus* Group, suggesting that these groups are of different clinical significance and are more suitable than species affiliation for determining food poisoning risk. A first-line, simple online tool (https://www.tools.symprevius.org/Bcereus/) to assign strains to the different phylogenetic groups is presented.

The *B. cereus* Group (*B. cereus sensu lato*) can induce diarrhea by producing heat-labile enterotoxins during growth in the small intestine (8) and emesis by producing a single heat-stable peptide toxin called cereulide (1). A suitable test for the detection of emetic toxin producers is available (7). For diarrhoeal syndrome, three enterotoxins have been described: the cytotoxin CytK (17), the nonhaemolytic enterotoxin Nhe (9) and the haemolysin HBL (2). Also, other factors may have a role in certain isolates. A way of evaluating the enterotoxicity produced by bacterial strains is to test cytotoxicity of culture filtrates on cell lines such as Vero cells (18) or Caco2 cells (4) which encompass all soluble factors.

Assessing the food poisoning power of *B. cereus sensu stricto* and other species of the Group (*B. thuringiensis*, *B. mycoides*, *B. pseudomycoides*, *B. weihenstephanensis* and *B. anthracis*) may not be feasible because they are not separate genomic species, some being distributed between few distinct phylogenetic groups (10), some other both belonging to a same phylogenetic groups (Table 1A). In contrast, the seven phylogenetic groups described in Table 1A covers the entire *B. cereus* Group (*sensu lato*), are based on genetic delimitation (10) and represent a more homogeneous basis to assess the food poisoning power. The aim of the present study was to determine food poisoning toxins and global cytoxicity associated with each of the seven phylogenetic groups to evaluate their food poisoning power.

A total of 391 independent strains (10) belonging to the *B. cereus* Group were used to determine presence of known toxin genes. In a first step total DNA was extracted for each strain as previously described (13). Detection of *hbl* genes and *nhe* genes was performed by PCR and Southern blots (to check false negative responses) as previously described (11). New primer pairs were also designated to detect *nhe* genes in the two most distant groups (I and VII), taking into account the sequence polymorphism observed between the sequence of genome NVH 391-98 (group VII) and that of other sequenced genomes (ATCC 14579, E33L, Ames, ATCC 10987 and KBAB4): $^{5'}$GTAAATGCTGCVGATAGYCAAAC$^{3'}$ / $^{5'}$GGCATV ATRTTYCCTGCTGC$^{3'}$ and $^{5'}$GGTTCRAAYGCTTTAGTAATGG$^{3'}$ / $^{5'}$ATTCCWGCRTCTTGACTAGC$^{3'}$, targeting *nheAB* genes and *nheB* gene respectively. Strains carrying the *cytK* gene and its two polymorphic forms *cytK-1* and *cytK-2* were detected using the duplex PCR (validated as without false positive or false negative response) described previously (12). Cereulide producers were detected using *ces* gene-specific PCR (7).

A subset of 97 strains, belonging to the different phylogenetic groups (II to VII), was characterized for cytotoxicity activity of culture filtrates. Cytotoxic activity was measured on Caco2 cells as described

previously (4). The Caco2 cell viability (*V)* was measured by DO_{620nm}, in % of the total viability (100% viability was DO_{620nm} obtained for Caco2 cells treated with non-inoculated BHI instead of culture filtrates), for serial 2-fold diluted culture filtrates (D = 0 to 1/32). The non-linear regression ($V = f(D)$) was obtained from duplicate values of the six tested dilutions. This curve and the *D* value for which *V* was 50% (SC50) were calculated using the GraphPad Prism software (GraphPad software incorporated, San Diego, USA). When no cytotoxic activity was recorded, SC50 was approximated to 1 to allow calculations. The cytotoxicity activity (CA) of culture filtrates was expressed as CA = 1-SC50. All the strains were tested in the course of two independent experiments.

Fisher's test was used to determine whether bacterial populations were significantly distant for the tested character, using the XLSTAT 2008 software package.

As two emetic strains (20) have been recently associated with other groups than the two usually known lineages (6) (21), we examined the distribution of the cereulide-producing strains over all seven phylogenetic groups. In our study, cereulide producers (7), were found only in group III, in subgroup III-2 containing the emetic strain F4810 and subgroup III-3 (Table 1B), which correspond to the known emetic lineages. None of the 137 tested strains in group VI (*B. thuringiensis* VI, *B. weihenstephanensis* and *B. mycoides*) carried the *ces* gene, which suggests that emetic *B. weihenstephanensis* strains, first observed by Thorsen *et al.* (20), are probably extremely rare.

Nhe genes were carried by 100% of the tested strains in each group and subgroup (Table 1B), in accordance with data from available sequenced genomes affiliated to the *B. cereus* Group (82 sequenced genomes). We can therefore now definitively assert that *nhe* genes are a constant part of the *B. cereus* Group strains. In contrast, *hbl* genes frequency varied from 40% to 97% between the phylogenetic groups I, II, IV, V and VI, and this operon was seldom carried by strains of the phylogenetic group III, particularly in subgroups III-2 and III-3 containing emetic strains and III-4 containing the *B. anthracis* strains (Table 1B). Thus rarity of *hbl* genes is not a specificity of the 'emetic' lineages as previously published (6) but also concern other sub-groups in the phylogenetic group III (III-4). As for *hbl* genes, the distribution of *cytK* gene was disrupted. However, it showed some interesting specificities (Table 1B) : *CytK-1* form was specific of the most distant group VII '*B. cytotoxicus*' (versus the 387 strains tested from the other groups); *cytK-2* form was particularly frequent in mesophilic groups III and IV (except for the 12 tested *B. anthracis* strains that

were negative in III-4 sub-group) whereas it was rare or absent in the psychrotolerant or moderately psychrotolerant groups (VI, II and V). *CytK* gene was also absent in group I strains.

The disrupted distribution of *hbl* and *cytK* genes is in accordance with what is observed from available sequenced genomes. It opens the way to new hypothetical mechanisms for spread history of enterotoxins, such as horizontal gene transfers (HGT) in the *B. cereus* Group. This is coherent with traces of HGT previously observed on *B. cereus* genome around *hbl* genes (14) or with existence of plasmids carrying enterotoxin-like genes (15, 16)**.** On this assumption, some phylogenetic groups might have failed to acquire *cytK* or *hbl* (for example group VI for *cytK* gene or sub-group III-2 for *hbl*) because they occupy very specific thermal niches unfavorable to cohabitation and exchange of genetic material with other phylogenetic groups.

Low or no cytotoxic activity of culture filtrates was recorded for all the strains of group VI and subgroup III-3 (Fig. 1). By contrast, higher cytotoxic activity ($p < 0.001$) was recorded for strains of the other groups (Fig. 1a), reaching very high values for a great number of strains in group III. Cytotoxic activity was heterogeneous in group III (Fig. 1b), with high cytotoxic activity in groups III-1 and III-2 and very low cytotoxic activity in subgroup III-3 ($p < 0.0001$). In addition, the cytotoxicity recorded for strains of subgroup IV-2 (Fig. 1b) was higher than for subgroups IV-1 and IV-3 ($p < 0.05$) in group IV. Finally, strain 391-98, representing group VII (‘*B. cytotoxicus’*), exhibited a high level of cytotoxic activity (mean = 0.75).

The enterotoxic potential conferred by each phylogenetic group, independently of species affiliation, has implications for health risk. Based on the frequency of highly enterotoxic strains (generally associated with diarrheal syndrome) recorded in each group, food poisoning risk should be the highest for group III (containing *B. cereus III*, *B. thuringiensis III* and *B. anthracis like strains*), particularly subgroups III-1, III-2 and III-4. Sub-groups III-2 and III-3 may be also dangerous for their emetic potential. This risk remains high for groups VII, IV and II, decreases with group V and seems very low for group VI. Strains belonging to this last group should be the safest with regard to diarrheal syndrome. This is consistent with the absence of strains isolated from foodborne disease in this group (10). Concerning emetic syndrome, the possible production of emetic toxin by some group VI strains, although presumably very rare, needs to be clarified. As *B. weihenstephanensis* and *B. mycoides* strains are delimited into group VI, we can also consider these two species as representing a low level of risk, on condition that they are reliably identified. For *B. cereus*

sensu stricto and *B. thuringiensis* species, the level of risk is impossible to determine without taking into account phylogenetic affiliation of strains (I to VII), as they can belong to the phylogenetic groups II to VI and thus reflect several different levels of food poisoning risk.

In conclusion, affiliation to a phylogenetic group provides a more accurate indication of the risk than affiliation to a current species of the *B. cereus* Group. Associating the phylogenetic number (I-VII) to the species name when identifying new isolates offers a first useful indicator of risk. The challenge is thus to propose reliable methods for identifying the seven phylogenetic groups.

As shown in this study, the validated duplex-PCR method (12) permits to identify *cytK-1*-carrying strains specific of the rare and hazardous group VII ('*B. cytotoxicus*'). A rapid method to assign bacterial isolates to groups I-VII is to compare *panC* gene sequence from new isolates with those of reference strains published in Guinebretiere *et al.* (10). For this purpose, we propose the online tool at the following link: https://www.tools.symprevius.org/Bcereus/english.php. As *panC* gene sequence was previously shown to reliably delimit the phylogenetic groups and sub-groups (10), this tool allows a rapid and reliable identification (100% exact responses) at the phylogenetic group level (I-VII). It compares a query sequence with those in a database (85 *panC* sequences for which the phylogenetic affiliation is known). Protocols and homology search algorithm are available from the site.

This phylogenetic approach takes a first step towards evaluating the potential risk associated with the *B. cereus* Group strains. It can in the future be extended by the presence or production of pathogenic markers. To date, the *ces* gene is the most relevant marker for the emetic syndrome. The toxins Nhe and HlyII have been directly or indirectly linked to the diarrheal syndrome (3, 19), but they do not yet explain the pathogenicity of all strains. Some new associated-virulence factors (5) have not been evaluated as pathogenic makers yet, and some other are doubtlessly still unknown. Further knowledge of virulence-associated factors secreted by the *B. cereus* Group strains, is thus currently needed for a more accurate detection of food poisoning strains.

ACKNOWLEDGMENTS

We thank the "Direction Generale de l'Alimentation, Ministère de l'Agriculture", INRA and AFSSA, for financial support under the projects "Aliment Qualité Sécurité 2000-R0013" and "Tiacer".

We are grateful to Caroline Delseny and Sylvain Courtillier for their skilful contributions.

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Table 1. Characteristics of the studied strains, including **(A)** the phylogenetic groups to which they belong in the *B. cereus* Group, previously defined (10) and **(B)** the distribution of the known food poisoning toxin genes among these groups (this study).

	A				B					
Phylogenetic group	**Species**	**subgroup**	**Other designation**	**Range of growth T°C**	***No. of tested strains***	**% of strains carrying :**				
						hbl	***cytK-2***	***cytK-1***	***nhe***	***ces***
I	*B. pseudomycoides*	BC10	I-1	10-43	*17*	41	**0**	0	100[a]	0
		BC13	I-2		*7*	86	**0**			
II	*B. cereus II, B. thuringiensis II*	BC06	II	7-40	*31*	61	**13**	0	100	0
III	*B. cereus III B. thuringiensis III,*	BC12	III-1	15-45	*15*	67	73	0	100	0
		BC05	III-2		*26*	**12**	31			**31**
		BC09	III-3		*14*	**14**	57			**7**
	B. anthracis, B. cereus III-4, B. thuringiensis III-4	BC08	III-4		*28*	**14**	39			0
IV	*B. cereus IV, B. thuringiensis IV*	BC04	IV-1	10-45	*34*	97	79	0	100	0
		BC03	IV-2		*32*	97	97			
		BC07	IV-3		*29*	86	79			
V	*B. cereus V, B. thuringiensis V*	BC11	V	8-40	*17*	88	**6**	0	100	0
VI	*B. weihenstephanensis B. mycoides, B. thuringiensis VI*	BC01	VI-1	5-37	*93*	83	**0**	0	100	0
		BC02	VI-2		*43*	60	**0**		100	
VII	*'B. Cytotoxicus' sp. nov.*	BC14	VII	20-50	*5*	**0**	0	**100**	100	0

[a] In group I, nine strains were tested for *nhe* genes instead of 24 strains

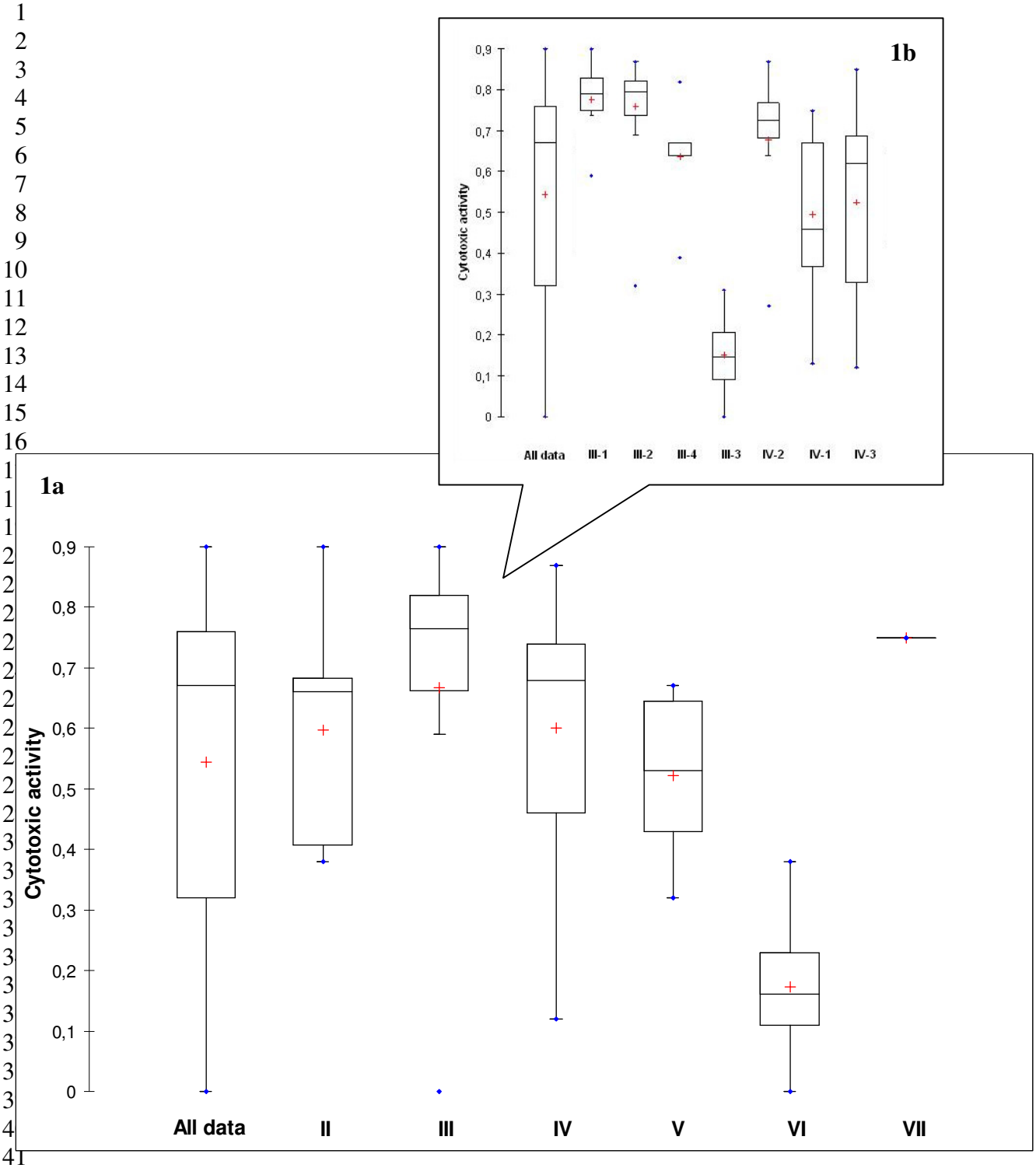

Figure 1. Cytotoxic activity of *B. cereus* culture filtrates against Caco2 cells.

1a, Data distribution of the global population (*B. cereus* Group) is represented by the first Box plot ('All data') whereas that into each sub-population (phylogenetic group II to VII) by the next box plots ('II' to 'VII'). **1b,** Detailed results for subgroups in the phylogenetic groups III and IV.