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Characterization of *Bacillus cereus*

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Abstract

Bacillus cereus is an ubiquitary microorganism that may cause foodborne disease. The two known types of disease, emetic and diarrheal, are caused by toxins: the emetic type is due to a single heat-stable toxin, and the diarrheal type to 3 or 4 heat-instable enterotoxins.

Since (entero-)toxins are the compounds that cause disease and since *B. cereus* may be part of the transient human flora, detection of *B. cereus* by microbiological methods does not suffice to determine definitively its involvement in foodborne disease. Therefore, further characterization to elucidate the presence of or the potential to form (entero-)toxins is of great importance. In this report methods for further characterization and their applications are described.

These methods can also be used to characterize *B. cereus* strains occurring in food. Thus, the the pathogenic potential of those strains and the possible health hazard may be determined.

The application of these methods may lead to re-evaluation of tolerance levels for *B. cereus* in food commodities.

Contents

Samenvatting

Bacillus cereus is een algemeen voorkomend micro-organisme dat verantwoordelijk kan zijn voor voedsel gerelateerde aandoeningen. Het vermogen van het micro-organisme om ziekte te verwekken hangt evenwel af van eigenschappen zoals de mogelijkheid tot het vormen van (entero)-toxine(n) die niet met microbiologische methoden kunnen worden gedetecteerd. Zodoende is verder onderzoek naar ziekteverwekkende eigenschappen niet alleen van belang om karakteristieken van stammen te identificeren, maar ook om het vóórkomen van ziekteverwekkende stammen op bepaalde voedsel-componenten na te gaan. Methoden die verdere karakterisering mogelijk maken staan beschreven in dit rapport.

Aan de hand van een "case study" wordt duidelijk gemaakt hoe de diverse methoden kunnen worden toegepast. Tevens wordt ingegaan op de toepassing en mogelijkheden van de methoden bij het onderzoek van stammen die op levensmiddelen voorkomen.

Onderscheid kunnen maken tusen ziekteverwekkende en niet- ziekteverwekkende *B. cereus* stammen zou aanleiding kunnen geven tot het bijstellen van de criteria die gesteld zijn ten aanzien van het mogen vóórkomen van *B. cereus* in levensmiddelen. Nader onderzoek met betrekking tot het tot expressie komen van genen coderend voor enterotoxinen en de vorming en het werkingsmechanisme van de enterotoxine complexen is hierbij van veel belang.

Summary

Bacillus cereus is an ubiquitary microorganism that may cause food borne disease. Pathogenicity, however, depends on various characteristics such as the ability to form (entero)-toxin(s) that can not be detected by microbiological methods. Further characterization of pathogenic properties is not only of importance to identify strains from outbreaks of food borne disease, but also to investigate whether possible pathogenic strains occur on specific types of food. Methods to establish pathogenic properties are described in this report.

With a case study the use of the methods as described in this report is clarified. Also the use of methods in research of strains occurring in/on food commodities is discussed.

The possibility to discriminate between pathogenic and non-pathogenic *B. cereus* strains could lead to a re-evaluation of tolerance levels for *B. cereus* in food commodities. Therefore, further research into the conditions for expression of genes coding for enterotoxins, the way of construction and pathogenic mechanism of enterotoxins is of great importance.

1.   Introduction

Bacillus (B.) species are ubiquitary organisms; they can readily be isolated from soil, water, dust and air. At this moment about 50 species have been described. Some *B.* species have applications in industrial (enzyme-production) or environmental (insecticide) applications, and some are pathogenic to man and/or animal (Kramer and Gilbert 1989). The most important pathogenic species belong to the *B. cereus* group which consists of *B. cereus*, *B. mycoides*, *B. thuringiensis*, *B. anthracis* and the recently described *B. weihenstephanensis* (Lechner et al. 1998) and *B. pseudomycoides* (Nakamura 1998). The natural habitat for most species is the soil, and direct contamination of agricultural products from soil is of importance with respect to foodborne infection or intoxication and food spoilage (Kramer and Gilbert 1989).

The most important *Bacillus* species with respect to food is *B. cereus*. It is a Gram-positive, rod-shaped, and sporeforming organism. Growth may occur from pH 4.5 to 9.3; water-activity must be higher than 0.92 for growth and the temperature range for growth (4ºC – 50ºC) is very wide (Kramer and Gilbert 1989). Besides food related illnesses *B. cereus* may also cause non-gastrointestinal disease like endocarditis and endophthalmitis (Drobniewski 1993).

Besides occurrence in the environment *B. cereus* may be isolated from stools of healthy people, probably due to dietary intake. Estimates on the prevalence in healthy individuals range from 15 – 40% of the population. Human carriage is not considered to be of any significance in food-borne disease (Turnbull and Kramer 1985;Ghosh 1978).

B. cereus is the etiologic agent of two types of food-borne disease, namely a toxico-infection with diarrhea as major clinical symptom and an intoxication causing vomiting. Both types are caused by toxins: the diarrheal type by protein toxins which must be formed in the intestinal tract by growing organisms (enterotoxins), the emetic type by a peptide toxin that is preformed in the food (emetic toxin or cereulide) (Agata et al.1994; Agata et al. 1995).

The diarrheal type of disease is characterized by diarrhea within 12 hours after consumption of the suspected food. Other symptoms are nausea and total malaise. The emetic type of disease is characterized by vomiting and nausea within two hours after the consumption of the suspected food. For both types of disease the total duration of clinical signs is about 24 hours (Kramer and Gilbert 1989).

Although within the scope of project V/250912/01/AB (Quantitative research of *B. cereus* within the scope of hazard characterization and exposure assessment) primarily the species causing the diarrheal type are being investigated, we will discuss also the vomiting type by giving an overview of techniques that can be used for the diagnosis of *B. cereus* toxico-infections and intoxications.

The diarrheal type of disease is caused by *B. cereus* species that are able to produce enterotoxins. Sofar four toxins have been described: 1) Haemolytic BL toxin (HBL), a haemolytic enterotoxin complex made of three proteins, 2) Non Haemolytic Enterotoxin (NHE), a non-haemolytic enterotoxin complex made of three proteins, 3) enterotoxin T, a single protein, and 4) cytotoxin K, also a single protein. Three of these toxins are related to food borne outbreaks; the fourth, enterotoxin T, is not (Granum and Lund 1997;Lund et al. 2000;Agata et al. 1995).

The current hypothesis is that HBL and NHE are formed and excreted as a three-component complex during exponential growth of the organism, not only in vitro but also during active growth in the small intestine. This implies that an entire three-component complex is derived from one strain and not composed of components from various strains (P.E.Granum, personal communication).

Besides the discrimination of *B. cereus* on the basis of toxin-production, also growth-temperature is a means of discrimination. *B. cereus* species may be subdivided according to their thermotolerance: the psychrotrophes that can grow at temperatures as low as 4ºC, but fail to grow at temperatures above 37ºC, and the mesophiles, able to grow at temperatures ranging from 10ºC to, sometimes even, 50ºC (Kramer and Gilbert 1989).

The purpose of the description of methods in this report is twofold. Firstly, the described methods enable researchers to determine whether *B. cereus* strains found in samples associated with outbreaks of food-borne disease are indeed the cause for the outbreak. Since *B. cereus* may be part of the transient flora in humans, the detection of the organism in stool samples of people suspected to have a food-borne disease is not conclusive for its involvement. Other virulence factors such as the presence or absence of genes coding for enterotoxins, the thermotolerance or the ability to produce emetic toxin must be investigated. The methods also enable investigators to compare strains isolated after an outbreak. Similarity of strains isolated from food samples and patients may lead to a more reliable basis for determining the involvement of *B. cereus* in an outbreak.

Secondly, the described methods provide tools to further characterize *B. cereus* strains found in/on food-commodities. With such increased knowledge it is possible to determine whether isolated strains pose a health risk or not. Since *B. cereus* is an ubiquitous organism it can be encountered in many types of food. However, the discovery of *B. cereus* in/on food commodities does not necessarily imply a health risk, since not all *B. cereus* strains are pathogenic. As pathogenicity depends on the presence and nature of virulence factors such as the ability to produce (entero)-toxins, the methods described in this report for the detection of (genes coding for) (entero)-toxins enable researchers to determine whether the strains found on/in food can be considered pathogenic and thus pose a health risk.

Finally, the application of the methods described in this report as an identification tool in a food borne outbreak of *B. cereus* is described.

2. Microbiological methods

2.1 Enumeration and identification

To date enumeration and identification of *B. cereus* is usually carried out by microbiological methods.

Various media and methods for isolation and identification have been described, but none of these methods is conclusive in identifying *B. cereus*, let alone the *B. cereus* group. The most current medium for the isolation of *B. cereus* is Mannitol-Egg Yolk-Polymyxin agar (MEYP) (Mossel et al. 1967). A selective medium with diagnostic value that is closely related to MEYP is polymyxin-egg yolk-mannitol bromothymol blue agar (PEMBA) (Holbrook and Anderson 1980). Both media use characteristics like the lecithinase reaction and the inability to use mannitol as carbon source as identification properties and the resistance of the organism to polymyxin as a selective property.

For further identification and confirmation of *B. cereus* several biochemical reactions are in use (fermentation of glucose, nitrate reduction, Voges Proskauer reaction) (Anonymous 1999) and (Anonymous 1991). Individually or combined, none of these reactions is conclusive for the identification of *B. cereus*.

Various enumeration and identification protocols have been described. The ISO-method requires MEYP-agar for enumeration and glucose fermentation, nitrate reduction, and Voges Proskauer as confirmation (Borge et al. 2001). The USDA/FSIS Microbiology Laboratory guidebook (Dey and Lattuada 1998) prescribes MEYP-agar for examination of samples and enumeration. For further confirmation hemolytic activity, motility, growth characteristics (rhizoidity), and protein toxin crystal identification are prescribed (Lattuada and McClain 1998). Also commercial preparations give no conclusive identification schemes for *B. cereus* as demonstrated by the API 50 CH[®] bacterial identification system.

Sofar no conclusive microbiological or biochemical media/reactions have been described for the identification of *B. cereus*.

2.2 Growth temperature of *B. cereus* strains

Determination of temperature-tolerance of *B. cereus* strains is useful to discriminate strains. The microbiological method consists of growing newly inoculated strains at various temperatures, especially at the critical temperatures 10ºC and 42ºC and determining whether growth occurs or not. Strains can be subdivided as follows:

- mesophilic strains: able to grow above 10ºC and at 42ºC, optimal growth at temperatures between 30ºC and 37ºC.
- psychrophilic strains: able to grow below 10ºC and at 42ºC, optimal growth at temperatures between 30ºC and 37ºC, the ultimate growth temperature 37ºC.

- psychrotrophic strains: able to grow below 10ºC and not at 42ºC, usually 37ºC is the ultimate growth temperature.

A special psychrotrophic subspecies of *B. cereus* is *B. weihenstephanensis*, which is able to grow below 7ºC but, like all psychrotrophic strains, not above 37ºC, and which has another characteristic feature that will be discussed in paragraph 4.2 (Lechner and others 1998).

2.3   Properties of spores

Although such information does not directly contribute to the determination or identification of *B. cereus*, the properties of spores may help to characterize *B. cereus* strains for risk assessment purposes. The most important factor with respect to spores is the D-value, which is the time needed at a certain temperature to reduce the amount of viable spores by 90%. It gives an indication of the effectiveness of a certain heat treatment with respect to the killing of spores and the possibility of survival of spores. Derived from the D-values at various temperatures is the Z-value, the number of degrees Celsius needed to increase or decrease the D-value 10-fold. The Z-value gives an indication how a process must be altered to secure the inactivation properties.

Another indication may be the acid resistance of spores. At this moment, research in our laboratory to determine the acid resistance (or rather the stability of spores under stomach conditions) is being performed but not finished yet.

3.   Methods to detect enterotoxin-genes

The genome of (micro-)organisms contains all information necessary for survival and growth. Each gene encodes for a certain protein which is involved directly or indirectly in the life cycle of the organism. The presence of genes can be established by using PCR-methods. The active transcription of a gene, i.e. the production of messenger RNA (mRNA) can be determined by Reversed Transcriptase PCR (RT-PCR). The production of proteins, encoded by a gene, can be detected for example by using antibodies. One of the enterotoxins discussed here, HBL, can be detected with monoclonal antibodies (Dietrich et al. 1999).

In this chapter we describe methods to detect the presence of toxin-genes and not methods to detect transcription of genes nor methods for the detection of the presence of HBL with monoclonal antibodies.

As mentioned in the introduction there are four known enterotoxins: HBL, NHE, enterotoxin-T and cytotoxin-K. With the exception of enterotoxin-T all are known to be involved in food-borne disease.

- HBL was the first toxin-complex to be identified. It consists of three proteins, namely L_1, L_2 and B, all of which are necessary to form an active enterotoxin within the cell. Each of these proteins is encoded by a single gene (Granum 1994).
  The detection of (parts of) the individual genes encoding for the three composing proteins is carried out by PCR. The L_1 and L_2 genes can be identified in a duplex PCR, the B-gene in a separate PCR. The methods we use have been published in 2001 (In 't Veld et al. 2001) and are described in detail in appendices 2 and 3. The actual production of the HBL-complex can be identified on sheep-blood agar plates by the transient formation of a double haemolysis around the colonies (In 't Veld et al. 2001).

- NHE also is a complex of three proteins, namely A, B and C; here too, all three proteins are necessary to form an active enterotoxin within the cell. Each protein is encoded by a single gene. The existence of NHE was first described in 1996 (Lund and Granum 1996). A multiplex PCR, described in appendix 4, can detect each of the genes (Granum et al. 1999). Here too, the absence of one or more of the genes can be determined too.

- Enterotoxin-T is a single protein and was first described as having enterotoxic properties in 1995 (Agata et al. 1995). It has not been related to foodborne outbreaks yet. But on the basis of its structure and its resemblance to other bacterial toxins it is named an enterotoxin. The presence of the gene encoding for enterotoxin-T is detected by PCR. The method, based on research by Ombui (Ombui et al. 1997), is presented in appendix 5.

- Cytotoxin-K was first discovered during the investigation of a French outbreak (Lund et al. 2000). It consists of a single protein, which resembles structurally the α-haemolysin of *Staphylococcus aureus* and the β-toxin from *Clostridium perfringens.* The gene encoding for this toxin can also be detected by PCR, for which a method is given in appendix 6.

To investigate the transcription of any of the genes mentioned in this section RT-PCRs can be employed. Since such methods are still under construction in our laboratory, they will not be described in this report.

4.   Methods to determine thermotolerance

B. cereus species can be discriminated on the basis of their thermotolerance, i.e. the ability to grow at certain temperatures.

There are several methods for the determination of thermotolerance, either based on growth or on genetic coding of thermotolerance.

4.1   Determination by growth

As mentioned in paragraph 2.2 three temperatures are of importance in the discrimination upon thermotolerance, 10ºC, 37ºC, and 42ºC.

Mesophilic strains are able to grow above 10ºC and at 42ºC, psychrophilic strains are able to grow below 10ºC and at 42ºC, and psychrotrophic strains are able to grow below 10ºC and not at 42ºC. Usually 37ºC is the ultimate growth temperature for psychrotrophic strains.

4.2   Detection of the cspA gene

Strains that are psychrotrophic may produce at least one protein that enables them to survive and grow at low temperatures. This protein is the cold shock protein A (cspA), and the gene which encodes for this protein is known. By PCR the presence of this gene can be determined. The possession of this gene is a prerequisite for *B. weihenstephanensis* strains, a subspecies of *B. cereus*. The presence of the gene in mesophilic strains has sofar not yet been established (Lechner et al. 1998). In appendix 7 the method for determination of the cspA gene has been described.

4.3   Determination of 16S rDNA signatures

Psychrophilic and mesophilic strains have different specific sequences (signatures) on the 16S ribosomal DNA (rDNA) (Von Stetten et al. 1998). On the basis of these different signatures thermotolerance of strains is easily identified. Other researchers found that the discrimination in psychrotrophic and mesophilic strains on the basis of the specific 16S rDNA sequence is not as black-and-white as suggested (Prüss et al. 1999). The rDNA sequences occur in multiple copies in the genome, sometimes up to ten copies. In a strain not all copies have necessarily the same 16S signature. The method for determination of the 16S rDNA signatures is described in detail in appendix 8.

5.   Methods to compare strains

Comparison of strains is not a prime tool for characterization of individual strains. It rather is a method for establishing the relationship between strains collected in an outbreak of food-borne disease or during different stages of a production process. Two methods will be described here:
-   fatty acid analysis and clustering
-   Random Amplified Polymorphic DNA-PCR (RAPD-PCR)

5.1   Fatty acid analysis and clustering

Fatty acid analysis is the (partial) identification of strains by characterization of the fatty acid composition of the cell membrane.

Clustering enables the comparison of fatty acid patterns to establish relationships among strains. Prerequisite is the investigation of all strains involved in one analytical run as slight differences in processing may influence the result.

In appendix 9 the methods for the fatty acid analysis and clustering have been described. The method is based on previously described techniques (Sasser 1990;Osterhout et al. 1991) and is performed by the Diagnostic Laboratory for Infectious Diseases and Perinatal Screening (LIS) of the National Institute for Public Health and the Environment.

A disadvantage of this method may be that the media used for growing the cultures before fatty acid extraction may influence the results. Different batches of the same medium may lead to slightly different analyses and subsequent clustering.

5.2   RAPD-PCR

In PCR the primers are adjusted to the sequence of the target DNA, and forward and reverse primers are used to amplify both strands of DNA after dissociation. In Random Amplified Polymorphic DNA (RAPD)-PCR a single, and usually, small primer (ca. 10 bases) is used, that may anneal to several places in the single stranded target DNA. It was first performed in 1990 (Williams et al. 1990), and ever since has found its way in the laboratory on a large scale. Upon using DNA from different strains it is possible to retrieve whether these strains are related or not. If they are related the patterns deriving from the RAPD-PCR after electrophoresis must be alike. If the strains are not related the resulting patterns will differ.

The results obtained with RAPD-PCR are even more valid when using several PCR's with different primers.

The basic method for RAPD-PCR and the primers are based on previous research (Nilsson et al. 1998;Hsueh et al. 1999) and are described in appendix 10.

6.   Methods to extract and detect emetic toxin

The emetic toxin, or cereulide, is a dodecadepsipeptide consisting of three units of [D-O-Leu D-Ala L-O-Val L-Val] (Agata et al. 1995;Isobe et al. 1995). The genome does not contain any code for the toxin. Probably due to the action of an enzyme complex the toxin is formed either inside or outside the cell. The toxin is very apolar, heat-resistant, and pH-resistant (Agata et al. 1994).

Extraction and purification of the toxin from pure cultures or food samples is based on these three properties (Andersson et al. 1998;Finlay et al. 1999). In appendix 11 the basic methods for extraction and purification of the toxin are described. Experiments to optimize the methods are currently performed in our laboratory in collaboration with the Laboratory for Organic Chemistry in our institute.

The toxin is known to have two modes of action: first it may attach to the nervus vagus inducing a vomiting reflex, and second it interferes with the metabolic action of mitochondriae. Based on this last action two different assays have been described, a boar sperm test (Andersson et al. 1998) and a cell test using HEp-2-cells (Finlay et al. 1999; Hughes et al. 1988;Szabo et al. 1991). The cell test, as used in our laboratory, is described in appendix 12. The controls of the test consist of treated culture fluid from an emetic toxin positive strain, treated culture fluid from an emetic toxin negative strain, and treated culture fluid without any bacterial strain added. Possible non specific interferences can be ruled out by using such controls. Although some researchers believe that the incubation time for this test, 40 hours, is too long and may result in aspecific reactions (M.S. Salkinoja-Salonen, personal communication), results from tests in our lab with the controls as described above show that shorter incubation times reveal less distinct differences in optical density (data not published). The 40-hour incubation time is necessary to obtain optimal results.

Confirmation of the presence of the toxin in extracts of cultures or food commodities can be performed by mass spectrometry (Mikkola et al. 1999;Andersson et al. 1998). Experiments for implementation of this mass spectrometric method or for a liquid chromatography-mass spectrometry (LC-MS) method are currently performed buy the Laboratory for Organic Chemistry in our institute.

7.   Application of methods in case of foodborne disease caused by *B.cereus*

To give an indication of the way the described methods can be used to determine the involvement of *B. cereus* in foodborne disease and to establish whether the emetic or diarrheal type of disease is concerned an example of an outbreak is presented.

Background:

In the summer of 2000 around 100 students out of a group of about 1200 were struck by foodborne disease while camping somewhere in the Netherlands. Within two hours after the consumption of a vegetarian rice dish they suffered from vomiting and abdominal pain. Fifteen students had serious complaints and three of them had to be admitted to hospital for observation.

The symptoms indicated a foodborne intoxication.

Samples, initial experiments and results:

At least 6 samples from food and 2 from vomit were collected and investigated bacteriologically by the Inspectorate for Health Protection, Commodities and Veterinary Health in Amsterdam. All samples grew *B. cereus*.

Further characterization of *B. cereus* strains:

From each of six cultures from food left-overs 10 colonies were subcultured for further investigation.

The microorganisms isolated from the food and the vomit were investigated for the presence of the enterotoxin-genes encoding HBL and NHE. The relationship between the strains was investigated by RAPD; thermotolerance based on 16S signature was investigated. Also the potential to form emetic toxin was determined using the cell test.

Results: All strains (subcultures from food and cultures from vomit) appeared to be mesophilic (data not shown). One subculture (383-6) exhibited the genes for all three NHE genes (marked by a black X in figure 1). All other cultures showed two of the three genes for the enterotoxin NHE. In all these last cases the gene for the C-component, which is represented by a fragment of 834 base pairs as the top band in control strain 26, was not detected.

One subculture from a foodsample (391-9) showed the genes for the enterotoxin HBL (marked by a black X in figures 2 and 3); strains 456-3 and 456-5 showed the B-component (marked by a black X in figure 3) but not the L-components (see figures 2 and 3, respectively). None of the other cultures showed any of the HBL-genes.

In figure 4 the result of RAPD-PCR, using primer BcRAPD-1, is shown. The two vomit-strains, each exhibiting a different profile, are shown in lanes a and b on the right hand

bottom of the picture. The subcultures from the food left-overs all exhibit either the a-pattern or the b-pattern. In table 1 the results of the cell test for the detection of emetic toxin are shown. Apart from three subcultures (383-4, 383-6 and 391-9) all strains exhibit emetic toxin activity according to the criteria for this test as described by Finlay et al. (Finlay et al. 1999). In table 2 a compilation of all results for each of the investigated strains is presented.

Conclusion:
The following conclusions were drawn:
Based on 1) the symptoms of an intoxication, 2) the culturing of *B. cereus*, 3) the near complete absence of HBL-genes, 4) the near complete absence of the gene for the C-component of NHE, 5) the near complete presence of emetic toxin activity in all strains in the cell test, and 6) the similariy in RAPD pattern between the vomit strains and the subcultures from food left-overs, *B. cereus* emetic toxin was held responsible for this outbreak.
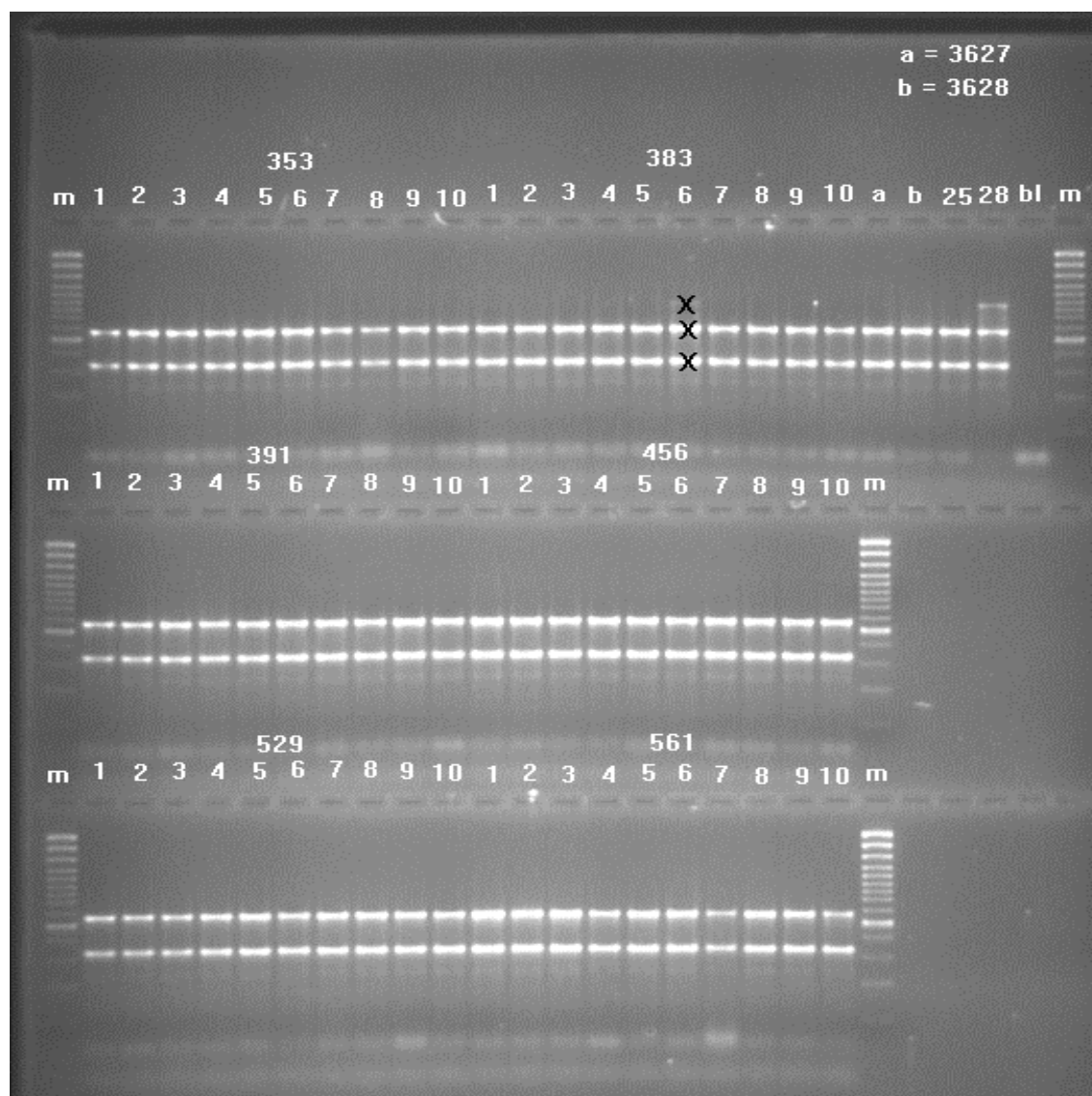
Figure 1: *Detection of genes encoding for NHE (non haemolytic enterotoxin)*
353 - 1 to 10: substrains 1 to 10 from foodsample 353
383, 391, 456, 529 and 561 as 353
a = 3627: strain collected from vomitsample 3627
b = 3628: strain collected from vomitsample 3628
25: controlstrain showing two of the three NHE-bands (B and A, from bottom to top)
28: controlstrain showing the three NHE-bands (B, A and C, from bottom to top)
bl: blank
m: marker (100 bp ladder MBI Fermentas®)
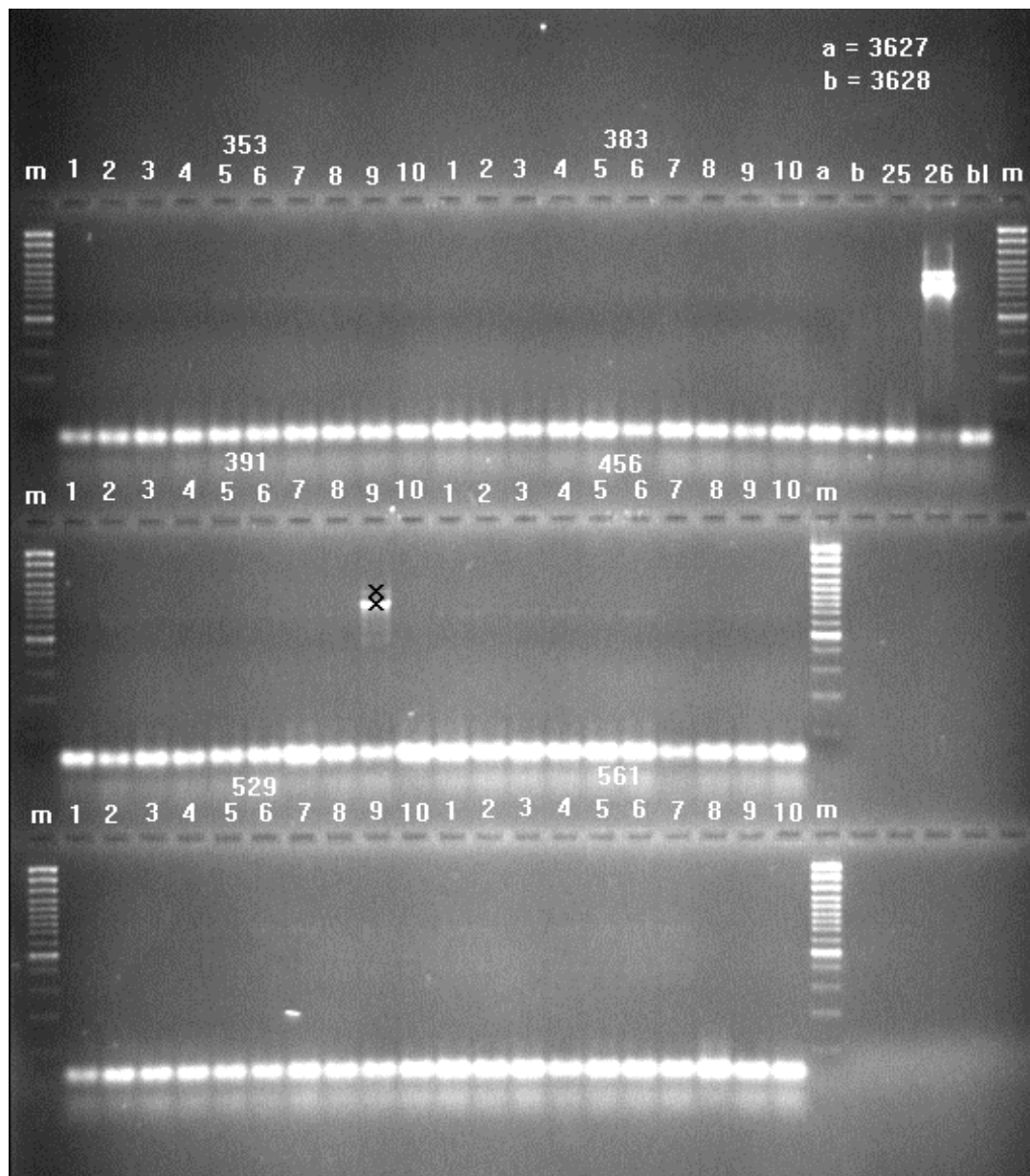
Figure 2:   *Detection of gene encoding for L-components of HBL (haemolytic BL enterotoxin)*

        353 - 1 to 10: substrains 1 to 10 from foodsample 353
        383, 391, 456, 529 and 561 as 353
        a = 3627: strain collected from vomitsample 3627
        b = 3628: strain collected from vomitsample 3628
        25: negative control (NCTC 11143), i.e.strain lacking the three HBL-genes
        26: positive control (NCTC 11145), i.e. strain containing all three HBL-genes
        bl: blank
        m: marker (100 bp ladder MBI Fermentas®)
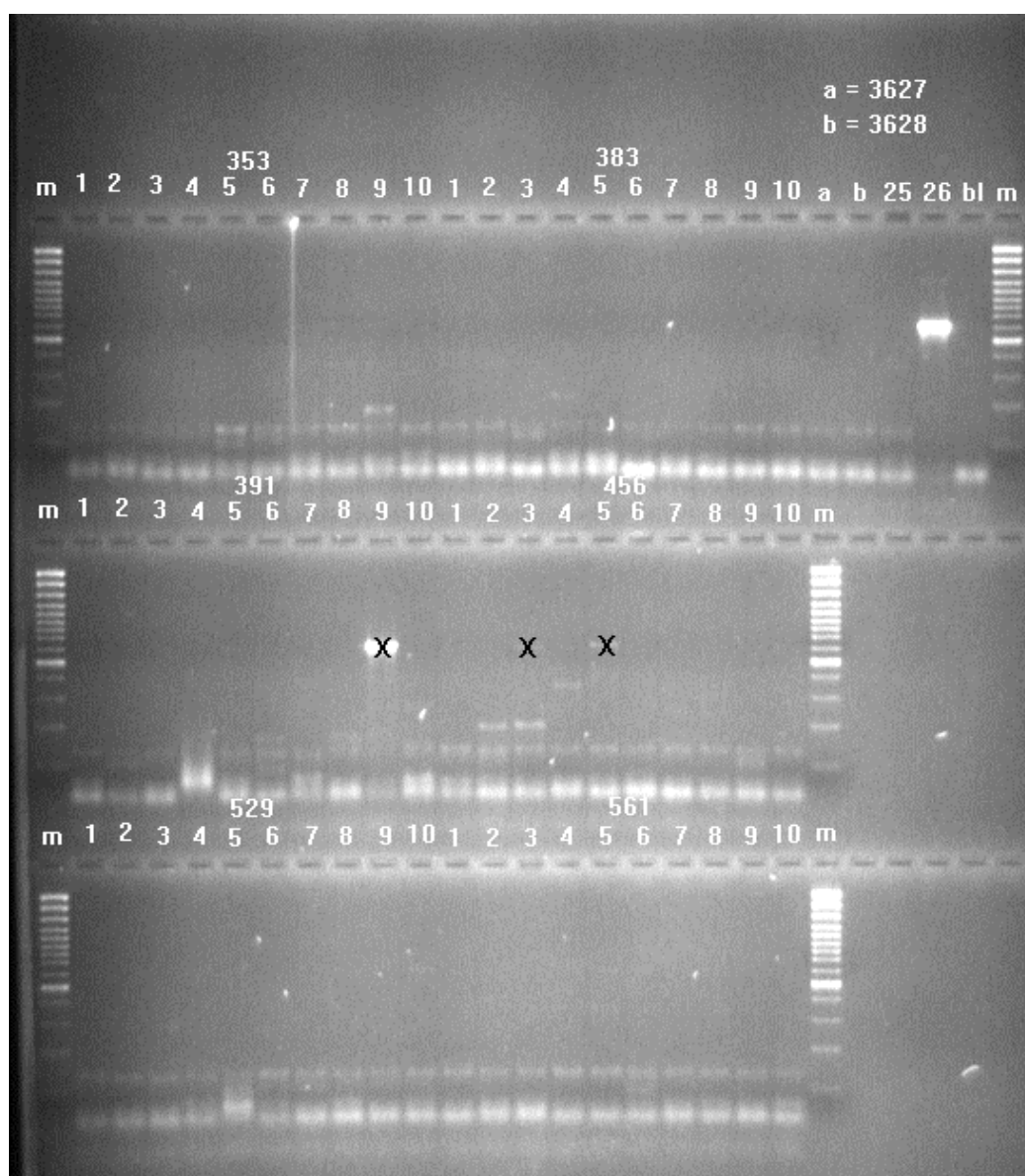
Figure 3:   *Detection of gene encoding for B-component of HBL (haemolytic BL enterotoxin)*
                353 - 1 to 10: substrains 1 to 10 from foodsample 353
                383, 391, 456, 529 and 561 as 353
                a = 3627: strain collected from vomitsample 3627
                b = 3628: strain collected from vomitsample 3628
                25: negative control (NCTC 11143), i.e.strain lacking the three HBL-genes
                26: positive control (NCTC 11145), i.e. strain containing all three HBL-genes
                bl: blank
                m: marker (100 bp ladder MBI Fermentas®)
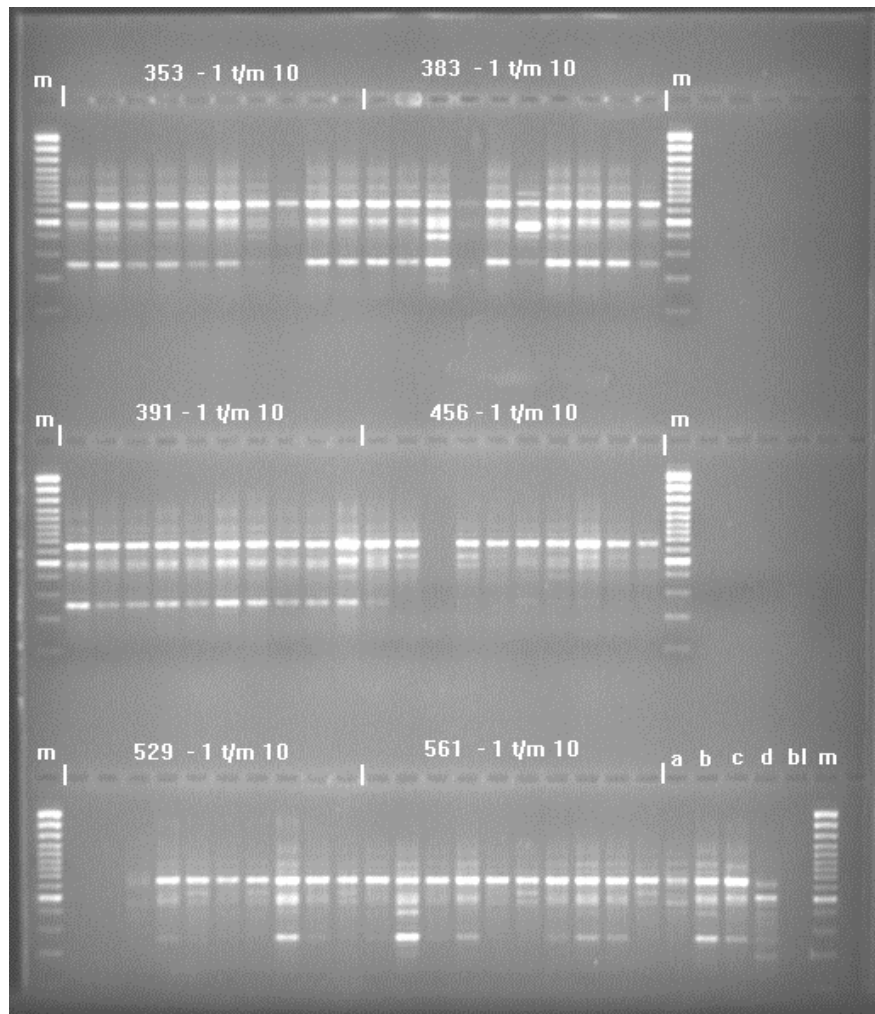
Figure 4:   *Comparison of outbreak strains by RAPD-PCR*
353 - 1 to 10: substrains 1 to 10 from foodsample 353
383, 391, 456, 529 and 561 as 353
a = 3627: strain collected from vomitsample 3627
b = 3628: strain collected from vomitsample 3628
c = *B. cereus* NCTC 11143
d = *B. cereus* NCTC 11145
bl: blank
m: marker (100 bp ladder MBI Fermentas®)

Table 1: Detection of emetic toxin in outbreak strains

353 - 1 to 10: substrains 1 to 10 from foodsample 353
383, 391, 456, 529 and 561 as 353
a = 3627: strain collected from vomitsample 3627
b = 3628: strain collected from vomitsample 3628
NCTC 11143 = emetic toxin positive controlstrain
NCTC 11145 = emetic toxin negative control strain

Strainnr.			Strainnr.			Strainnr.	
353-1	+		383-1	+		391-1	+
353-2	+		383-2	+		391-2	+
353-3	+		383-3	+		391-3	+
353-4	+		383-4	-		391-4	+
353-5	+		383-5	+		391-5	+
353-6	+		383-6	-		391-6	+
353-7	+		383-7	+		391-7	+
353-8	+		383-8	+		391-8	+
353-9	+		383-9	+		391-9	-
353-10	+		383-10	+		391-10	+
Strainnr.			Strainnr.			Strainnr.	
456-1	+		529-1	+		561-1	+
456-2	+		529-2	+		561-2	+
456-3	+		529-3	+		561-3	+
456-4	+		529-4	+		561-4	+
456-5	+		529-5	+		561-5	+
456-6	+		529-6	+		561-6	+
456-7	+		529-7	+		561-7	+
456-8	+		529-8	+		561-8	+
456-9	+		529-9	+		561-9	+
456-10	+		529-10	+		561-10	+
Strainnr.			Strainnr.				
3627	+		NCTC 11143	+		bl	-
3628	+		NCTC 11145	-			

Table 2: Compilation of results as shown in Figures 1 to 4 and Table 1

RAPD types strains 3627 and 3628 are resp. A and B, other strains compared to those.

RAPD types X and Y: divergent from A and B

? = no reaction in RAPD-PCR

Food strain	HBL	NHE	Hep2-test	RAPD		Food strain	HBL	NHE	Hep2-test	RAPD		Food strain	HBL	NHE	Hep2-test	RAPD
	L1 L2 B	A B C					L1 L2 B	A B C					L1 L2 B	A B C		
353-1	- - -	+ + -	+	B		383-1	- - -	+ + -	+	B		391-1	- - -	+ + -	+	B
353-2	- - -	+ + -	+	B		383-2	- - -	+ + -	+	B		391-2	- - -	+ + -	+	B
353-3	- - -	+ + -	+	B		383-3	- - -	+ + -	+	X		391-3	- - -	+ + -	+	B
353-4	- - -	+ + -	+	B		383-4	- - -	+ + -	-	(B)		391-4	- - -	+ + -	+	B
353-5	- - -	+ + -	+	B		383-5	- - -	+ + -	+	B		391-5	- - -	+ + -	+	B
353-6	- - -	+ + -	+	B		383-6	- - -	+ + -	-	Y		391-6	- - -	+ + -	+	B
353-7	- - -	+ + -	+	A		383-7	- - -	+ + -	+	B		391-7	- - -	+ + -	+	B
353-8	- - -	+ + -	+	A		383-8	- - -	+ + -	+	B		391-8	- - -	+ + -	+	B
353-9	- - -	+ + -	+	B		383-9	- - -	+ + -	+	B		391-9	+ + +	+ + -	-	B
353-10	- - -	+ + -	+	B		383-10	- - -	+ + -	+	B		391-10	- - -	+ + -	+	B

Food strain	HBL	NHE	Hep2-test	RAPD		Food strain	HBL	NHE	Hep2-test	RAPD		Food strain	HBL	NHE	Hep2-test	RAPD
	L1 L2 B	A B C					L1 L2 B	A B C					L1 L2 B	A B C		
456-1	- - -	+ + -	+	A		529-1	- - -	+ + -	+	?		561-1	- - -	+ + -	+	A
456-2	- - -	+ + -	+	A		529-2	- - -	+ + -	+	?		561-2	- - -	+ + -	+	B
456-3	- - -	+ + -	+	?		529-3	- - -	+ + -	+	A		561-3	- - -	+ + -	+	A
456-4	- - -	+ + -	+	A		529-4	- - -	+ + -	+	B		561-4	- - -	+ + -	+	B
456-5	- - -	+ + -	+	A		529-5	- - -	+ + -	+	A		561-5	- - -	+ + -	+	A
456-6	- - -	+ + -	+	A		529-6	- - -	+ + -	+	A		561-6	- - -	+ + -	+	A
456-7	- - -	+ + -	+	A		529-7	- - -	+ + -	+	A		561-7	- - -	+ + -	+	B
456-8	- - -	+ + -	+	A		529-8	- - -	+ + -	+	B		561-8	- - -	+ + -	+	B
456-9	- - -	+ + -	+	A		529-9	- - -	+ + -	+	A		561-9	- - -	+ + -	+	B
456-10	- - -	+ + -	+	A		529-10	- - -	+ + -	+	A		561-10	- - -	+ + -	+	A

Vomit culture	HBL	NHE	Hep2-test	RAPD
	L1 L2 B	A B C		
3627	- - -	+ + -	+	A
3628	- - -	+ + -	+	B

8.   Application of methods to assess food safety

B. cereus occurs in/on various food commodities. Usually reports are published concerning the thermotolerance of strains occurring in food commodities (Nortje et al. 1999;Larsen and Jorgensen 1997;Giffel et al. 1996). No information on occurrence of genes coding for enterotoxins or the ability to produce emetic toxin (the virulence factors) is available. Therefore it is impossible to indicate whether the strains found in/on food commodities are potentially pathogenic, information that is essential for quantitative microbiological risk assessment.

The methods described in appendices 2 to 6, concerning the detection of genes coding for various (entero)toxins, and the methods described in appendices 11 and 12, concerning the purification and detection of emetic toxin, are helpful tools to nominate *B. cereus* strains as potentially pathogenic or not.

Such information is also helpful to investigate whether certain types of food contain certain types of *B. cereus* strains. In other words whether certain types of food always contain potentially pathogenic strains, and other types of food never.

Research as described above will provide more or less qualitative information with respect to dose-response relations. At this moment no tools for quantitative information are available. Only for the detection of emetic toxin more quantitative methods, based on described methods (Andersson et al. 1998), will be available soon. In case of foodborne toxico-infections information on dose response relation can be obtained in a indirect way. Numbers of "pathogenic" (i.e. ability to produce enterotoxins) *B. cereus* in the suspected food in relation to burden of disease in patients can provide more usefull information.

When quantitative methods become available these will not only provide information on the pathogenic potential but also on the pathogenic potency of *B. cereus* strains. Combined with data on biodiversity information will become available on the occurrence of potentially pathogenic strains in combination with food type.

9.   Discussion

Isolation and biochemical identification of bacterial strains in foodborne disease outbreaks may not be enough to decide on the involvement of a certain organism as cause of illness. This problem is endorsed when the organisms occur in the transient intestinal flora, like *B. cereus*. Also, isolation and biochemical identification of large numbers of *B. cereus* in food is not conclusive to indicate the food as hazardous to health in case of consumption. In both cases additional techniques are necessary to identify *B. cereus* in the suspected food and in the patient as identical and to characterize them as potentially pathogenic.

In foodborne outbreaks, the RAPD-method in combination with the PCR-tests for the genes of the HBL and NHE enterotoxins and the HEp-2-test for the detection of interference of emetic toxin with the metabolic action of mitochondriae are useful tools to show relationship between organisms from suspected food and patients, and to assess the pathogenic potency.

As can be seen from table 2 in chapter 7 two dominant RAPD types of *B. cereus* were present in the 6 suspected food samples. The same RAPD types of *B. cereus* were found in the two patients vomit samples, RAPD type A in vomit sample 3627 and RAPD type B in vomit sample 3628. Nearly all isolates from the dominant RAPD types lack the genes of the HBL enterotoxin, nearly all isolates from the dominant RAPD types possess 2 of the 3 genes of the NHE enterotoxin, and nearly all isolates from the dominant RAPD types showed emetic toxin to interfere with the metabolic action of mitochondriae in the HEp-2-test.

Based on the present knowledge (Wijnands et al. in preparation), food, contaminated with *B. cereus*, must be considered hazardous to health in case of consumption if 1) not only, more or less, large numbers of vegetative cells or spores are present, but also if one or more of the following tests is positive: PCRs for the presence of all HBL genes, PCR for the presence of all NHE genes, PCR for the presence of cytotoxin-K gene, and HEp-2 cell test for the detection of the interference of the emetic toxin with the metabolic action of mitochondriae; and 2) small numbers of microorganisms are present and the HEp-2 cell test for the detection of the interference of the emetic toxin with the metabolic action of mitochondriae is positive.

Only when the strains contain all three genes of the HBL or the NHE enterotoxin, they have the potency to cause foodborne illness. This is also the case when they have the gene for cytotoxin K and the ability to produce the emetic toxin, as showed by activity in the HEp-2-test. At this moment it is still unknown under what conditions the genes are expressed and the toxins formed.

When strains do not possess all genes of the HBL or the NHE enterotoxin, or do not possess the gene for cytotoxin K or the potential to produce activity as measured by the HEp-2-test, strains can not be regarded as pathogenic for foodborne disease.

In all comments the enterotoxin T has been omitted as a potential pathogenic property as this enterotoxin has never yet been related to any foodborne outbreak. If in the future such might

be the case also the detection of the gene for this enterotoxin may help to characterize *B. cereus* strains.

Methods to study growth temperature, properties of spores and to determine thermotolerance are necessary in risk assessment. The methods to compare strains can also be used in tracing sources of contamination with *B. cereus* in the food production process of food commodities.

The methods described in this report may have far-reaching consequences. It may lead to re-evaluation of the tolerance level set for the presence of *B. cereus* in food commodities. The current tolerance level is set at 10^5 microorganisms/g food, regardless of the potential pathogenicity of the strains found in food. With the methods described here it is possible to distinguish between pathogenic and non pathogenic strains. For definitive discrimination into pathogenic and non-pathogenic *B. cereus* strains it remains of importance to develop methods that can establish the transcription from the enterotoxin genes into messenger RNA and methods that can determine the formation of the enterotoxins. Also knowledge on the working mechanism of the enterotoxin complexes is of significance for definitive discrimination into pathogenic and non-pathogenic *B. cereus* strains.

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Appendix 2

PCR for the detection of the L1 and L2 genes from the HBL-complex

Primers
L1a: 5'-ATA TTC ACC TTA ATC AAG AGC TGT CAG G-3'
L1b: 5'-CCA GTA AAT CTG TAT AAT TTG CGC CC-3'
L2a: 5'-TAT CAA TAC TCT CGC AAC ACC AAT CG-3'
L2b: 5'-GTT TCT CTA AAT CAT CTA AAT ATG CTC GC-3

PCR mixture
-   2.5 µl 10x PCR buffer (Roche Molecular Biochemicals),
-   1.7 mM MgCl$_2$,
-   200 µM of each deoxynucleoside triphosphate,
-   300 µM of each primer,
-   2.6 U of AmpliTaq Polymerase (Roche Molecular Biochemicals),
-   1 µl template DNA (= 1 µl overnight BHI-culture), and
-   sterile MilliQ water to a final volume of 25 µl,.

PCR-program
94ºC   3 minutes
94ºC   1 minute
64ºC   1 minute   2 cycles
72ºC   3 minutes
94ºC   1 minute
62ºC   1 minute   2 cycles
72ºC   3 minute
94ºC   1 minutes
60ºC   1 minute   2 cycles
72ºC   3 minute
94ºC   1 minute
58ºC   1 minute   2 cycles
72ºC   3 minutes
94ºC   1 minute
56ºC   1 minute   2 cycles
72ºC   3 minutes
94ºC   1 minute
54ºC   1 minute   30 cycles
72ºC   3 minutes
72ºC   10 minutes
Keep at 4ºC until further analysis

Control-strains
B. cereus NCTC 11143 HBL-L1 and HBL-L2 negative
B. cereus NCTC 11145 HBL-L1 and HBL-L2 positive

<u>Amplicon size</u>
L1-gene: 809 basepair (bp)
L2-gene: 976 bp

Based on Heinrichs et al. (1993) and Ryan et al. (1997).

Appendix 3

PCR for the detection of the B gene from the HBL-complex

Primers
B1F: 5'-ACG AAC AAT GGA GAT ACG GC-3'
B2R: 5'TTG GTA GAC CCA AAA TAG CAC C-3'

PCR mixture
-   2.5 µl 10x PCR buffer (Roche Molecular Biochemicals),
-   1.7 mM MgCl$_2$,
-   200 µM of each deoxynucleoside triphosphate,
-   300 µM of each primer,
-   2.6 U of AmpliTaq Polymerase (Roche Molecular Biochemicals),
-   1 µl template DNA (= 1 µl overnight BHI-culture), and
-   sterile MilliQ water to a final volume of 25 µl,.

PCR-program
94ºC   3 minutes
94ºC   1 minute
64ºC   1 minute   2 cycles
72ºC   3 minutes
94ºC   1 minute
62ºC   1 minute   2 cycles
72ºC   3 minute
94ºC   1 minutes
60ºC   1 minute   2 cycles
72ºC   3 minute
94ºC   1 minute
58ºC   1 minute   2 cycles
72ºC   3 minutes
94ºC   1 minute
56ºC   1 minute   2 cycles
72ºC   3 minutes
94ºC   1 minute
54ºC   1 minute   30 cycles
72ºC   3 minutes
72ºC   10 minutes
Keep at 4ºC until further analysis

Control-strains
B. cereus NCTC 11143 HBL-B-negative
B. cereus NCTC 11145 HBL-B-positive

Amplicon size
B-gene: 600 bp

Based on Heinrichs et al.(1993) and Ryan et al. (1997).

Appendix 4

PCR for the detection of the A, B and C genes from the NHE-complex

Primers
A-45-2F: 5'-GCT CTA TGA ACT AGC AGG AAA C-3'
A-45-3R: 5'-GCT ATT TAC TTG ATC TTC AAC G-3'
B-39-1F: 5'-CGG TTC ATC TGT TGC GAC AGC –3'
B-39-3R: 5'-GAT CCC ATT GTG TAC CAT TGG-3'
C-39-F: 5'-CCT TAT AAA GAG AAT AGG TG-3'
C-39-2R: 5'CGA CTT CTG CTT GTG CTC CTG-3'

PCR mixture
-     5 µl 10x PCR buffer (Roche Molecular Biochemicals),
-     1.5 mM MgCl$_2$,
-     200 µM of each deoxynucleoside triphosphate,
-     300 µM of each primer,
-     2.5 U of AmpliTaq Polymerase (Roche Molecular Biochemicals),
-     1 µl template DNA (= 1 µl overnight BHI-culture), and
-     sterile MilliQ water to a final volume of 50 µl,.

PCR-programme
94ºC   5 minutes
94ºC   1 minute
48ºC   1 minute   30 cycles
72ºC   1 minutes
72ºC   5 minutes
Keep at 4ºC until further analysis

Control-strain
B. cereus 1230-88

Amplicon size
nheA: 540 bp
nheB: 312 bp
nheC: 834 bp

Based on Granum et al. (1999).

Appendix 5

PCR for the detection of the gene encoding enterotoxin-T

<u>Primers</u>
BceT-F: 5'-TTA GTT TCA ACA GCG CGT ATC GGT-3'
BceT-R: 5'-ATA CAC ATG CAA ATG CTC CGG AC-3'

<u>PCR mixture</u>
- 5 µl 10x PCR buffer (Roche Molecular Biochemicals),
- 1.5 mM MgCl$_2$,
- 200 µM of each deoxynucleoside triphosphate,
- 300 µM of each primer,
- 2.5 U of AmpliTaq Polymerase (Roche Molecular Biochemicals),
- 1 µl template DNA (= 1 µl overnight BHI-culture), and
- sterile MilliQ water to a final volume of 50 µl,.

<u>PCR-programme</u>
94ºC   5 minutes
94ºC   1 minute ⎤
63ºC   1 minute ⎥ 30 cycles
72ºC   1 minutes ⎦
72ºC   5 minutes
Keep at 4ºC until further analysis

<u>Controlstrain</u>
B. cereus B4ac

<u>Amplicon size</u>
bce-T: 741 bp

Based on Ombui et al. (1997).

Appendix 6

PCR for the detection of the genes encoding the cytotoxin-K

Primers
Bc-cytK-FC: 5'-GTA ACT TTC ATT GAT GAT CC-3'
Bc-cytK-RC: 5'-GAA TAC ATA AAT AAT TGG TTT CC-3'

PCR mixture
- 5 µl 10x PCR buffer (Roche Molecular Biochemicals),
- 1.5 mM $MgCl_2$,
- 200 µM of each deoxynucleoside triphosphate,
- 300 µM of each primer,
- 2.5 U of AmpliTaq Polymerase (Roche Molecular Biochemicals),
- 1 µl template DNA (= 1 µl overnight BHI-culture), and
- sterile MilliQ water to a final volume of 50 µl,.

PCR-programme
94ºC   5 minutes
94ºC   1 minute
48ºC   1 minute   30 cycles
72ºC   1 minutes
72ºC   7 minutes
Keep at 4ºC until further analysis

Controlstrain
B. cereus 391-98

Amplicon size
Cyt-K: 480 bp

Based on Hardy et al. (2001) and Lund et al. (2000).

Appendix 7

PCR for the detection of the gene encoding the cold shock protein A (cspA)

Primers
Bc-cspA-F1:   5'-GAG GAA ATA ATT ATG ACA GTT-3'
Bc-cspA-R1:   5'-CTT C(T)TT GGC CTT CTT CTA A-3'

PCR mixture
- 5 µl 10x PCR buffer (Roche Molecular Biochemicals),
- 1.75 mM $MgCl_2$,
- 200 µM of each deoxynucleoside triphosphate,
- 300 µM of each primer,
- 2.5 U of AmpliTaq Polymerase (Roche Molecular Biochemicals),
- 1 µl template DNA (= 1 µl overnight BHI-culture), and
- sterile MilliQ water to a final volume of 50 µl,.

PCR-programme
95ºC   5 minutes
95ºC   15 seconds
55ºC   30 seconds   30 cycles
72ºC   30 seconds
72ºC   2 minutes
Keep at 4ºC until further analysis

Controlstrain
Psychrotrophic *B. cereus*

Amplicon size
csp-A: 160 bp

Based on Francis et al. (1998) and Stenfors and Granum (2001).

Appendix 8

PCR for the determination of the 16S rDNA thermotolerance signature

Primers
Primer MF: 5'-ATA ACA TTT TGA ACC GCA TG-3'
Primer UR: 5'-CTT CAT CAC TCA CGC GGC-3'
Primer UF: 5'-CAA GGC TGA AAC TCA AGG GA-3'
Primer PR: 5'-GAG AAG CTC TAT CTC TAG A-3'

PCR mixture
-   5 µl 10x PCR buffer (Roche Molecular Biochemicals),
-   1.5 mM MgCl$_2$,
-   200 µM of each deoxynucleoside triphosphate,
-   300 µM of each primer,
-   2.5 U of AmpliTaq Polymerase (Roche Molecular Biochemicals),
-   1 µl template DNA (= 1 µl overnight BHI-culture), and
-   sterile MilliQ water to a final volume of 50 µl,.

PCR-programme
94ºC   2 minutes
94ºC   15 seconds ⌐
55ºC   15 seconds │── 30 cycles
72ºC   15 seconds ⌐
72ºC   2 minutes
Keep at 4ºC until further analysis

Controlstrain
Psychrotrophic *B. cereus*

Amplicon size
psychrotrophic signature: 130 bp
mesophilic signature: 250 bp

Based on Von Stetten et al. (1998).

Appendix 9

Analysis and clustering of whole cell fatty acid composition

Analysis
A loopfull bacteria were harvested after 24 h incubation on Trypticase Soy Broth Agar (TSBA: 30g/l trypticase soy broth BBL 11768, 15 g agar/l, pH 7,3 prepared by SVM, Bilthoven The Netherlands), at 28° C, aerobic atmosfeer.
Saponification, methylation and extraction were performed as described by Sasser [A].
The gas chromatography system comprised a Hewlett Pacard (HP) 6890 gas chromatograph equipped with a flame ionization detector and an autosampler, HP Chemstation 5.03 software; connected with the Microbial Identification System (MIS): Sherlock System Software, Aerobic Bacteria Library TSBA40, MIDI Data Export (MIDI, Newark DE, USA). Fatty acid methylesters were separated on a fused-silica capillary column (25 m by 0.2 mm) HP Ultra 2 (crosslinked 5% PHME Siloxane, HP 19091B-102). The MIS controlled operating parameters were as follows: injector temperature, 250° C; detector temperature, 300° C; oven temperature , programmed to equilibrate at 170° C for 3 min at the beginning of the cycle , then raised from 170 to 270° C at 5° C/min and from 270 to 310° C at 30° C/min.
Data analysis. Fatty acids were identified on basis of equivalent chain length data.

Clustering
Clustering methods used were Unweighted Pair-Group Method Arithmetic averages (UPGMA) as described by Romsburg on the relative (% of the total) peak area of named peaks. Differences between the individual clusters are expressed in Euclidian Distance.

Based on Sasser (1990), Romsburg (1990) and Osterhout et al. (1991).

Appendix 10

RAPD-PCR for typing of *B. cereus*

Primers
BcRAPD-1:   5'-CCG AGT CCA-3'

PCR mixture
- 2.5 µl 10x PCR buffer (Roche Molecular Biochemicals),
- 1.75 mM MgCl$_2$,
- 200 µM of each deoxynucleoside triphosphate,
- 300 µM primer,
- 2.5 U of AmpliTaq Polymerase (Roche Molecular Biochemicals),
- 1 µl template DNA (= 1 µl overnight BHI-culture), and
- sterile MilliQ water to a final volume of 25 µl,.

PCR-programme
94ºC   3 minutes
94ºC   45 seconds ⌐
30ºC   2 minutes ⊢   4 cycles
72ºC   1 minute   ⌐
94ºC   45 seconds ⌐
36ºC   1 minute   ⊢   26 cycles
72ºC   2 minutes ⌐
75ºC   10 minutes
Keep at 4ºC until further analysis

Controlstrains
B. cereus NCTC 11143 and *B. cereus* NCTC 11145

Other possible primers:
BcRAPD-2:   5'-ACG CGC CCT-3'
BcRAPD-3:   5'-CCGGCCGCC-3'
BcRAPD-5:   5'-CGG CCA CTG T-3'
OPA-1:   5'-CAG GCC CTT C-3'
ERIC-1:   5'-GTG AAT CCC CAG GAG CTT ACA-3'

Based on Nilsson et al. (1998) and Hsueh et al. (1999).

Appendix 11

Extraction and purification of *B. cereus* emetic toxin

Materials:
- Trypton soy agar plates (TSA)
- Brain Heart Infusion broth (BHI)
- Incubator 28ºC
- Methanol
- Evaporation instrument with N_2-supply
- Chloroform
- Methanol-water 60-40
- Methanol-water 80-20
- Sep-Pak C_{18} cartridges (Waters Co., Milford, Mass., USA)

Methods:
1) Extraction from pure cultures:
    a) Grow the strains of interest on tryptic soy agar for 10 days at 28ºC or in BHI for 18 hours.
    b) Collect 500 mg of cellmaterial and transfer to a glass bottle.
    c) Add 100 ml methanol and mix thoroughly for 30 minutes.
    d) Collect the supernatant.

1) Extraction from inoculated rice samples:
    a) Boil rice.
    b) Aliquot the boiled rice into portions of 10 g.
    c) Inoculate the rice with the strain(s) of interest.
    d) Incubate at room temperature for two days.
    e) Heat the samples by placing them at 80ºC for 30 minutes.
    f) Incubate the rice for another two days at room temperature.
    g) Add 10 ml methanol to each rice sample to extract the emetic toxin.
    h) Mix thoroughly for 30 minutes.
    i) Collect the supernatant.

1) Extraction from food samples:
    a) Weigh portions of 10 gr of food sample in duplicate.
    b) Add 10 ml methanol to each sample to extract the emetic toxin.
    c) Mix thoroughly for 30 minutes.
    d) Collect the supernatant.

1) Purification for HEp-2 assay:
    a) Evaporate the supernatant (1d, 2i, 3d) to dryness under N_2.
    b) Dissolve the retentate in 10 ml incomplete MEM (MEM without L-glutamate and phenol-red), Gibco cat.nr. 51200-038.
    c) Proceed as described in V/250912/AB-021.

1) Purification for mass spectrometry:

a) Evaporate the supernatant (1d, 2i, 3d) to dryness under N_2.
b) Dissolve the dry material in chloroform.
c) Collect the soluble fraction and evaporate to dryness under N_2.
d) Dissolve the dry material in methanol-water 60-40.
e) Inject the solution into a Sep-Pak C_{18} cartridge.
f) Wash with methanol-water 80-20.
g) Elute the purified material with methanol..

Based on Andersson et al. (1998).

Appendix 12

Determination of emetic toxin produced by *B. cereus* by determining vacuolation and metabolic staining

Materials:
1. Positive control strain, DSM 4312 (= F4810/72) (Finlay et al. 1999)
2. Negative control strain, DSM 4313 (= F4433/73) (Finlay et al. 1999)
3. Skimmed milk medium 10% (SMM) Oxoid cat.nr.LP 0031
4. Erlenmeyer flasks 500 ml
5. Incubator 30ºC
6. Orbitary shaker
7. Autoclave
8. 96-wells microtitre plates (sterile, with individual lid), Costar cat. nr.3599
9. Eagle's MEM complete medium [Eagle's Minimal Essential Medium (Gibco 21581-20), 50,000 IU penicillin, 50,000 μg streptomycin, 0.085% sodiumbicarbonate solution, 2 mM L-glutamin, 10% foetal calf serum (FCS)]
10. HEp-2 cells in Eagle's MEM complete medium
11. Trypsin solution 0.05%
12. Counting chamber, type Bürker
13. Microscope
14. Incubator 37ºC, 5% CO_2
15. MTT (= 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), Acros
16. Incomplete MEM (= MEM medium without L-glutamine and phenol-red), Gibco cat.nr. 51200-038
17. Dimethylsulfoxide
18. Micro plate reader for 570 nm

Methods:
- Culture the strains of interest plus the control strains in 10% semi skimmed milk medium (Oxoid), overnight at 30ºC
- Transfer the overnight culture to 500 ml erlenmeyer flasks containing 50 ml SMM-medium, in triplicate
- Incubate for 18 hours at 30ºC with orbital shaking (200 rpm)
- Centrifuge 50 ml samples of the supernatants (4500xg, 40 minutes, 4ºC.)
- Autoclave the supernatants (121ºC, 15 minutes)
- Dilute all supernatants 1:8 in complete Eagle's MEM
- Make two-fold serial dilutions in triplicate in complete Eagle's MEM in 96-wells microtiterplates (100 μl per well). Pipette 200 μl 1:8 dilution in column 1 and make twofold dilutions with a multichannel pipette
- Trypsinize and suspend HEp-2 cells in incomplete MEM at 10^6/ml
- Add 100 μl cell-suspension per well by using a multichannel pipette. CHANGE THE TIPS FOR EACH COLUMN TO BE FILLED.
- Incubate the microtiterplates at 37ºC and 5% CO_2.
- Monitor the appearance of vacuolation regularly up to 40 hrs.
- Remove after 40 hours of incubation medum by inverting the plates

- Add 50 µl MEM (lacking supplements and phenol-red) containing 5 mg/ml MTT
- Incubate the plates at 37ºC for 3 hrs
- Remove the medium
- Add 50 µl DMSO per well to solubilize the intracellular formazan
- Read the absorbance at 570 nm with a micro plate reader
- The mean endpoint titer was recorded as the reciprocal of the highest dilution giving colorimetric reading lower than that of the negative control.

Based on Finlay et al. (1999), Szabo et al. (1991) and Hughes et al. (1988).