Molecular Detection of Foodborne Pathogens

Chapter 2: Bacillus

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Introduction:

The genus *Bacillus* includes Gram-positive spore-forming rod-shaped bacteria that are very diverse, from physiology to the ecological niche, from DNA sequence to gene regulation. Several species within the genus *Bacillus* have been associated to foodborne diseases, or have been shown to carry toxin determinants: among others, *B. weihenstephanenesis*, *B. pumilus*, *B. mojavensis*, *B. licheniformis* and *B. subtilis*[1-5]. However, the best-known foodborne pathogen within the genus is *B. cereus*. All these *Bacillus* species, owing to endospore formation, can survive heat treatment and in the absence of a competitive microbiota, can multiply in cooked food during storage, producing toxins under suitable conditions[6-8]. Consumption of such contaminated food might lead to intoxication with acute symptoms shortly after ingestion. Bacterial cells and/or spores in the food can survive the passage through the acidic environment of the stomach and infect the intestine with subsequent toxin production and symptoms which last hours to days after food consumption[5].

In this chapter, we will focus on the food-poisoning implications of *Bacillus* species. Before giving details on the food poisoning potential, the type of toxins and the method of detection, we will briefly introduce the complex ecology and the identification challenges that characterize several *Bacillus* species. We will use as a paradigm the *B. cereus* group that shows a 'bivalent face' as far as its important impact on human activity[9,10].

B. cereus is the most important foodborne pathogen in this group and will be the major subject of this chapter. This bacterium has been shown to be involved in food-poisoning illnesses[11-13] as well as in different kinds of clinical infections[14-18].

B. thuringiensis is an insect pathogen widely used as biopesticide and differently from *B. cereus* has a very useful impact on human activities being widely used in agriculture for insect pest biocontrol.

B. anthracis is the etiological agent of anthrax, a fatal disease for human and animals and has been sometimes associated with foodborne anthrax cases[19-21]. Three forms of anthrax can be distinguished depending on the route of infection: cutaneous, gastrointestinal and inhalational

(pulmonary). Each form may progress to fatal systemic anthrax. The gastrointestinal form is extremely rare and occurs frequently after the ingestion of undercooked meat from animals with *B. anthracis*. The means by which *B. anthracis* crosses membrane barriers to establish infection remains unknown. *B. anthracis* probably invades the mucosa through preexisting lesions, or, in the absence of mucosal damage, through the Peyer's patch as a result of the uptake by M cells or dendritic cells. The disease is characterized by fever, nausea, vomiting, abdominal pain and bloody diarrhea[19,22].

B. weihenstephanensis, a psychrotolerant species capable of growing at temperatures as low as 4-6°C is implicated in food spoilage[23,24].

In addition to *B. cereus*, *B. thuringiensis*, *B. anthracis* and *B. weihenstaephanenesis*, the *B. cereus* group encompasses two other species, *B. mycoides* and *B. pseudomycoides* that are typically isolated from soil and plant rhizosphere.

The six species are known to be strictly related phylogenetically, as has been shown by DNA-DNA hybridization studies[23,25-27] and the sequencing of the ribosomal RNA genes[23,28-30]. However, a marked variability is always observed when large collections of strains are examined by DNA fingerprinting methods that target the whole genome[31-35] and/or discrete genes[36-40]. Hence, the phylogenetic and taxonomic relationship among these species is still open to debate. It has been proposed previously that *B. anthracis*, *B. cereus*, and *B. thuringiensis* represent a single species, this conclusion having been reached through genome sizing and mapping[32-35], multilocus enzyme electrophoresis[41], multilocus sequence typing (MLST)[42,43], and genome sequencing[44]. The genetic variability observed within/between these species has raised several interesting questions and posed a challenge to microbiologists: i) What is the evolutionary pathway of these species and how are they differentiated during evolution? ii) What is the genetic baseline driving the different ecotypes and the virulence?

The genetic baseline of the *B. cereus* group ecotypes:

The ecology of these bacteria is still far to be clear, despite several analyses have recently shown that a major environmental niche for these bacteria is the invertebrate intestinal tract[45-47]. In a general model, these species commonly live associated to the intestine of invertebrates assuming a symbiotic life style, but occasionally they escape such an ecotype becoming invasive for other animal hosts, that are in specific insects, arthropods and nematodes for *B. thuringiensis*, mammals and humans for *B. anthracis* and *B. cereus*[46]. Apart from this general scheme, the ecology of these species outside the invertebrate host is not completely clear, and several environments where these bacteria have been commonly associated to, have been questioned to be really able to continuously support the life cycle of these bacteria. It has been shown that with respect to other soil inhabiting bacteria, like those of the *B. subtilis* group that have genomes harboring many genetic determinants for the metabolism of plant-derived sugars, *B. cereus* group bacteria are rich in genes for protein metabolism. This suggests that they evolved in relation to nutrient rich environments like animal guts or animal tissues and fluids rather than the plant-environment[44,48]. Specialization on different 'animal' niches has lead to different evolution of the species in the *B. cereus* group. For instance, *B. anthracis* has been proposed to diverge from the other members of the group by specializing as a lethal mammal pathogen. It has been supposed that *B. anthracis* has evolved along two possible pathways[49]: one considers *B. anthracis* to be a relatively ancient organism with a low growth rate, determined by the previously mentioned ecological constraints and evolving separately from a common ancestor with *B. cereus* and other relatives. In a second pathway, it has been supposed to be derived relatively recently from *B. cereus*, through the acquisition and rearrangement of plasmids resulting in the actual pXO plasmid pattern responsible of lethal virulence. Such a divergence from the *B. cereus* ancestor has been proposed to occur rather recently, between 13,000 to 26,000 years ago[50]. A recent divergence from *B. cereus* is supported by i) the very marked similarity between *B. anthracis* and certain strains of *B. cereus*[41,51]; ii) the presence in *B. anthracis* genome of several *B. cereus*-typical virulence genes that are, however, not expressed[52]. The lack of gene expression in *B. anthracis* has been shown to be due to a non-sense mutation in PlcR, a

pleiotrophic regulator driving the transcription of several *B. cereus* virulence factors (such as phospholipases C, proteases and enterotoxins) except cereulide[53-55]. Recently, it has been shown that under anaerobiosis, at least two separate pathways, i.e., the PlcR-dependent and the Fnr-dependent pathways, control the expression of enterotoxin genes[56,57,58]. The activity of PlcR depends on PapR, a secreted signaling peptide re-imported into the bacterial cell through the Opp system[59] while the activity of Fnr depends on the availability of O_2 and NO. In *B. anthracis*, a non-functional PlcR is the result of counter selection, due to its disadvantageous effects on the overall fitness of the cell. Mignot et al.[52] showed that a functional PlcR is incompatible with the plasmid borne AtxA-controlled virulence regulon, determining a dramatic effect on the sporulation. When the two regulators are simultaneously transcribed *B. anthracis* cells loose the ability to sporulate, a feature that heavily affects the overall survival capacity of the bacterium in the environment.

Species discrimination in the *B. cereus* group:

The similarity among such closely related bacteria as the species of the *B. cereus* group posed serious challenges for the species discrimination. This is an important point to be addressed, considering the differences in the virulence potential of these microorganisms. While *B. anthracis* can be differentiated by biochemical tests from most of *B. cereus* and *B. thuringiensis*, a problem still exists for isolates borderline between species such as, for instance, the pathogenic *B. cereus* G9241, for which these tests fail to recognize its pathogenic potential[60]. This strain has been confirmed to be a *B. cereus* harboring a virulence plasmid very similar to plasmid pXO1 of *B. anthracis*, and a second plasmid encoding for a capsule synthesis. However, such a second plasmid and the capsule coding genes were completely different from the pXO2 plasmid and the typical capsule of *B. anthracis*[60]. Thus, considering such a virulence potential of strains genetically near neighbor of *B. anthracis*, approaches that might rapidly identify these strains are of great interest. Indeed the characterization of these strains is actually providing useful clues for the understanding of the evolution pattern of these species and the mechanisms they have adopted for regulating the

virulence by the way of plasmid/chromosome interaction[52]. From a safety perspective, these strains could represent alternative hosts for *B. anthracis* toxin genes[60]. Several *B. cereus* strains resulted strictly related to *B. anthracis*. For example, Keim et al.[49] and Radnedge et al.[61] individuated by Amplified Fragment Length Polymorphism (AFLP), *B. cereus* and *B. thuringiensis* strains closely related to *B. anthracis*. Radnedge et al.[61] tried to identify by suppression subtractive hybridization genomic regions of *B. anthracis* absent in these closely related strains. Besides AFLP, several other methods based on whole genome fingerprinting have been used for typing *B. anthracis* and the identification of species borderline strains, like, among others, rep-PCR[10] and multi locus sequence typing (MLST)[42], or, recently, comparative genomics[62] and microarray analysis[63]. Alternative approaches have been based on length or sequence polymorphisms in variable number tandem repeats in multiple loci (Multi Locus VNTR Analysis, MLVA[64]) or on signature Single Nucleotide Polymorphisms (SNPs) in the genome[65]. MLVA has been used as a gold standard for subtyping *B. anthracis* isolated worldwide [see among others 64, 66-68]. Together with MLVA, SNP analysis is greatly contributing in typing and tracing *B. anthracis* isolates, especially by the whole genome SNP analysis[50]. Besides those identified in the whole genome, SNPs in housekeeping genes have been nicely exploited to identify strains related to certain species or genetic types. For example, Prüss et al.[69] showed that certain nucleotides in the 16S rRNA gene and their relative prevalence among the different ribosomal operons in the genome correlate with the psychrotolerance of *B. cereus* strains and hence are signature of the species *B. weihenstephanensis*.

Besides strain *B. cereus* G9241, several other isolates have been shown to be borderline between *B. cereus* or *B. thuringiensis* and *B. anthracis*. In population genetic studies among a strain collection of the *B. cereus* group species it was found by multilocus enzyme electrophoresis (MEE) and MLST[41,42] that the strains could be grouped in two main groups, the first including soil and dairy isolates, while the second those with pathogenic potential. This second group included, besides *B. anthracis*, most of the strains isolated from patients in clinical environments and, among these strains, the *B. cereus* strains isolated from periodontitis in humans. Helgason et al.[41,42] clearly

showed that in the species *B. cereus/B. thuringiensis* some genetic types exist having a close relationship with *B. anthracis*. For instance, besides the previously mentioned strain G9241, *B. cereus* Zebra Killer (ZK) and *B. thuringiensis* 97-27 (subsp. *Konkukian*, serotype H34)[70] resulted closely related to *B. anthracis* according to AFLP and MLST[71]. The proteome of all these three isolates resulted more similar to that of *B. anthracis* than to the non-virulent *B. cereus* ATCC 14579 [72 and references therein]. In a study aiming to determine a strategy for the identification of *B. cereus* group isolates near neighbor of *B. anthracis*[43], two *B. cereus* and one *B. thuringiensis* strains resulted closely related to *B. anthracis*, according to restriction site insertion (RSI)-PCR[36] patterns of the 16S-23S rRNA gene spacers, rep-PCR profiles and MLST analysis[43].

Despite several approaches based on comparative genome sequencing and SNPs analysis by microarray applications, have been recently proposed for the identification of strains close to *B. anthracis* and other virulent strains in the *B. cereus* group, the gold standard is actually represented by MLST. This approach is rather simple and straightforward and take advantage from the availability of databases for the comparison of new isolates with historical or well characterized strains[42,73]. Several MLST schemes based on sequencing of different gene sets have been proposed[42,43,73-77]. However, most of the published studies are referring to that developed by Priest et al.[74]. From this MLST scheme *B. anthracis* fall within the clade 1 that also include many virulent *B. cereus* near neighbors of *B. anthracis* including emetic strains.

Emetic and diarrhoeal illnesses caused by foodborne *B. cereus*:

This bacterium is a common soil inhabitant that has been recognized as a food poisoning species since the beginning of the century. The first confirmed outbreak of *B. cereus* food poisoning occurred in Norway in 1950 after the consumption of a contaminated vanilla sauce, which had been prepared a day in advance before consumption and stored at room temperature[78]. *B. cereus* is widespread in the environment including soil, water and phylloplane[79]. It is also often present in a variety of foods including rice, cereals, spices, vegetables, meat, milk and pasteurized milk

products[80-87]. Toxins produced by vegetative cells of *B. cereus* can be the causative agents of two types of gastrointestinal diseases, respectively associated to emesis and diarrhea. In general, both types of food-borne illnesses are relatively mild and usually do last not more than 24 h. Nevertheless, more severe forms have occasionally been reported, including two deaths after the ingestion of food contaminated with high amounts of the emetic toxin and three deaths caused by the diarrhoeal necrotic enterotoxin CytK1[12,55,88,89]. The emetic syndrome has been frequently associated with cereal foods including rice and pasta. Diarrhoeal toxins have been found in many foods, including milk, vegetables, and meat products[90]. The minimal cell density required to provoke both types of diseases is estimated in the range 10^5-10^8 CFU/g of ingested food (CFU = Colony-Forming Units). However, there are some reports of emetic syndrome associated with foods containing only 10^3 CFU/g food[91,92].

The diarrhoeal syndrome is caused by heat-labile hydrophilic enterotoxins produced during the vegetative growth of *B. cereus* in the small intestine, and is characterized by abdominal pain and diarrhoea that are manifested after 8 to 16 h incubation. Five chromosome-encoded enterotoxins have been characterized: two protein complexes hemolysin BL (HBL) and non-hemolytic enterotoxin (NHE), and three enterotoxic proteins: cytotoxin K (CytK), enterotoxin T (BcET) and enterotoxin FM/S (EntMF/S)[48,55,93-95]. CytK is a 34 kDa hemolytic pore-forming toxin with homology to the β-barrel pore-forming toxins, including staphylococcal β-hemolysin. To date, only three strains containing *cytk-1* gene, encoding the more toxic variant of the CytK protein, have been identified: *B.cereus* strains NVH391/98, NVH883/00, and INRAAF2. Phylogenetic analysis showed that these strains comprise a novel cluster of thermophilic strains genetically distant from the other *B. cereus* group strains[96,97]. Strain NVH 391/98, isolated in 1998 in France at a level of 3×10^5 g^{-1} from a vegetable puree, has been implicated in bloody diarrhoea that caused the death of three persons[55,98]. Recently, the complete genome sequence of this strain revealed the presence of two new important diarrheic toxin operons that may have contributed to the toxicity of this strain in the fatal incident[99].

HBL and NHE are tripartite toxins, in which all three components are necessary for maximal cytotoxic activity[100,101]. NHE consists of three components with molecular masses of 39, 45, and 105 kDa. HBL contains the protein components B (37.5 kDa), L1 (38.2kDa), and L2 (43.5 kDa)[102]. A previous study indicated that the cytotoxicity of *B. cereus* strains was dominated by the amount of secreted NHE and suggested that NHE is the most important toxin that contributes to food poisoning[103]. Recently, it has been shown that NHE acts as a pore-forming toxin inducing cell lysis; and that HBL and NHE constitute a superfamily of pore-forming cytotoxins[104]. It has been suggested that BcET and EntFM have either an unknown type of enterotoxic action or none at all[105].

The emetic syndromes characterized by nausea and vomiting, and induced from 1 to 5 h after consumption of the contaminated food, are associated with the 1.2-kDa cereulide toxin[12,88,93,106]. Cereulide concentrations ranging from 0.01 to1.28 μg g^{-1} were reported in foods implicated in the emetic type of food poisoning[106,107]. The chemical structure and characteristics of this toxin have been studied in detail and recently the molecular basis for its synthesis has been described[91,92,107-109]. Cereulide is a hydrophobic, non ribosomally synthesized small cyclic dodecadepsipeptide [(D-O-Leu-D-Ala-L-OVal-L-Val)$_3$]. The 24-kb (nt 15094 to 38668) cereulide synthesis gene cluster (*ces*) encodes seven proteins involved in the synthesis of the cereulide toxin and is located on a 270 kb plasmid[13,109,110]. Cereulide is heat- and protease-resistant and pH-stable. At low K^+ concentration, it acts as an ionophore through mitochondrial membranes and interferes with oxidative phosphorylation[111-114]. The diversity of the cereulide producers was investigated by Ehling-Schulz et al.[115], who concluded that, like *B. anthracis*, the cereulide-producing strains represent a recently emerged virulent emetic clone of *B. cereus*. These emetic toxin-producing *B. cereus* strains form a distinct cluster within *B. cereus* and harbor some specific characters, such as inability to hydrolyze starch and to ferment salicin, poor ability to grow at temperatures below 10 °C, growth at 48°C, high heat resistance and low ability of their spores to germinate at 30°C. However, individual cereulide-producing strains differed up to 1000-fold in their capacity to produce cereulide[116]. *B. cereus* may be differentiated into 18 serotypes based upon flagellar (H)

antigens. Serovar H1 was considered the predominant serovar implicated in the emetic food poisoning syndrome[117]. However, a recent study showed that strains of serovars H3 and H12 are also sources of emetic-type food poisoning and that emetic toxin producers belong to two phylogenetically distinct *B. cereus* clusters[118]. Taken together with the discovery of emetic *B. weihenstephanensis*[4], these data suggest that cereulide-producing strains are progressively diversifying. The fact that the capacity for cereulide production is shared by two phylogenetically distinct *B. cereus* clusters and by *B. weihenstephanensis* strains, suggests that the 270 kb plasmid carrying the operon responsible for cereulide production, named pCER270[13,109,110], can undergo to lateral transfer. Indeed, plasmid transfer between members of the *B. cereus* group has been shown to occur[119-122].

The true incidence of *B. cereus* food poisoning is unknown for a number of reasons. These include the differences reporting procedures between countries and the relatively short duration of both disease syndromes (< 24 h). In addition, the frequency at which single people are affected is usually not monitored. Another reason for this underestimation is the misdiagnosis of the disease, which is symptomatically similar to other types of food poisoning. For example, the symptoms caused by emetic *B. cereus* resemble those caused by *Staphylococcus aureus*, while those caused by diarrhoeal *B. cereus* resemble those caused by *Clostridium perfringens*[91,92]. In some cases, epidemiological investigations of *B. cereus* outbreak could not be achieved due to delayed notification, which prevented the acquisition of suspected food samples, or to the lack of a reference laboratory and/or specific diagnostic protocols[123]. For instance, between 1991 and 2005, 153 outbreaks were caused by *B. cereus* in Taiwan, representing 11.2% of the total outbreaks of the period[124]. In the USA, from 1982 to 1997, 8781 foodborne-disease outbreaks were reported to the Centers for Disease Control and Prevention (CDC). Of these, 2246 (26%) involved at least five individuals and had sufficient clinical information reported to be considered confirmed foodborne outbreaks. A specific etiology was confirmed by laboratory testing in 697 (31%) outbreaks; among which *B. cereus* accounted for 1% (10 out of 697 outbreaks with a known etiology)[125]. Between

1980 and 1997, 2715 cases of *B. cereus* food poisoning in England and Wales were reported to the Public Health Laboratory Service[126]. In Italy and Germany few *B. cereus* foodborne disease outbreaks have been reported. In June 2000, 173 people presented symptoms of intoxication (nausea and watery diarrhoea) after they attended banquettes in Pisa, Italy. A microbiological investigation was performed and HBL-producing *B. cereus* strains were recovered from stool samples of 19 patients (out of 23 who required hospitalization), foods (more than 10^2 CFU) and from the rolling board of the confectioner's shop which was hypothesized to be the source of contamination of all food samples analyzed[127]. In 2006, 57 persons (out of 149 participants to a wedding banquet) had a foodborne illness in Salerno, Italy. Ricotta cheese contaminated with *B. cereus* was supposed to be the responsible of this outbreak, although diagnostic certainty was not achieved, as no leftovers were available[123]. In June 2006, two emetic food poisoning outbreaks involving 17 children who became sick one hour after eating a rice dish with vegetables, and one student who consumed cooked cauliflower, stored at room temperature for 1.5 days and then reheated, were reported in Germany[128]. In France and Belgium, diarrhoeal and emetic *B. cereus* fatal food poisoning cases were reported[12,55]. In African countries very few is known about the real incidence of *B. cereus* food poisoning outbreaks, and this could be due to the lack of surveillance data and under-reporting of the disease. In Kenya, 37 food poisoning outbreaks were reported to the Ministry of Health from various parts of the country in the period 1970 to 1993. Only 13 of these involving 926 people were confirmed to be due to particular etiological agents, among which *B. cereus*. However, authors suggested that foodborne disease outbreaks are many more than those recorded by the Ministry of Health due to under-reporting, inadequate investigation of outbreaks and inadequate diagnostic facilities[129]. The type of illness most commonly encountered in a country is influenced by the average diet type and nutrition habits of the population. For example, while in Japan, the emetic illness is reported more frequently than the diarrhoeal one, in Europe and North America the diarrhoeal illness is more frequent[112].

Other foodborne virulent *Bacillus* sp.:

Besides *B. cereus*, other species in the genus *Bacillus* have been shown to be implicated in food poisoning. Among these, *B. weihenstephanensis, B. subtilis, B. licheniformis, B. pumilus, B. mojavensis, B. firmus, B. megaterium, B. simplex*, and *B. fusiformis* have been shown to produce toxins and should be considered of food safety concerns[2,3,5,7,90]. In recent years, several authors reported on food poisoning incidents implicating *Bacillus* species other than the *B. cereus*: (i) The baby milk powder containing toxigenic *B. licheniformis* involved in fatal illness of an infant[1], which were later shown to produce the toxin lichenysin A[130]. (ii) a *B. pumilus*-contaminated rice in a Chinese restaurant intoxicated three individuals. *S*train NVH891-05 isolated from the contaminated rice showed to grow well at low temperatures (10-15°C) and to produce large amounts of a toxic cyclic acylheptapeptide, named pumilacidin[5]. Heat-stable toxin-producing *B. licheniformis* and *B. pumilus* were also isolated from mastitic milks. The toxins inhibited mobility of boar sperm cells and disturbed the plasma membrane permeability barrier without affecting the mitochondria[131]. The *B. licheniformis* isolates were shown to have ribopattern similar to strains implicated in the infant fatal case of food poisoning, and harbored three lichenysin A synthetase genes *lchAA, lchAB* and *lchAC*[131]. (iii) Duca et al.[132] reported on a case of infant emesis resulting from consumption of an infant cereal product contaminated with *B. cereus* and *B. subtilis* spores. However, the emetic toxin production was not demonstrated and the *B. cereus* strain was not isolated from the afflicted infants. The emesis has than been attributed to the high bacterial load ingested, in which the presence of *B. subtilis* could have been a contributing factor. *B. subtilis* has been associated with emesis following the consumption of large numbers (2×10^6) of cells[133]. Moreover, the detergent surfactin was shown to be toxic towards boar sperm cells through pore formation and destruction of the cell with subsequent loss of mitochondrial activity[134,135].

With regard to the other *Bacillus* species, toxin production was confirmed and in vitro toxicity towards cell lines was demonstrated. For instance, two emetic *B. weihenstephanensis* strains were able to grow and produce cereulide at 8°C[4]. The complete genome sequencing of strain

B. weihenstephanensis KBAB4 revealed the presence of a Nhe-like toxin on a 400 kb plasmid[99]. To our knowledge, up to now there were no reports on food poisoning cases related to these species. However, the risk of food poisoning from psychrotolerant emetic strains in refrigerated foods, and from cyclic lipopeptides-producing strains needs to be further investigated.

The loss of cytotoxicity by *B. mojavensis* following the abolition of surfactin synthesis[5] supports a role of this molecule in potential virulence. It has been shown that strains of *B. firmus, B. megaterium, B mojavensis, B. fusiformis* and *B. simplex*[2,3] can produce heat-stable toxins with physical characteristics and mode of action similar to those of the *B. cereus* emetic toxin.

Detection of *B. cereus* toxins:

The detection of cereulide in suspected contaminated food samples, or in vomit and fecal samples from ill patients or in the isolates obtained from those samples, represents a necessary clue to confirm *B. cereus* as the causative agent. At present, different tests exist and are commonly used for the detection of diarrhoeal toxins like the vascular permeability reaction (VPR), the ligated rabbit ileal loop and cell cytotoxicity assays. VPR consists in injecting cell-free culture supernatants (0.1 ml) intradermally into rabbits weighting 2.5 to 3.0 kg. After 3 h, 4 ml of 2% Evans blue dye solution is injected intravenously. Measurements of perpendicular diameters of the zones of light and dark blue, and necrosis when present, are made after 1 h[136]. The ligated rabbit ileal loop assay consists in the injection of the material to be tested (e.g. the bacterial culture supernatant) into 5 cm ileal loop of female New Zealand white rabbits. The reaction is considered positive if the ratio of the volume of fluid accumulation to loop length is > 0.5[94]. In the cytotoxicity assays filtered supernatant is supplemented to a cell line and the effects of the treatment on the cells is evaluated. A number of cell lines are susceptible to the diarrhoeal toxins. The most commonly used are Vero (monkey kidney) and CHO (Chinese hamster ovary) cell lines, although other cell lines have been used including HeLa S3, Human Embryonic Lung (HEL) and McCoy cell lines[136,137]. Gray et al.[90] developed an innovative assay using a murine hybridoma Ped-2E9 cell model. Culture supernatants

containing enterotoxins are added to a Ped-2E9 cell line and analyzed for cytotoxicity with an alkaline phosphatase release assay. The assay was shown to be rapid (results obtained within 1 h) and to have 25- to 58-fold-higher sensitivity than the CHO assay. Although cell cytotoxicity assays are an inexpensive and convenient method for diarrhoeal toxin detection, they present many drawbacks. *B. cereus* produces many extracellular virulence factors such as phospholipases and sphingomyelinase, which may be cytotoxic to the cell lines[38,138,139]. The test is time-consuming since it needs an enrichment step and requires the constant maintenance of cell lines.

Commercial kits have been developed for the detection of the diarrhoeal toxins of *B. cereus*. Two immunological assays are commercially available: the *B. cereus* Enterotoxin Reverse Passive Latex Agglutination (BCET-RPLA) kit (Oxoid, Basingstoke, UK) and the Tecra *Bacillus* Diarrhoeal Enterotoxin Visual Immunoassay (BDE-VIA) kit (Tecra Diagnostics, Frenchs Forest, Australia) for detection of the L2 component of HBL (HblC) and the 41-kDa subunit of NHE, respectively. Both kits require the culture of *B. cereus* isolates for 6 to 18 h prior to testing. The culture supernatants are then tested for the enterotoxin-related proteins. To our knowledge, commercial kits are not yet available for the detection emetic toxin of *B. cereus*. Cereulide is traditionally assessed using a HEp-2 tissue culture assay by observing vacuole formation[106,140,141] or colorimetrically, by using the 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) metabolic staining assay[142,143]. An assay based on the uncoupling of respiratory activity of rat liver mitochondria has been developed for emetic toxin[112]. Another bioassay, based on the loss of motility of boar spermatozoa, has also been developed[144,145]. However, this assay has been found to be not enough specific, since other toxins were shown to affect sperm motility[146]. Recently, a Computer Aided Semen Analysis (CASA) study of the boar semen motility has been demonstrated to be an appropriate assay for detection of cereulide, which induces motility ceasing at concentrations lower than 20 ng ml^{-1}, in comparison with other toxins that have the same effect at much higher concentrations. Since the assay detected cereulide indirectly, the presence of cereulide in positive samples was verified by HPLC-MS[147-149].

Detection of *B. cereus* cells in food and environmental samples:

Standard isolation and enumeration of *B. cereus* from foods, the environment and clinical settings is generally performed by using conventional selective plating media. Two standard media are recommended by food authorities: polymyxin-egg yolk-mannitol-bromothymol blue (PEMBA, Oxoid) or mannitol-egg yolk-polymyxin (MEYP, Oxoid) agar[150]. When grown on PEMBA, *B. cereus* produces typical crenated colonies that retain the turquoise blue of the pH indicator (bromothymol blue) due to their inability to produce acid via mannitol fermentation. A zone of egg-yolk precipitation is produced through lecithinase activity, which involves the cleaving of lecithin (phosphatidylcholine) into phosphorylcholine and diglyceride. Polymyxin B is used in the media as a selective agent to suppress Gram-negative bacteria[151]. Recently, two new chromogenic plating media, *B. cereus* group plating medium (BCM, Biosynth AG, Switzerland) and Chromogenic *B. cereus* agar (CBC; Oxoid) were developed based on the activity of two different hydrolyzing enzymes: the phosphatidyl inositol phospholipase C (PI-PLC) for BCM, and the β-D-glucosidase for CBC. On BCM, 5-bromo-4-chloro-3-indoxylmyoinositol-1-phosphate is cleaved by PI-PLC and gives to *B.cereus* colonies a homogeneous blue-turquoise color. The colonies are sometimes surrounded by a blue halo. CBC contains 5-bromo-4-chloro-3-indolyl- β-glucopyranoside that is cleaved by β-D-glucosidase and results in white colonies with a blue-green centre. However, in addition to being time-consuming and unable to indicate the toxin production capability of the isolates, the performance of these culture-dependent detection methods are debated. In fact, some *B. cereus* strains, especially highly toxic strains with atypical growth characteristics, lack one or more of the key characteristics on these media, a feature that could lead to their misidentification[152]. In addition, presumptive *B. cereus* isolates must then be tested by several biochemical and microscopic procedures to confirm whether they are true *B. cereus*, due to the phylogenetically close relationship between the different species. Crystal formation is one key test that positively identifies *B. thuringiensis*, while rhizoid colony morphology is a phenotypic character of the species

B. mycoides and *B. pseudomycoides*[27]. Thus, acrystalliferous variants of *B. thuringiensis* or nonrhizoid variants of *B. mycoides/B. pseudomycoides* may be misidentified as *B. cereus*. Moreover, the difficulty in clearly identifying isolates within the *B. cereus* group is also increased by the presence of enterotoxin-producing *B. thuringiensis* strains [153-156; Raddadi et al., unpublished]. Blood-agar medium could also be used to select HBL-producing *B. cereus* strains that show discontinuous hemolytic patterns[157]. Based on monoclonal antibodies that detect specifically the B component of HBL and the nheA component of NHE, Moravek et al.[158] described a colony immunoblot assay (CIA) that enables the identification of HBL and NHE-producing *B. cereus* isolates grown on blood agar within 24 h.

Along the years, several biosensor-based techniques for the detection of foodborne pathogens and bioterrorism agents have been developed and reported in the literature. A biosensor is an analytical device that integrates a biological sensing element with an electrical transducer to quantify a biological event into an electrical output. Recently, Pal et al.[159] used immunochemical techniques for the development of a *B. cereus* biosensor. The approach utilizes the concept of a direct charge transfer (DCT) of electrons in a voltage controlled switch format for bacterial detection. The biosensor uses rabbit polyclonal anti-*B. cereus* antibodies as the biological sensing element and polyaniline nanowires as the electrical transducer. The biosensor was shown to have high sensitivity, being able to detect the presence of *B. cereus* at concentrations as low as 10^1 CFU/ml, and rapid with a detection time being only 6 min.

In addition to these different detection methods, molecular detection systems for foodborne pathogenic bacteria in general, as well as for diarrhoeal and emetic *B. cereus* strains, have been developed along the years. These include PCRs (conventional, multiplex and real-time) and oligonucleotide DNA microarrays. Jin et al.[160] developed DNA microarrays for the detection and identification of intestinal pathogens using two universal PCR primers to amplify two variable regions of bacterial 16S and 23S ribosomal RNA genes. Two oligonucleotide microarrays based on 16S rRNA gene sequences of predominant human intestinal bacterial species were reported for the

detection of intestinal bacteria in fecal samples collected from human subjects, and for the detection of foodborne pathogenic bacteria, respectively[161,162]. However, these tools could not be useful for the discrimination among the bacteria of the *B. cereus* group due to the close phylogenetic relationship between the different species that harbor mostly 16S rRNA gene sequences. Recently, Park et al.[163] developed a multiplex PCR that targets *gyrB* and *groEL* genes as diagnostic markers for the simultaneous detection and identification of the *B. cereus* group bacteria from food samples. A 400 bp PCR fragment was amplified from the *groEL* gene for all the bacteria of the *B. cereus* group, while amplicons of 253, 475, 299 and 604 bp were yielded for the *gyrB* gene from *B. anthracis, B. cereus, B. thuringiensis* and *B. mycoides*, respectively.

The polymerase chain reaction has been extensively used to amplify all the diarrhoeal toxin-producing genes[13,89,127,154,164], and of genes encoding for the emetic toxin cereulide, after their identification and sequencing[91,92,108,115]. However, the presence of a toxin gene does not necessarily indicate that the bacterium is capable of producing the protein in concentrations high enough to determine the disease. For example, different emetic *B. cereus* strains were shown to have 1000-fold difference in their capacity to express cereulide[116]. Another major drawback of conventional PCR is the requirement for post-PCR analysis by gel or capillary electrophoresis that is time-consuming, and bears the risk of false-positive results due to laboratory contamination. Thus, introduction of more rapid and sensitive detection systems, such as quantitative Real-Time PCR (RT-PCR), is required. In principle, two different chemistries are available for real-time detection of PCR products: fluorescent probes that bind specifically to certain DNA sequences (Taq Man-based RT-PCR) and fluorescent dyes that intercalate in any double-stranded DNA (SYBR green I-based RT-PCR). Based on the sequence of the cereulide synthetase (*ces*) gene, Fricker et al.[128] developed novel diagnostic assays that were applied successfully to identify the causative agent of two recent emetic food-poisoning outbreaks in Germany. The methods developed were a Taq Man-based RT-PCR assay targeting a highly specific sequence of the *ces* gene, and a duplex SYBR green I-based RT-PCR assay for one-step differentiation between emetic *B. cereus* and *S. aureus*. Yang et al.[124]

developed a SYBR green-based RT-PCR assay for the rapid (analysis is performed in less than 3 h) quantitative detection of *B. cereus* in food (cooked rice, milk and chicken meat) and fecal samples, using the *nheB* gene as target. Combined with the most probable number (MPN) technique (MPN-RT-PCR assay) for the enumeration of low loads of contamination, the assay was performed in less than 8 h and the detection limit was as low as 1 CFU ml^{-1}. The MPN-RT-PCR assay could be used as an alternative method for detecting low levels of contamination. It provides stronger quantification than the traditional enrichment step, allows the dilution of PCR inhibitors and the discrimination of viable and dead cells. In fact, when applying RT-PCR to the detection of pathogens in foods without enrichment, the method usually consistently detects 10^2 to 10^3 CFU or more per g of sample[124,128,165]. This decrease in the sensitivity of RT-PCR, which theoretically can reach one copy of target gene detected per reaction, is mainly due to PCR inhibitors such as lipids, proteases and divalent cations present in dairy products[166,167]. To overcome this problem and enhance the sensitivity of the detection, most available detection systems require selective enrichment steps, especially for low pathogen concentrations. However, this could prevent the appreciation of original pathogen contamination levels. Recently, Fukushima et al.[165] developed a method for the rapid separation and concentration of *B. cereus* cells (including pathogens) from food matrices that can be used prior to real-time quantitative PCR and viable-cell counting. Using these combined methods, the target organisms in the food samples can be concentrated up to 250-fold and detected at cell concentrations as low as 10^1 CFU g^{-1}.

In the following paragraphs, we will report on procedures that can be adopted for the detection and identification of *B. cereus* using modern molecular methods. Principles and procedures, from sample preparation to application of detection techniques, are illustrated and discussed.

Sample preparation:

With sample preparation is intended the ensemble of procedures applied for bacterial isolation and enumeration and total DNA extraction from food samples and individual isolated bacteria. For molecular detection of foodborne pathogenic bacteria, sample preparation includes mainly DNA extraction from food matrices both for microbiological quality evaluation and risk assessment studies, as well as for epidemiological diagnosis of food poisoning illnesses. In the latter case, the incriminated bacterium is also isolated on selective medium and further identified by biochemical and or molecular methods.

B. cereus isolation and enumeration:

To enumerate *B. cereus* spores, 25 g or 25 ml of food samples are homogenized with 225 ml of Peptone Saline Solution (PSS) containing 8.5 g l^{-1} NaCl and 1 g l^{-1} of neutralized bacteriological peptone. Ten milliliters of the homogenate (diluted 1:10) are transferred into sterile tubes, incubated at 80 °C for 10 min, and then cooled in melting ice prior to further serial dilutions in PSS, until 1:1000 dilution. In order to detect and enumerate low levels of *B. cereus* spores, a three-tubes MPN procedure could be performed using as growth medium tryptone soya broth (TSB, Oxoid) supplemented with 100 mg l^{-1} of polymyxin B sulfate. After incubation at 30 °C for 24 h, tubes are examined visually for turbidity. A loopful of culture from positive MPN tubes is streaked onto one the chromogenic *B. cereus* group selective media (MEYP; BCM; CBC) plates and incubated at 30 °C for 24–48 h. Colonies with a typical *B. cereus* morphotype are purified on tryptone soya agar (TSA; Oxoid).

In order to detect and enumerate high levels of spores of *B. cereus*, the spread-plate procedure is carried out as follows: 1 ml volume of the 1:1000 dilution is spread plated in duplicate onto one of the three *B. cereus* group selective media agar plates and incubated at 30 °C for 24-48 h. The plates with 15-150 typical *B. cereus* colonies are counted. One to five randomly selected typical colonies are purified. The total counts of *B. cereus* spores are based on percentage of

colonies tested that were confirmed as *B. cereus*, and expressed as CFU g^{-1} of sample. The purified isolates are stored in nutrient broth (Oxoid) with 20% glycerol at -80°C until use[84,162].

DNA isolation:

DNA template for PCR reactions can be prepared from the purified isolates by a simple boiling method as following. Cells are collected by centrifugation (5000 × g for 2 min) from 1 ml of a 4 h-culture grown in nutrient broth at 30°C,. The cell pellet is washed once with 500 µl of sterile MilliQ water or TE buffer (pH = 8), centrifuged, resuspended in100 µl of sterile MilliQ water or TE buffer and boiled for 10 to15 min. The boiled samples are centrifuged at 10,000 × g for 5 min to precipitate cell debris and the supernatants, which contain nucleic acids, are collected and stored at -20 °C until use. There are also several commercial kits available for total DNA extraction from overnight grown pure bacterial cultures such as AquaPure genomic DNA isolation kit (Bio-Rad) or QIAamp DNA stool mini kit (QIAGEN). The phenol-chloroform extraction protocol is also useful to obtain pure high molecular weight genomic DNA[168].

Total DNA from food samples can be isolated using either the AquaPure genomic DNA isolation kit (Bio-Rad), the QIAamp DNA Mini Kit (QIAGEN), the Wizard DNA Purification resin-based kit (Promega), or the NucleoSpin food kit (Macherey-Nagel) according to the manufacturer's instructions or a simple boiling method. Yang et al. [124] extracted DNA using the QIAamp DNA stool mini kit (QIAGEN) with some modifications to the manufacturer's instructions. For pure cultures, bacteria were placed in brain heart infusion broth and incubated overnight at 30°C. DNA was extracted from 200 µl of each individual culture. For cultures in spiked food or fecal samples, 200 µl or 200 µg of spiked samples were added to 1.4 ml of buffer ASL and heated for 15 min at 99°C. After heating, the manufacturer's steps were followed up to the proteinase K step. At this time, 30 µl of proteinase K (600 mAU ml^{-1}) was placed into a new microcentrifuge tube, and 400 µl of the supernatant obtained from Inhibit Extreatment step was

added, followed by 400 µl of buffer Al and 400 µl of ethanol. Since the total volume of the resulting lysate exceeded the volume of the spin column, the lysate was applied twice to the spin column. Finally, after the washing steps 40 µl of buffer AE was added to elute the DNA.

Procedures for molecular identification and detection:

The traditional methods for strain identification looking to metabolism and physiology can be time consuming and laborious. As well, the culture-dependent techniques for direct isolation and identification of *B. cereus* suffer the same problems. In addition, these techniques do not allow the differentiation among the *B. cereus* group isolates due to the close phylogenetic relationship between the species in this group and the presence of borderline isolates between *B. cereus* and *B. anthracis*. This could be of high risk from a human safety point of view. In fact, although *B. cereus* has been implicated in some fatal cases, the illnesses caused by this pathogen are usually mild. On the contrary, up to 40% of people having gastrointestinal anthrax die after antibiotic treatment[21]. Although rare (less than 1% of reported anthrax cases), foodborne anthrax cases have been reported after ingestion of *B. anthracis*-contaminated meat[21]. The gastrointestinal anthrax disease is characterized by fever, nausea, vomiting, abdominal pain and bloody diarrhea[19,22], symptoms that are somehow similar to intoxication with *B. cereus*. On the other hand, while *B. anthracis* could be differentiated from *B. cereus* based on biochemial traits, a problem still exists for the *B. cereus*/*B. thuringiensis* borderline strains, which can determine disease symptoms similar to *B. anthracis*. Recently a strain isolated from humans, identified as *B. cereus* on the basis of phenotypic and molecular data, caused symptoms similar to those of the anthrax disease and it was shown to harbor a virulence plasmid very similar to pXO1, while a capsule plasmid completely different from pXO2[60]. These isolates could be discriminated from *B. anthracis* by different molecular techniques such as RT-PCR assays that target the nonsense mutation in the pleiotrophic regulator gene *plcR*[53,169-171]. However, the approach requires expensive RT-PCR platforms, limiting equipment in most laboratories in developing countries where anthrax is endemic[67]. Recently, Gierczyński et

al.[172] developed a simple and cost effective restriction site insertion-PCR (RSI-PCR)[36]-based assay as an alternative to this RT-PCR approach. The assay precisely detects the *B. anthracis*-specific nonsense mutation in *plcR* gene by restriction digestion with the endonuclease *Ssp*I. The requirements of this assay are limited to standard PCR equipment and an agarose or a polyacrylamide gel electrophoresis (PAGE) apparatus, that makes it available for most routine diagnostic laboratories world-wide. The nonsense mutation in the *B. anthracis* gene *plcR* generates a termination codon (UAA) in place of glutamic acid (GAA) in position 209 of the amino acid sequence of the functional PlcR[53]. In order to detect thymidine (T), the key nucleotide in the *plcR* nonsense mutation of *B. anthracis*, an artificial *Ssp*I specific cleavage site can be introduced into a PCR product generated using the 60 bp-long AplR primer (5'-ATGTCATACTATTAATTTGACACGATAGTTCAATAGCTTTATTTGCATGACAAAGCGAAT-3'), modified at the 3' end to specifically introduce the *Ssp*I cleavage site in the amplified *plcR* fragment. At positions 58 and 59 of the AplR primer, two adenine (A) bases determine, when the amplicon is generated, the incorporation in the PCR product of an artificial cleavage site (AATATT) for *Ssp*I only in the *plcR* amplicon deriving from *B. anthracis*. These two adenines replaced cytosine (C) and thymine (T), which complement G and A at positions 1147 and 1146 (positions referred to sequence accession number AF132086), respectively, of the truncated *plcR* of *B. anthracis* strain 9131[53]. Primer AplF (5'-GCTCAATCAACAATTGGCAGG-3') can be used in combination with primer AplR for the amplification of a 278 bp fragment of the *plcR* gene[172]. The PCR is carried out in 25 μL reaction mixture, containing 1 U of Taq DNA polymerase (Polgen) and 1× PCR buffer (10 mM Tris-HCl pH 8.9, 2 mM $MgCl_2$, 50 mM KCl) supplied with the polymerase, 10 pmol of each the afore mentioned primer, 0.2 mM of each deoxynucleotide and 5-10 ng of genomic template DNA. A hot start step at 95°C for 5' before adding Taq DNA polymerase would avoid formation of primer dimers whose formation could be favored by the length of the forward primer used here (60 bp). The thermal program consists of an initial denaturation step at 94 °C for 3 min followed by 35 cycles of denaturation at 94 °C for 40 s, annealing at 58°C for 30 s and

extension at 72 °C for 30 s and a final extension at 72 °C for 5 minutes. Enzyme digestion can be performed in a 20 µl mixture containing 6 µl of PCR product and 5-7 U of the enzyme *Ssp*I using thermal conditions and reaction buffer recommended by the enzyme manufacturer. The digestion products can be separated in 4% high resolution agarose (MP Biomedicals) in TAE (40 mM Tris-acetate pH 8.0, 1 mM EDTA) at a constant voltage of 6 V cm^{-1} for 3 h. DNA can be visualized by conventional ethidium bromide staining. It is also possible to separate the digestion products onto 8% non denaturing polyacrylamide gel (Applichem) using a conventional chamber for vertical electrophoresis. In this case, DNA is visualized by either ethidium bromide staining or silver staining. Further details of the procedures to be adopted for RSI-PCR analysis can be found in Daffonchio et al.[36], Daffonchio et al.[43] and Gierczyński et al.[172]. This test was applied to 47 strains of *B. anthracis* that were isolated from four different countries (France, Georgia, Poland and Russia), including vaccinal strains, and all gave a *Ssp*I restriction profile with two fragments of about 60 and 220 bp as expected[172]. It is important to notice that some *B. thuringiensis* strains gave aspecific *Ssp*I restriction profile of about 120 and 160 bp. However, these strains were shown to have a native *Ssp*I restriction site that splits PCR product into fragments of 120 and 158 bp. In addition, some *B. cereus* tested in this assay gave a *plcR* amplified fragment of the expected length (290 bp) but was not cleaved by the restriction enzyme[172].

With regard to *B. cereus* toxins, two commercial kits are available for the detection of the diarrhoeal HBL and NHE (see introduction), while no kits are actually available for the detection of the emetic toxin cereulide, due to its low antigenic characteristics[173], or the necrotic toxin CytK1. Over the years, to allow the rapid detection of the genetic determinants of these toxins, different molecular methods have been developed, including microarray[174] conventional PCR and RT-PCR. Ngamwongsatit et al.[156] developed a multiplex PCR method using new specific PCR primers for detection of eight enterotoxin genes (*hblCDA, nheABC, cytK,* and *entFM*) in a single PCR reaction. This multiplex PCR amplification is performed in a final volume of 20 µl containing 5 µl of template DNA prepared by the boiling method, 1 × PCR buffer (10 mM Tris-HCl pH 8.3 and 50

mM KCl), 1.5 mM $MgCl_2$, 200 mM of each dNTP, 0.2 to 0.4 mM of primers and 5U of Taq DNA polymerase. In the study of Ngamwongsatit et al.[156], reactions were carried out in an iCycler (BioRad, Richmond, USA) with initial denaturation at 95°C for 5 min, followed by 30 cycles of 94°C for 45 s, 54°C for 1 min, 72°C for 2 min and a final extension at 72°C for 5 min. Amplicons can be separated on 1.5% agarose gels. Amplified fragments of predicted size of 1018, 935, 884, 759, 695, 618, 565, and 486 bp for *hblD, nheB, hblA, nheA, hblC, nheC, cytK*, and *entFM* genes, respectively, are obtained from *B. cereus/B. thuringiensis*. By using this assay, Ngamwongsatit et al.[156] categorized the 616 isolates they analyzed four groups. All eight genes were detected in group I. Group II and III lacked *hblCDA* and *cytK*, respectively, while in group IV, both *hblCDA* and *cytK* were missing. However, the applicability of the method was not tested on food samples, which often contain PCR inhibitors. This multiplex PCR is an informative and easy-to-handle tool for the detection of enterotoxin genes in individual isolates purified from food samples. However its efficiency for the direct detection of toxin-producing *B. cereus/B. thuringiensis* in food samples should be tested to be used as a tool for risk assessment. Yang et al.[124] developed a SYBR green-based RT-PCR for the detection of *B. cereus* group cells in different food and fecal samples. They choose the *nhe* gene that codify for the diarrhoeal toxin NHE, because this gene was shown to be present in 100% of *B. cereus* and *B. thuringiensis* strains[156]. Another reason of the choice is related to the fact that NHE toxin is the most important toxin in *B. cereus* diarrhoeal food poisoning[103]. The RT-PCR developed by Yang et al.[124] is performed in a 25 µl reaction mixture containing 12.5 µl of SYBR *Premix Ex Taq* (2 ×) (TaKaRa Bio Inc.), 0.5 µl of ROX reference dye II (50 ×), 10 µM of each primer and 2.5 µl of template DNA extracted by boiling. Two primer pairs (consensus primers: SG-F3, 5'-GCACTTATGGCAGTATTTGCAGC-3', and SG-R3, 5'-GCATCTTTTAAGCCTTCTGGTC-3'; degenerate primers: mSG-F3, 5'-GCACTKATGGCAGTATTTRCR GC-3', and mSG-R3, 5'-GCATCTTTYARGCCTTCTGGTC-3') are used in the assay in order to overcome false-negative results due to the sequence polymorphisms characterizing this gene. Yang et al. [124] performed PCR amplification on an

Mx3000P apparatus (Stratagene). The thermal protocol consists in initial denaturation at 95°C for 30 s, followed by 40 cycles at 95°C for 5 s and 60°C for 30 s. Amplified products can be examined by gel electrophoresis to confirm that the results reported by the Mx3000P correlated with the amplification of a product with the expected size (152 bp). The performance of the two primer pairs was evaluated with 10 ng of purified DNA from 60 strains of the *B. cereus* group and was shown to be specific for bacteria of this group. This method was shown to be highly sensitive detecting as less as 10^2 to 10^7 CFU g^{-1} of *B. cereus* in cooked rice and chicken samples, and thus was supposed to be sufficient to be used as a quick and routine technique for the quantification of *B. cereus* group cells. However, a problem still exists for the detection of low-level contamination. In this case, an enrichment step could be incorporated in order to allow bacterial pathogens to multiply and reach detectable levels. However, this kind of method prevents the quantification of original contamination levels. In order to solve the problem of low-level contamination detection, the RT-PCR can be coupled to a three-tube MPN assay. This method was applied to 30 food samples, and was shown to have a detection limit of as low as 1 CFU of *B. cereus* ml^{-1}, which is more sensitive than an RT-PCR assay without enrichment[124]. This MPN RT-PCR assay provides stronger quantification than the traditional enrichment and is important for the discrimination of viable and dead cells. This method is useful for detection and quantification of cells of the *B. cereus* group and not only of the *B. cereus* species, since *nhe* gene is widely distributed among bacteria in this group.

The first conventional PCR-based assay for the detection of emetic *B. cereus* strains has been described in 2004[91,92]. Primers EM1F (5'-GACAAGAGAAATTTCTACGAGCAAGTACAAT-3') and EM1R (5P-GCAGCCTTCCAATTACTCCTTCTGCCACAGT-3') amplify a fragment of 635 bp from emetic *B. cereus* genomic DNA. The specificity of the assay was assessed using a panel of 178 bacterial strains; neither false-positive nor false-negative signals were detected[91,92]. Recently, after the biosynthetic pathway for nonribosomal synthesis of cereulide and the related genes have been deciphered[115,176], a TaqMan-based as well as a SYBR green I-based duplex RT-PCR assays that

target a highly specific part of the cereulide synthetase (*ces*: accession no. DQ360825) genes were developed by Fricker et al.[128] for the detection of emetic *B. cereus* and its differentiation from *S. aureus*. The target probe for emetic *B. cereus* was labeled at the 5' end with the reporter dye 6-carboxyfluorescein (FAM), and the IAC probe was labeled at the 5'end with 5-hexachloro-6-carboxyfluorescein (HEX). Both probes were labeled at the 3' ends with tetramethyl-6-carboxyrhodamine (TAMRA). For the TaqMan-based PCR assay, reactions were carried out in a 25-µl final volume containing 12.5 µl Brilliant Q PCR Multiplex Mastermix (Stratagene), 0.5 µM of each primer (ces_TaqMan_for: 5'-CGCCGAAAGTGATTATACCAA-3'; ces_TaqMan_rev: 5'-TATGCCCCGTTCTCAAACTG-3'; IAC_for: 5'-GCAGCCACTGGTAACAGGAT-3'; IAC_rev: 5'-GCAGAGCGCAGATACCAAAT-3'), 0.2 µM of each probe (ces_TaqMan_probe: 5'-FAM-GGGAAAATAACGAGAAATGCA-TAMRA-3'; IAC_probe: 5'-HEX-AGAGCGAGGTATGTAGGCGG-TAMRA-3'), approximately 170 copies of plasmid DNA pUC19 (Fermentas), and 5 µl of DNA template. Controls containing 5 µl of only Tris-EDTA buffer were included in each run to detect any contamination. The amplification was carried out in a Stratagene MX3000P PCR system (Stratagene) and the thermal cycling consisted in 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 55°C for 60 s. The maximum ramp rate of the Stratagene MX3000P RT-PCR system was 2.5°C s^{-1}. The SYBR green I RT-PCR reactions were carried out in a 25-µl PCR mixture containing 12.5 µl SYBR Premix Ex Taq (Takara Bio, Inc.), 1 to 5 µl of template DNA (depending on extraction method), 0.3 µM for each *B. cereus* primer (ces_SYBR_F: 5'-CACGCCGAAAGTGATTATACCAA-3'; ces_SYBR_R: 5'-CACGATAAAACCACTGAGATAGTG-3'), and 0.12 µM for each *S. aureus* primer (sa_SYBR_F: 5'-CGTGTTGAACGTGGTCAAATCA-3'; sa_SYBR_R: 5'-CACCTTCGTCTTTTGATAATACG-3'). The cycling conditions for a Stratagene MX3000P RT-PCR system were 95°C for 10 s, followed by 45 cycles at 95°C for 10 s and 60°C for 30 s. The same conditions were used with the SmartCyclerII system (Cepheid), but with the ramp rate set on

maximum (10°C s^{-1}) for the simplex detection of emetic *B. cereus* strains and altered to 3°C s^{-1} for simplex and duplex detection involving the primers specific for *S. aureus*. The real-time assays developed were shown to detect 10^1 to 10^3 CFU g^{-1} of emetic *B. cereus* directly from food samples without the need for further enrichment steps. Lower cell numbers (1 *B. cereus* CFU g^{-1}) can be detected after a short enrichment time (4 to 6 h). After 6 h of enrichment, it was possible to detect 10^0 *B. cereus* CFU/g and 10^3 CFU *S. aureus*/g of food by the SYBR green duplex assay when emetic *B. cereus* was present in the same enrichment. Foods incriminated in emetic outbreaks were reported to have 10^5 to 10^8 CFU *B.cereus*/g or between 10^5 and 10^6 *S. aureus* CFU/g[177]. Thus samples from emetic food poisonings can be processed and analyzed within short time (1.5 to 6 h). However, once the presence of *B. cereus* is detected by this assay, a further analysis of the emetic toxin cereulide is still needed due to its heat stability, especially in the cases of reheated foods. Three methods for detection of the emetic toxin have been described including a cytotoxicity assay using HEp2 or CHO cells, HPLC-MS analysis, and a sperm-based bioassay. The cytotoxicity assay could be performed from pure *B. cereus* isolates as following: cells are grown in 20 ml of skim milk medium for 18 h, and, after autoclaving, an aliquot of the preparation is serially diluted (two fold) in 96-well plates by using Earle's minimal essential cell culture medium supplemented with 1% fetal calf serum, 1% (vol/vol) sodium pyruvate (100 mM), 2% (vol/vol) L-glutamine (200 mM), 0.2% (vol/vol) penicillin-streptomycin (10,000 U/ml), and 2% ethanol as a diluent. Immediately after this, HEp-2 cells (0.15 ml; 10^5 cells per well) are added, and the plates are incubated for 48 h at 37°C in a 5% CO_2 atmosphere. Toxicity titers are determined by using the cell proliferation reagent WST-1[103,175]. The cytotoxicity assay could also be performed directly from food samples. In this case, 5 g (or 5ml) of food sample are homogenized in 5 ml sterile Milli-Q water and autoclaved (20 min at 121°C). Ten microliters of the cell-free supernatant are then tested in a HEp-2 cell culture assay as described above. Both cytotoxicity assay and sperm-based bioassay are semi-quantitative, and the conclusive identification of cereulide is only provided by HPLC-MS analysis[146,147].

Conclusions:

It is virtually impossible to ensure that foods are free from spore forming bacteria such as the ubiquitous bacterium *B. cereus*. In most food poisoning cases, illness is associated with the heating of precooked food held for too long at inappropriate storage temperature. However, if food is cooked and stored correctly, *B. cereus* should not constitute serious problems. Toxin-producing *Bacillus* spp. seem to be rare among isolates from water, food, and food environments, and none of the toxins detected are similar to the *B. cereus* enterotoxins or to cereulide. However, although rare, *Bacillus* spp. outside the *B. cereus* group might still be involved in food poisoning through foods that are considered safe by the public. Thus, it is interesting to develop detection methods of *Bacillus* spp. in food ingredients and in the food production plants. Several molecular detection methods are actually available for *B. cereus* and its toxins. These include conventional PCR, multiplex PCR and RT-PCR. However, although highly specific and sensitive, these molecular methods have some drawbacks. For example, the conventional PCR could be used for qualitative detection of the presence of the foodborne pathogenic bacterium, but it is not usable for risk assessment evaluation since further confirmation methods, such as DNA hybridization or sequencing, are always needed. This method bears also the risk of false-positive results due to laboratory contamination. The multiplex PCR could be a good alternative to detect different pathogens or different toxins of the same pathogenic bacterial species simultaneously. However, in same cases it is possible to get false negative results due to the presence of different primer pairs in the same reaction mix, or due to different annealing temperatures. In addition, this method is qualitative and do not allow quantification of the pathogenic bacterium or of its toxins. In order to quantify the presence of a specific pathogenic bacterium or its toxins, TaqMan or SYBR green-based RT-PCRs could be applied. However, although highly sensitive, this technique could be biased by the presence of PCR inhibitors in complex matrices (such as foods and faecal samples) and cannot discriminate between DNA originating from alive and dead cells. This method allows the detection of 10^2 to 10^4 CFU g^{-1} of sample and thus lower levels of the contaminants cannot be

detected, requiring an enrichment step. Enrichment steps are time-consuming and could overestimate the initial level of contamination. Another drawback of RT-PCR is the necessity of expensive and sophisticated real-time platforms and reagents that cannot be available in most laboratories especially in developing countries.

The confirmation of *B. cereus* as the causative agent responsible for food-borne disease is dependent upon a combination of different clues like food consumption history, nature of the symptoms and detection of the bacterium in the implicated food and/or the patient vomit or faeces. It is also necessary to demonstrate either that the same serotype isolated from the vomit/faeces is also present in the implicated food, or that the isolate is toxigenic.

The rapid detection of microbes in food samples is becoming more critical and the development of rapid and sensitive methods is of great interest for human safety. Molecular techniques can be used to confirm the identity and the nature of *B. cereus* isolates. These include multiplex PCR that allow the detection of the different enterotoxin genes and RT-PCR for both diarroheal and emetic toxin genes. However, there are still some limitations in the application of these methods. For example, the DNA template used for PCR amplification of the genes does not necessary derive from viable cell and thus also dead bacterial cells are quantified by the RT-PCR. Also, the amplification of the genes by PCR could not necessary be a confirmation of the incrimination of a *B. cereus* strain as a food poisoning agent because the capability of the *B. cereus* isolates to express emetic toxin genes was shown to vary 1000-fold between the different strains. In addition, several studies showed that, although the diarrhoeal toxin genes were amplified by PCR from different strains, their expression, or at least the expression of all the subunits of the three component toxins was not always confirmed. On the other hand, it is important to take into account the presence of PCR inhibitors in food matrices which could lead to false-negative results, and that the sensitivity of quantitative RT-PCR is affected by the presence of DNA polymorphisms of the toxin genes.

In conclusion, from a food safety perspective, important foodborne pathogens within the genus Bacillus are the species of the B. cereus group and in particular *B. cereus*. However, other species within the genus should be considered when examining suspected samples, since several strains of *Bacillus* species other than those of the *B. cereus* group have been described to produce lipophilic compounds such as surfactants with potential toxicity for humans. As far as the detection of *B. cereus*, a combination of molecular methods, including PCR amplification of the toxin-encoding genes and cytotoxicity assays represent the major tools necessary for risk assessment evaluation.

References:

1. Salkinoja-Salonen, M. S., et al., Toxigenic strains of *Bacillus licheniformis* related to food poisoning, Appl. Environ. Microbiol., 65, 4637, 1999.
2. Taylor, J. M. W. et al., Heat-stable toxin production by strains of *Bacillus cereus*, *Bacillus firmus*, *Bacillus megaterium*, *Bacillus simplex* and *Bacillus licheniformis*, FEMS Microbiol. Lett., 242, 313, 2005.
3. From, C. et al., Toxin-Producing Ability among *Bacillus* spp. outside the *Bacillus cereus* group, Appl. Environ. Microbiol., 71, 1178, 2005.
4. Thorsen, L. et al., Characterization of emetic *Bacillus weihenstephanensis*, a new cereulide-producing bacterium, Appl. Environ. Microbiol., 72, 5118, 2006.
5. From, C. et al., Cytotoxicity in *Bacillus mojavensis* is abolished following loss of surfactin synthesis: implications for assessment of toxicity and food poisoning potential, Int. J. Food Microbiol., 117, 43, 2007.
6. Brown, K. N., Spore resistance and ultra heat treatment processes, J. Appl. Bacteriol., 76, 67, 1994.
7. Østensvik, Ø. et al., Cytotoxic *Bacillus* spp. belonging to the *B. cereus* and *B. subtilis* groups in Norwegian surface waters, J. Appl. Microbiol., 96, 987, 2004.

8. Cronin, U. P., and Wilkinson, M. G., *Bacillus cereus* endospores exhibit a heterogeneous response to heat treatment and low-temperature storage, Food Microbiol., 25, 235, 2008.
9. Daffonchio, D., Cherif, A., and Borin, S., Homoduplex and heteroduplex polymorphisms of the amplified ribosomal 16S-23S internal transcribed spacers describe genetic relationships in the "*Bacillus cereus* group", Appl. Environ. Microbiol., 66, 5460, 2000.
10. Cherif, A. et al., Genetic relationship in the *'Bacillus cereus* group' by rep-PCR fingerprinting and sequencing of a *Bacillus anthracis*-specific rep-PCR fragment, J. Appl. Microbiol., 94, 1108, 2003.
11. Slaten, D. D., Oropeza, R. I., and Werner, S. B., An outbreak of *Bacillus cereus* food poisoning - Are caterers supervised sufficiently?, Public Health Rep., 107, 477, 1992.
12. Dierick, K. et al., Fatal family outbreak of *Bacillus cereus*-associated food poisoning, J. Clin. Microbiol., 43, 4277, 2005.
13. Ehling-Schultz, M. et al., Toxin gene profiling of enterotoxic and emetic *Bacillus cereus*. FEMS Microbiol. Lett., 260, 232, 2006.
14. Lequin, M. H. et al., *Bacillus cereus* meningoencephalitis in preterm infants: neuroimaging characteristics, Am. J. Neuroradiol., 26, 2137, 2005.
15. John, A. B. et al., Intractable *Bacillus cereus* bacteremia in a preterm neonate, J. Trop. Pediatr., 53, 131, 2007.
16. Sue, D. et al., Capsule production in *Bacillus cereus* strains associated with severe pneumonia, J. Clin. Microbiol., 44, 3426, 2006.
17. Pillai, A., Thomas, S., and Arora, J., *Bacillus cereus*: the forgotten pathogen. Surg. Infect., 7, 305, 2006.
18. Altiparmak, U. E., et al., Postoperative endophthalmitis caused by *Bacillus cereus* and *Chlamydia trachomatis*, J. Cataract. Refract. Surg., 33, 1284, 2007.

19. Beatty, M. E., et al., Gastrointestinal anthrax: review of the literature, Arch. Intern. Med., 163, 2527, 2003.
20. Ichhpujani, R. L. et al., An outbreak of human anthrax in Mysore (India), J. Commun. Dis., 36, 199, 2004.
21. Babamahmoodi, F. et al., Three rare cases of anthrax arising from the same source, J. Infect., 53, 175, 2006.
22. Glomski, I. J., et al., Primary involvement of pharynx and peyer's patch in inhalational and intestinal anthrax, PLoS Pathog., 3, e76, 2007.
23. Lechner, S., et al., *Bacillus weihenstephanensis* sp. nov. is a new psychrotolerant species of the *Bacillus cereus* group, Int. J. Syst. Bacteriol., 48, 1373, 1998.
24. Baron, F., et al., Isolation and characterization of a psychrotolerant toxin producer, *Bacillus weihenstephanensis*, in liquid egg products, J. Food Prot., 70, 2782, 2007.
25. Nakamura, L. K., DNA relatedness among *Bacillus thuringiensis* serovars, Int. J. Syst. Bacteriol., 44, 125, 1994.
26. Nakamura, L. K., and Jackson, M. A., Clarification of the taxonomy of *Bacillus mycoides*, Int. J. Syst. Bacteriol., 45, 46, 1995.
27. Nakamura, L. K., *Bacillus pseudomycoides* sp. nov., Int. J. Syst. Bacteriol., 48, 1031, 1998.
28. Ash, C. et al., Phylogenetic heterogeneity of the genus *Bacillus* revealed by comparative analysis of small-subunit-ribosomal RNA sequences, Lett. Appl. Microbiol., 13, 202, 1991.
29. Ash, C., et al., Comparative analysis of *Bacillus anthracis*, *Bacillus cereus*, and related species on the basis of reverse transcriptase sequencing of 16S rRNA, Int. J. Syst. Bacteriol., 41, 343, 1991.

30. Ash, C., and Collins, M. D., Comparative analysis of 23S ribosomal RNA gene sequences of *Bacillus anthracis* and emetic *Bacillus cereus* determined by PCR-direct sequencing. FEMS Microbiol. Lett., 94, 75, 1992.

31. Brousseau, R., et al., Arbitrary primer polymerase chain reaction, a powerful method to identify *Bacillus thuringiensis* serovars and strains, Appl. Environ. Microbiol., 59, 114, 1993.

32. Carlson, C. R., Grønstad, A. and Kolstø, A.-B., Physical maps of the genomes of three *Bacillus cereus* strains, J. Bacteriol., 174, 3750, 1992.

33. Carlson, C. R., and Kolstø, A.-B., A complete physical map of a *Bacillus thuringiensis* chromosome, J. Bacteriol., 175, 1053, 1993.

34. Carlson, C. R., Caugant, D. A., and Kolstø, A.-B., Genotypic diversity among *Bacillus cereus* and *Bacillus thuringiensis* strains, Appl. Environ. Microbiol., 60, 1719, 1994.

35. Carlson, C. R., Johansen, T., and Kolstø, A.-B., The chromosome map of *Bacillus thuringiensis* subsp. *canadensis* HD224 is highly similar to that of the *Bacillus cereus* type strain ATCC 14579, FEMS Microbiol. Lett., 141, 163, 1996.

36. Daffonchio, D. et al., Restriction Site Insertion-PCR (RSI-PCR) for rapid discrimination and typing of closely related microbial strains, FEMS Microbiol. Lett., 180, 77, 1999.

37. Daffonchio, D. et al., A randomly amplified polymorphic DNA marker specific for the *Bacillus cereus* group is diagnostic for *Bacillus anthracis,* Appl. Environ. Microbiol., 65, 1298, 1999.

38. Kim, Y. R, Czajka, J., and Batt, C. A., Development of a fluorogenic probe-based PCR assay for detection of *Bacillus cereus* in nonfat dry milk. Appl. Environ. Microbiol., 66, 1453, 2000.

39. Schraft, H., and Griffith, M. W., Specific oligonucleotide primers for detection of lecitinase-positive *Bacillus* spp. by PCR, Appl. Environ. Microbiol., 61, 98, 1995.

40. Schraft, H. et al., Epidemiological typing of *Bacillus* spp. isolated from food, Appl. Environ. Microbiol., 62, 4229, 1996.
41. Helgason, E. et al., *Bacillus anthracis, Bacillus cereus*, and *Bacillus thuringiensis*-one species on the basis of genetic evidence, Appl. Environ. Microbiol., 66, 2627, 2000.
42. Helgason, E. et al., Multilocus sequence typing scheme for bacteria of the *Bacillus cereus* group, Appl. Environ. Microbiol., 70, 191, 2004.
43. Daffonchio, D. et al., Strategy for identification of *Bacillus cereus* and *Bacillus thuringiensis* strains closely related to *Bacillus anthracis*, Appl. Environ. Microbiol., 72, 1295, 2006.
44. Ivanova, N. et al., Genome sequence of *Bacillus cereus* and comparative analysis with *Bacillus anthracis*, Nature, 423, 87, 2003.
45. Margulis, L., et al., The *Arthromitus* stage of *Bacillus cereus*: intestinal symbionts of animals, Proc. Natl. Acad. Sci. USA, 95, 1236, 1998.
46. Jensen, G.B. et al., The hidden lifestyles of *Bacillus cereus* and relatives, Environ. Microbiol., 5, 631, 2003.
47. Swiecicka I. and Mahillon J., Diversityof commensal Bacillus cereus sensu latoisolated from the commonsowbug (Porcellio scaber, Isopoda), FEMS MIcrobiol. Ecol., 56, 132, 2006.
48. Gohar, M. et al., A comparative study of *Bacillus cereus*, *Bacillus thuringiensis* and *Bacillus anthracis* extracellular proteomes. Proteomics, 5, 3696, 2005.
49. Keim, P. et al., Molecular evolution and diversity in *Bacillus anthracis* as detected by amplified fragment length polymorphism markers, J. Bacteriol., 179, 818, 1997.
50. Van Ert, M. N. et al., Global genetic population structure of *Bacillus anthracis*, PLoS ONE, 2, e461, 2007.

51. Helgason, E. et al., Genetic structure of population of *Bacillus cereus* and *B. thuringiensis* isolates associated with periodontitis and other human infections. J. Clin. Microbiol., 38, 1615, 2000.
52. Mignot, T. et al., The incompatibility between the PlcR- and AtxA-controlled regulons may have selected a nonsense mutation in *Bacillus anthracis*, Mol. Microbiol., 42, 1189, 2001.
53. Agaisse, H. et al., PlcR is a pleiotropic regulator of extracellular virulence factor gene expression in *Bacillus thuringiensis*, Mol. Microbiol., 32, 1043, 1999.
54. Okstad, O.A. et al., Sequence analysis of three *Bacillus cereus* loci carrying PIcR-regulated genes encoding degradative enzymes and enterotoxin, Microbiol., 145, 3129, 1999.
55. Lund, T., De Buyser, M. L., and Granum, P. E., A new cytotoxin from *Bacillus cereus* that may cause necrotic enteritis, Mol. Microbiol., 38, 254, 2000.
56. Duport, C. et al., Control of enterotoxin gene expression in *Bacillus cereus* F4430/73 involves the redox-sensitive ResDE signal transduction system, J. Bacteriol., 188, 6640, 2006.
57. Zigha, A. et al., Anaerobic cells of *Bacillus cereus* F4430/73 respond to low oxidoreduction potential by metabolic readjustments and activation of enterotoxin expression, Arch. Microbiol., 185, 222, 2006.
58. Zigha, A. et al., The redox regulator Fnr is required for fermentative growth and enterotoxin synthesis in *Bacillus cereus* F4430/73, J. Bacteriol., 189, 2813, 2007.
59. Slamti, L., and Lereclus, D., A cell-cell signaling peptide activates the PlcR virulence regulon in bacteria of the *Bacillus cereus* group, EMBO J., 21, 4550, 2002.
60. Hoffmaster, A. R., et al., Identification of anthrax toxin genes in a *Bacillus cereus* associated with an illness resembling inhalation anthrax, Proc. Natl. Acad. Sci. USA, 101, 8449, 2004.

61. Radnedge, L. et al., Genome differences that distinguish *Bacillus anthracis* from *Bacillus cereus* and *Bacillus thuringiensis*, Appl. Environ. Microbiol., 69, 2755, 2003.

62. Read, T. D. et al., The genome sequence of *Bacillus anthracis* Ames and comparison to closely related bacteria. Nature, 423, 81, 2003.

63. Zwick, M.E. et al., Microarray-based resequencing of multiple *Bacillus anthracis* isolates. Genome Biol 6: R10, 2005.

64. Keim, P. et al., Multiple-locus variable-number tandem repeat analysis reveals genetic relationships within *Bacillus anthracis*. J. Bacteriol., 182, 2928, 2000.

65. Read, T. D. et al., Comparative genome sequencing for discovery of novel polymorphisms in *Bacillus anthracis*. Science, 296, 2028, 2002.

66. Gierczyński, R. et al. Intriguing diversity of *Bacillus anthracis* in eastern Poland - the molecular echoes of the past outbreaks. FEMS Microbiol. Lett. 239, 235, 2004.

67. Merabishvili, M et al., Diversity of *Bacillus anthracis* strains in Georgia and of vaccine strains from the former Soviet Union, Appl. Environ. Microbiol., 72, 5631, 2006.

68. Ciammaruconi, A. et al. Fieldable genotyping of *Bacillus anthracis* and *Yersinia pestis* based on 25-loci Multi Locus VNTR Analysis. BMC Microbiol 8, 21, 2008.

69. Prüss, B. M. et al., Correlation of 16S ribosomal DNA signature sequences with temperature-dependent growth rates of mesophilic and psychrotolerant strains of the *Bacillus cereus* group, J. Bacteriol., 181, 2624, 1999.

70. Hernandez, E. et al., Super-infection by *Bacillus thuringiensis* H34 or 3a3b can lead to death in mice infected with the influenza A virus, FEMS Immunol. Med. Microbiol., 29, 177, 2000.

71. Hill, K.K. et al., Fluorescent amplified fragment length polymorphism analysis of *Bacillus anthracis*, *Bacillus cereus*, and *Bacillus thuringiensis* isolates, Appl. Environ. Microbiol., 70, 1068, 2004.

72. Rasko, D. A. et al., Genomics of the *Bacillus cereus* group of organisms, FEMS Microbiol. Rev., 29, 303, 2005.

73. Tourasse, N. J., and Kolstø, A. B. SuperCAT: a supertree database for combined and integrative multilocus sequence typing analysis of the *Bacillus cereus* group of bacteria (including *B. cereus*, *B. anthracis* and *B. thuringiensis*). Nucleic Acids Res. 36, D461, 2007.

74. Priest, F. G. et al., Population structure and evolution of the Bacillus cereus group. J. Bacteriol. 186, 7959, 2004.

75. Candelon, B., Two distinct types of rRNA operons in the Bacillus cereus group. Microbiology, 150, 601, 2004.

76. Ko, K. S. et al. Population structure of the *Bacillus cereus* group as determined by sequence analysis of six housekeeping genes and the *plcR* gene. Infect. Immun., 72, 5253, 2004.

77. Sorokin, A., et al. Multiple-locus sequence typing analysis of *Bacillus cereus* and *Bacillus thuringiensis* reveals separate clustering and a distinct population structure of psychrotrophic strains. Appl. Environ. Microbiol., 72, 1569, 2006.

78. Haug, S., Food poisoning caused by aerobic spore forming bacilli, J. Appl. Bacteriol., 18, 591, 1955.

79. Altayar, M., and Sutherland, A. D., *Bacillus cereus* is common in the environment but emetic toxin producing isolates are rare. J. Appl. Microbiol., 100, 7, 2006.

80. in't Veld, P. H. et al., Detection of genes encoding for enterotoxins and determination of the production of enterotoxins by HBL blood plates and immunoassays of psychrotrophic strains of *Bacillus cereus* isolated from pasteurised milk, Int. J. Food Microbiol., 64, 63, 2001.

81. Smith, D. P. et al., Detection of *Bacillus cereus* on selected retail chicken products, J. Food Prot., 67, 1770, 2004.

82. Collier, F. A., Elliot, S. L., and Ellis, R. J., Spatial variation in *Bacillus thuringiensis/cereus* populations within the phyllosphere of broad-leaved dock (*Rumex obtusifolius*) and surrounding habitats, FEMS Microbiol. Ecol., 54, 417, 2005.
83. Wijnands, L. M. et al., Prevalence of potentially pathogenic *Bacillus cereus* in food commodities in The Netherlands, J. Food Prot., 69, 2587, 2006.
84. Reyes, J. E. et al., Prevalence of *Bacillus cereus* in dried milk products used by Chilean School Feeding Program, Food Microbiol., 24, 1, 2007.
85. King, N. J., Whyte, R., and Hudson, J. A., Presence and significance of *Bacillus cereus* in dehydrated potato products, J. Food Prot., 70, 514, 2007.
86. Choo, E. et al., Prevalence and genetic diversity of *Bacillus cereus* in dried red pepper in Korea, J. Food Prot., 70, 917, 2007.
87. Valero, M., Hernández-Herrero, L. A and Giner, M. J., Survival, isolation and characterization of a psychrotrophic *Bacillus cereus* strain from a mayonnaise-based ready-to-eat vegetable salad, Food Microbiol., 24, 671, 2007.
88. Mahler, H., Pasi, A. and Kramer, J. A., Fulminant liver failure in association with the emetic toxin of *Bacillus cereus*, New. Engl. J. Med., 336, 1142, 1997.
89. Guinebretière, M.-H. et al., Rapid discrimination ofcytK-1 andcytK-2 genes in *Bacillus cereus* strains by a novel duplex PCR system, FEMS Microbiol. Lett., 259, 74, 2006.
90. Gray, K. M. et al., Rapid Ped-2E9 cell-based cytotoxicity analysis and genotyping of *Bacillus* species. J. Clin. Microbiol., 43, 5865, 2005.
91. Ehling-Schulz, M., Fricker, M., and Scherer, S., Identification of emetic toxin producing *Bacillus cereus* strains by a novel molecular assay, FEMS Microbiol. Lett., 232, 189, 2004.
92. Ehling-Schulz, M., et al., *Bacillus cereus*, the causative agent of an emetic type of food-borne illness, Mol. Nutr. Food Res., 48, 479, 2004.

93. Agata, N. et al., A novel dodecadepsipeptide, cereulide is an emetic toxin of *Bacillus cereus*, FEMS Microbiol. Lett., 129, 17, 1995.

94. Beecher, D. J., Schoeni, J. L. and Wong, A. C., Enterotoxic activity of hemolysin BL from *Bacillus cereus*, Infect. Immun., 63, 4423, 1995.

95. Lund, T. and Granum, P. E., Characterization of a nonhaemolytic enterotoxin complex from *Bacillus cereus* isolated after a foodborne outbreak, FEMS Microbiol. Lett., 141, 151, 1996.

96. Auger, S., et al., The genetically remote pathogenic strain NVH391-98 of the *Bacillus cereus* group is representative of a cluster of thermophilic strains, Appl. Environ. Microbiol., 74, 1276, 2008.

97. Fagerlund, A., et al., Toxin production in a rare and genetically remote cluster of strains of the *Bacillus cereus* group, BMC Microbiol., 7, 43, 2007.

98. Hardy, S. P., Lund, T., and Granum, P. E., CytK toxin of *Bacillus cereus* forms pores in planar lipid bilayers and is cytotoxic to intestinal epithelia, FEMS Microbiol. Lett., 197, 47, 2001.

99. Lapidus, A. et al., Extending the *Bacillus cereus* group genomics to putative food-borne pathogens of different toxicity, Chem. Biol. Int., 171, 236, 2008.

100. Beecher, D. J., and Macmillan, J. D., Characterization of the components of hemolysin BL from *Bacillus cereus*, Infect. Immun., 59, 1778, 1991.

101. Lindbäck, T. et al., Characterization of the *Bacillus cereus* Nhe enterotoxin, Microbiol., 150, 3959, 2004.

102. Dietrich, R. et al., Production and characterization of monoclonal antibodies against the hemolysin BL enterotoxin complex produced by *Bacillus cereus*, Appl. Environ. Microbiol., 65, 4470, 1999.

103. Moravek, M. et al., Determination of the toxic potential of *Bacillus cereus* isolates by quantitative enterotoxin analyses, FEMS Microbiol. Lett., 257, 293, 2006.

104. Fagerlund, A. et al., *Bacillus cereus* Nhe is a pore-forming toxin with structural and functional properties similar to the ClyA (HlyE, SheA) family of haemolysins, able to induce osmotic lysis in epithelia, Microbiol., 154, 693, 2008.

105. Choma, C., and Granum, P. E., The enterotoxin T (BcET) from *Bacillus cereus* can probably not contribute to food poisoning, FEMS Microbiol. Lett., 217, 115, 2002.

106. Agata, N., Ohta, M., and Yokoyama, K., Production of *Bacillus cereus* emetic toxin (cereulide) in various foods, Int. J. Food Microbiol., 73, 23, 2002.

107. Agata, N. et al., A novel dodecadepsipeptide, cereulide, isolated from *Bacillus cereus* causes vacuole formation in HEp-2 cells, FEMS Microbiol., Lett., 121, 31, 1994.

108. Horwood, P. F., Burgess, G. W., and Oakey, H. J., Evidence for non-ribosomal peptide synthetase production of cereulide (the emetic toxin) in *Bacillus cereus*, FEMS Microbiol. Lett., 236, 319, 2004.

109. Hoton, F. M. et al., The cereulide genetic determinants of emetic *Bacillus cereus* are plasmid-borne, Microbiol., 151, 2121, 2005.

110. Rasko, D. A. et al., Complete sequence analysis of novel plasmids from emetic and periodontal *Bacillus cereus* isolates reveals a common evolutionary history among the *B. cereus*-group plasmids, including *Bacillus anthracis* pXO1, J. Bacteriol., 189, 52, 2007.

111. Jaaskelainen, E. L. et al., In vitro assay for human toxicity of cereulide, the emetic mitochondrial toxin produced by food poisoning *Bacillus cereus*, Toxicol. In Vitro, 107, 737, 2003.

112. Kawamura-Satò, K. et al., Quantitative analysis of cereulide, an emetic toxin of *Bacillus cereus*, by using rat liver mitochondria, Microbiol. Immunol., 49, 25, 2005.

113. Rajkovic, A. et al., Heat resistance of *Bacillus cereus* emetic toxin, cereulide, Lett. Appl. Microbiol., 46, 536, 2008.

114. Teplova, V. V. et al., The higher toxicity of cereulide relative to valinomycin is due to its higher affinity for potassium at physiological plasma concentration, Int. J. Food Microbiol., 109, 132, 2006.

115. Ehling-Schulz, M. et al., Identification and partial characterization of the nonribosomal peptide synthetase gene responsible for cereulide productionin emetic *Bacillus cereus*, Appl. Environ. Microbiol., 71, 105, 2005.

116. Carlin, F. et al., Emetic toxin-producing strains of *Bacillus cereus* show distinct characteristics within the *Bacillus cereus* group, J. Food. Prot., 69, 847, 2006.

117. Gilbert, R. J., and Parry, J. M., Serotypes of *Bacillus cereus* from outbreaks of food poisoning and from routine foods, J. Hyg. (Lond)., 78, 69, 1977.

118. Vassileva, M. et al., A new phylogenetic cluster of cereulide-producing *Bacillus cereus* strains, J. Clin. Microbiol., 45, 1274, 2007.

119. Hu, X. et al., Transfer and expression of the mosquitocidal plasmid pBtoxis in *Bacillus cereus* group strains, FEMS Microbiol. Lett., 245, 239, 2005.

120. Van der Auwera, G. A. et al., Plasmid exchanges among members of the *Bacillus cereus* group in foodstuffs, Int. J. Food Microbiol., 113, 164, 2007.

121. Van der Auwera, G. A., Timmery, S., and Mahillon, J., Self-transfer and mobilisation capabilities of the pXO2-like plasmid pBT9727 from *Bacillus thuringiensis* subsp. *konkukian* 97-27, Plasmid, 59, 134, 2008.

122. Yuan, Y. M. et al., Kinetics of plasmid transfer among *Bacillus cereus* group strains within lepidopteran larvae, Arch. Microbiol., 187, 425, 2007.

123. Panico, M. G., Caporale, V., and Agozzino, E., Investigating on a foodborne outbreak: analysis of the critical points, Ann. Ig., 18, 191, 2006.

124. Yang, I. C. et al., Development of rapid Real-Time PCR and Most-Probable-Number Real-Time PCR assays to quantify enterotoxigenic strains of the species in the *Bacillus cereus* group, J. Food Prot., 70, 2774, 2007.

125. Hedberg, C. W. et al., The use of clinical profiles in the investigation of foodborne outbreaks in restaurants: United States, 1982-1997, Epidemiol. Infect., 136, 65, 2008.

126. Ripabelli, G. et al., Epidemiological typing of *Bacillus cereus* by amplified fragment length polymorphism, Lett. Appl. Microbiol., 30, 358, 2000.

127. Ghelardi, E. et al., Identification and characterization of toxigenic *Bacillus cereus* isolates responsible for two food-poisoning outbreaks, FEMS Microbiol. Lett., 208, 129, 2002.

128. Fricker, M. et al., Diagnostic Real-Time PCR assays for the detection of emetic *Bacillus cereus* strains in foods and recent food-borne outbreaks, Appl. Environ. Microbiol., 73, 1892, 2007.

129. Ombui, J. N., and Nduhiu, J. G., Prevalence of enterotoxigenic *Bacillus cereus* and its enterotoxins in milk and milk products in and around Nairobi, East. Afr. Med. J., 82, 280, 2005.

130. Mikkola, R. et al., Toxic lactonic lipopeptide from food poisoning isolates of *Bacillus licheniformis*, Eur. J. Biochem., 267, 4068, 2000.

131. Nieminen, T. et al., Toxinogenic *Bacillus pumilus* and *Bacillus licheniformis* from mastitic milk, Veter. Microbiol., 124, 329, 2007.

132. Duca, Le H. et al., Cases of emesis associated with bacterial contamination of an infant breakfast cereal product, Int. Journal. Food Microbiol., 102, 245, 2005.

133. Kramer, J. M. et al., Identification and characterization of *Bacillus cereus* and other *Bacillus* species associated with foods and food poisoning. In: Corry, J. E. L., Roberts, D., and Skinner, F. A. (Eds.), Isolation and identification methods for food poisoning organisms. Academic Press, London, 261, 1982.

134. Suominen, I. et al., Toxic *Bacillus pumilus* from indoor air, recycled paper pulp, Norway spruce, food poisoning outbreaks and clinical samples, Syst. Appl. Microbiol., 24, 267, 2001.

135. Hoornstra, D. et al., A new method for in vitro detection of microbially produced mitochondrial toxins, Toxicol. In Vitro, 17, 745, 2003.

136. Christiansson, A. et al., Toxin production by *Bacillus cereus* dairy isolates in milk at low temperatures, Appl. Environ. Microbiol., 55, 2595, 1989.

137. Fletcher, P., and Logan, N. A., Improved cytotoxicity assay for *Bacillus cereus* diarrhoeal enterotoxin, Lett. Appl. Microbiol., 28, 394, 1999.

138. Gilmore, M. S., et al., A *Bacillus cereus* cytolytic determinant, cereolysin AB, which comprises the phospholipase C and sphingomyelinase genes: nucleotide sequence and genetic linkage. J. Bacteriol., 171, 744, 1989.

139. Gohar, M., et al., Two-dimensional electrophoresis analysis of the extracellular proteome of *Bacillus cereus* reveals the importance of the PlcR regulon, Proteomics, 2, 784, 2002.

140. Hughes, S. et al., Potential application of a HEp-2 cell assay in the investigation of *Bacillus cereus* emetic-syndrome food poisoning. FEMS Microbiol. Lett., 52, 7, 1988.

141. Shinagawa, K. et al., Emesis of rhesusmonkeys induced by intragastric administration with the HEp-2 vacuolation factor (cereulide) produced by *Bacillus cereus*. FEMS Microbiol. Lett., 130, 87, 1995.

142. Finlay, W. J., Logan, N. A., and Sutherland, A. D., Semiautomated metabolic staining assay for *Bacillus cereus* emetic toxin, Appl. Environ. Microbiol., 65, 1811, 1999.

143. Rowan, N. J. et al., Putative virulence factor expression by clinical and food isolates of *Bacillus* spp. after growth in reconstituted infant milk formulae, Appl. Environ. Microbiol., 67, 3873, 2001.

144. Andersson, M. A. et al., A novel sensitive bioassay for detection of *Bacillus cereus* emetic toxin and related depsipeptide ionophores. Appl. Environ. Microbiol., 64, 1338, 1998.

145. Andersson, M. A. et al., Sperm bioassay for rapid detection of cereulide-producing *Bacillus cereus* in food and related environments, Int. J. Food Microbiol., 94, 175, 2004.

146. Rajkovic, A., Uyttendaele, M., and Debevere, J., Computer aided boar semen motility analysis for cereulide detection in different food matrices, Int. J. Food Microbiol., 114 92, 2007.

147. Haggblom, M. M. et al., Quantitative analysis of cereulide, the emetic toxin of *Bacillus cereus* produced under various conditions, Appl. Environ. Microbiol., 68, 2479, 2002.

148. Rajkovic, A. et al., Influence of type of food on the kinetics and overall production of *Bacillus cereus* emetic toxin, J. Food Prot., 69, 847, 2006.

149. Rajkovic, A. et al., Dynamics of boar semen motility inhibition as a semi-quantitative measurement of *Bacillus cereus* emetic toxin (Cereulide), J. Microbiol. Methods, 65, 525, 2006.

150. Schulten, S. M. et al., Evaluation of the ISO 7932 standard for the enumeration of *Bacillus cereus* in foods, Int. J. Food Microbiol., 57, 53, 2000.

151. Evans, M. E., Feola, D. J., and Rapp, R. P., Polymyxin B sulfate and colistin: old antibiotics for emerging multiresistant gram-negative bacteria, Annals Pharmacoth., 33, 960, 1999.

152. Fricker, M., Reissbrodt, R., and Ehling-Schulz M., Evaluation of standard and new chromogenic selective plating media for isolation and identification of *Bacillus cereus*, Int. J. Food Microbiol., 121, 27, 2008.

153. Damgaard, P. H., Diarrhoeal enterotoxin production by strains of *Bacillus thuringiensis* isolated from commercial *Bacillus thuringiensis*-based insecticides, FEMS Immun. Med. Microbiol., 12, 245, 1995.

154. Hansen, B. M., and Hendriksen, N. B., Detection of enterotoxic *Bacillus cereus* and *Bacillus thuringiensis* strains by PCR analysis, Appl. Environ. Microbiol., 67, 185, 2001.

155. Swiecicka, I. et al., Haemolytic and nonhaemolytic enterotoxin genes are broadly distributed among *Bacillus thuringiensis* isolated from wild mammals, Microbial. Ecol., 52, 544, 2006.

156. Ngamwongsatit, P. et al., Broad distribution of enterotoxin genes (*hblCDA, nheABC, cytK,* and *entFM*) among *Bacillus thuringiensis* and *Bacillus cereus* as shown by novel primers, Int. J. Food. Microbiol., 121, 352, 2008.

157. Beecher, D. J. and Wong, A. C. L., Identification and analysis of the antigens detected by two commercial *Bacillus cereus* diarrheal enterotoxin immunoassay kits, Appl. Environ. Microbiol., 60, 4614, 1994.

158. Moravek, M. et al., Colony immunoblot assay for the detection of hemolysin BL enterotoxin producing *Bacillus cereus,* FEMS Microbiol. Lett., 238, 107, 2004.

159. Pal, S. et al., Nanowire labeled direct-charge transfer biosensor for detecting *Bacillus* species, Biosen. Bioelect., 22, 2329, 2007.

160. Jin, D.Z. et al., Detection and identification of intestinal pathogens in clinical specimens using DNA microarrays, Mol. Cell. Probes, 20, 337, 2006.

161. Wang, R. F. et al., Design and evaluation of oligonucleotide-microarray method for the detection of human intestinal bacteria in fecal samples, FEMS Microbiol. Lett., 213, 175, 2006.

162. Wang, et al., Development and application of an oligonucleotide microarray for the detection of food-borne bacterial pathogens, Appl. Microbiol. Biotechnol., 76, 225, 2007.

163. Park, S. H. et al., Simultaneous detection and identification of *Bacillus cereus* group bacteria using multiplex PCR, J. Microbiol. Biotechnol., 17, 1177, 2007.

164. Jackson, S. G., Rapid screening test for enterotoxin-producing *Bacillus cereus*, J. Clin. Microbiol., 31, 972, 1993.

165. Fukushima, H. et al., Rapid separation and concentration of food-borne pathogens in food samples prior to quantification by viable-cell counting and Real-Time PCR, Appl. Environ. Microbiol., 73, 92, 2007.

166. Feng, P., Impact of molecular biology on the detection of foodborne pathogens, Mol. Biotechnol., 7, 267, 1997.

167. McKillip, J. L., and Drake, M., Real-time nucleic acid–based detection methods for pathogenic bacteria in food, J. Food Prot., 67, 823, 2004.

168. Sambrook, J., Fritsch, E. F., and Maniatis, T., Molecular cloning: a laboratory manual, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. 1989.

169. Slamti, L. et al., Distinct mutations in PlcR explain why some strains of the *Bacillus cereus* group are nonhemolytic, J. Bacteriol., 186, 3531, 2004.

170. Easterday, W. R. et al., Specific detection of *Bacillus anthracis* using a TaqMan mismatch amplification mutation assay, Biotechniques, 38, 731, 2005.

171. Easterday, W. R. et al., Use of single nucleotide polymorphisms in the plcR gene for specific identification of *Bacillus anthracis*, J. Clin. Microbiol., 43, 1995, 2005.

172. Gierczyński, R. et al., Specific *Bacillus anthracis* identification by a plcR–targeted restriction site insertion-PCR (RSI-PCR) assay, FEMS Microbiol. Lett., 272, 55, 2007.

173. Melling, J., and Capel, B. J., Characteristics of *Bacillus cereus* emetic toxin. FEMS Microbiol. Lett., 4, 133, 1978.

174. Liu, Y. et al., Confirmative electric DNA array-based test for food poisoning *Bacillus cereus*, J. Microbiol. Methods, 70, 55, 2007.

175. Ngamwongsatit, P. et al., WST-1-based cell cytotoxicity assay as a substitute for MTT-based assay for rapid detection of toxigenic *Bacillus* species using CHO cell line, J. Microbiol. Methods, 73, 211, 2008.

176. Magarvey, N. A., Ehling-Schulz, M., and Walsh, C. T., Characterizationof the cereulide NRPS α-hydroxy acid specifying modules: activation of α-keto acids and chiral reduction on the assembly line, J. Am. Chem. Soc., 128, 10698, 2006.

177. Bennett, R. W., Staphylococcal enterotoxin and its rapid identificationin foods by enzyme-linked immunosorbent assay-based methodology, J. Food Prot., 68, 1264, 2005.