Toxin genes and cytotoxicity levels detected in *Bacillus cereus* isolates collected from cooked food products delivered by Swiss Army catering facilities

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Abstract

Heated food is known to be often contaminated with *B. cereus,* leading to cases of diarrhoeal or emetic diseases. Battalion kitchens or army catering facilities present a food safety risk, as temperature abuse and long storage time can result in serious public health problems affecting a high number of served people. In contrast to civil catering facilities, no microbiological monitoring systems are currently implemented in Swiss military kitchens. In this study toxin gene profiles and cytotoxicity levels of 21 isolates of *B. cereus* originating from six different food categories were determined. Nearly all isolates (95%) harbored the *nhe* gene, whereas no *hbl* could be detected. Seven isolates displayed the *cytK2* gene and one cereulide-producer was isolated out of vegetables. While most isolates displayed low cytotoxicity, highly cytotoxic strains were detected, with three isolates even exceeding the cytotoxicity level of the reference strain for high-level toxin production, underpinning that cytotoxicity cannot be deduced only from presence or absence of toxin genes. These findings further underline the importance of rapid cooling of foods or maintenance over 65°C before serving. This is especially important in mass catering facilities, such as military kitchens, in which food is often prepared a long time in advance.

Introduction

Bacillus cereus sensu lato (*B. cereus s.l.*) is a gram-positive bacterium that can be found in different environments and a wide range of foods such as rice, dairy products, spices, and vegetables (Kotiranta *et al.,* 2000; Kramer and Gilbert, 1989). *B. cereus s.l.* is a pathogen that can cause both infection and intoxication. Two different types of *B. cereus* gastrointestinal disease are known, which are caused by different toxins. Cereulide, a heat-stable cyclic peptide, causes foodborne intoxications with the key symptoms of emesis and nausea, whereas the heat-labile enterotoxins – non-hemolytic enterotoxin (Nhe), hemolysin BL (Hbl), and cytotoxin K (CytK) – produced in the gut of the consumer (foodborne infection) cause diarrhoea (Stenfors Arnesen *et al*., 2008). While the emetic syndrome is often associated with starchy foods (Stenfors Arnesen *et al*., 2008), the diarrhoeal form is frequently related to meat, sauces, vegetables and milk products (Kotiranta *et al*., 2000). Although most cases of illness are self-limiting within a short period of time, severe infections leading to death have been reported (Dierick *et al.*, 2005; Naranjo *et al.*, 2011). Bacterial counts of 10^5 to 10^8 CFU/g are considered sufficient to cause harmful effects (Stenfors Arnesen *et al.*, 2008). However, strong strain-specific differences in toxin production have been demonstrated (Guinebretière *et al.,* 2010) and foodborne diseases caused by an infective dose as low as 10^3 to 10^4 bacteria per gram have been reported (Andersson *et al.*, 1995).

The production of highly resistant spores capable of surviving strong environmental stresses represents a main risk factor for the contamination of food products with *B. cereus*. At harvest, spores can be attached to plant materials and can subsequently be transferred to food-processing equipments. Due to their strong adhesive properties and the formation of biofilms, spores are difficult to remove (Andersson *et al.*, 1995). Besides cross-contamination, spores can also survive cooking or pasteurization processes and subsequently germinate and multiply when food is not properly refrigerated (Kramer and Gilbert, 1989). Moreover, the growth possibility of *B. cereus* ranges from thermophilic to psychrotrophic strains, making it difficult to meet food safety regulations (Guinebretière *et al.*, 2008).

Mass catering facilities, such as military kitchens represent a challenge for food safety. Temperature control is crucial, given that often a considerable amount of time passes between cooking and consumption, especially in the case of meals that are served on the field. In particular, heating processes, storage, and recontamination possibilities need to be strictly controlled. Kleer *et al.* have shown that *B. cereus* is a food safety problem, especially in the catering environment (Kleer *et al.*, 2001). They also showed that *B. cereus* was responsible for 52% of diarrhoea outbreaks from 1994-1997 in the German Armed Forces. Furthermore, Ernst *et al.* reported a prevalence of 14% for *B. cereus* on surfaces in catering facilities in the German Army, pointing out that cross-contamination is a real problem during food preparation (Ernst *et al.*, 2001). In case of the Swiss Army, the food safety concept is based on the food law and comprises three key factors: education, good hygiene practices and self-control. Recently, two studies were performed providing first baseline data about the microbiological quality of cooked foods from battalions' kitchens and Swiss Army catering facilities (Hunziker, 2016; Ruf and Stephan, 2017). The aim of the present study was to further characterize 21 isolates of *B. cereus* originating from cooked food collected from these army kitchens. Molecular characterization was based on toxin gene profiling. Moreover, as the occurrence of enterotoxin genes does not allow for conclusions regarding toxin levels produced, the cytotoxic potential of all isolates was determined.

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Materials and Methods

Origin of bacterial strains

Twenty-one isolates of *B. cereus* had previously been isolated from cooked food collected from regiment kitchens in Switzerland in 2014 and 2015. Three isolates originated from meat dishes, six from starchy side dishes, five from vegetables, two from sauces, one from soup, and four from teas.

DNA extraction and toxin gene profiling

DNA was extracted from all isolates using the GenElute Bacterial Genomic DNA Kit according to the manufacturer's instructions (Sigma-Aldrich, St. Louis, MO). All isolates were screened for the different toxin genes using a PCR approach as previously described by Ehling-Schulz *et al.* (Ehling-Schulz *et al*., 2006) with minor modifications. The GoTaq PCR system was used at the following cycling conditions: i) 2 min at 95°C, ii) 30× [45 s at 95°C, 45 s at 51°C, 2 min at 72°C]; iii) 5 min at 72°C. In order to distinguish between *cytK1* and *cytK2,* a duplex PCR was performed (Guinebretière *et al.*, 2006). The following strains were used as positive controls for the different toxin gene PCRs: WSBC10028 (*nhe*, *cytK*, *ces*), CH-35 (*nhe*, *hbl*, *cytK*), NVH0391/98 (*cytK1*), NVH0674/98 (*cytK2*).

Cytotoxicity assay

Cytotoxicity of all isolates was determined in a Vero cell assay using WST-1 (Sigma-Aldrich, St. Louis, MO) as described elsewhere (Moravek *et al.*, 2006). Cell-free culture supernatants were produced by growing the strains in 30 ml CGY broth in an Erlenmeyer flask. The cultures were adjusted to an OD_{600} of 0.05 using an overnight culture of the isolate. The day cultures were grown to an OD of 7 at 30°C (120 rpm shaking) and were subsequently centrifugated at 11000 rpm for 10 min and filtrated through 0.2 µm sterile filtres. Aliquots of 1 mL supernatants were supplemented with 10 uL 0.1 M Na2 EDTA and stored at -80°C. Reference strains for low (RIVM Bc90) and high-level of toxin production (NVH 0075-95) were included in every run.

Results

Nearly all strains (95%) harbored the *nhe* gene, whereas no *hbl* gene could be detected. Seven strains displayed the *cytK2* gene which originated from different food categories. Moreover, one cereulide-producing strain was isolated out of vegetables. Tables 1 and 2 give an overview of the results of PCR-based screening for toxin genes carried by the isolates.

While all isolates were cytotoxic, a wide range of cytotoxicity levels could be detected (Figure 1). There was no correlation between food product and cytotoxic potential of the isolate. Three strains exceeded the cytotoxic level of the reference strain for high-level toxin production. They originated from pork sausage, tomato sauce, and French browned potatoes (*Pommes Risolées*). The product showing the highest *B. cereus* counts was tomato sauce.

Discussion

EFSA stated that foodborne illness caused by *B. cereus* has been associated with almost all categories of food products (European Food Safety Authority, 2005). In the present study, *B. cereus* was detected in a wide range of different foods, which is consistent with its ubiquitous presence.

The finding that nearly all isolates in this study harbored the *nhe* gene is in accordance with other studies (Guinebretière *et al.,* 2010; Moravek *et al*., 2006). However, it is uncommon that no *hbl* gene could be detected, given that this gene is normally

Table 1. Toxin genes detected in the 21 *B. cereus* isolates.

Strain ID	Source	Toxin genes			
		nhe	*hbl*	*cytK*	*ces*
CH_5	Minced meat	+	-	+ (*cytK2*)	-
CH_6	Cauliflower	+	-	-	-
CH_7	Rice	+	-	+ (*cytK2*)	-
CH_8	Tea	+	-	+ (*cytK2*)	-
CH_11	White wine risotto	+	-	-	-
CH_12	Älplermagronen	+	-	-	-
CH_14	Pork sausage	+	-	+ (*cytK2*)	-
CH_17	Mashed potatoes	+	-	-	-
CH_18	Pasta	+	-	-	-
CH_20	Fruit Tea	+	-	-	-
CH_21	Tea	+	-	-	-
CH_22	Cherry Tea	+	-	-	-
CH_27	Tomato sauce	+	-	-	-
CH_28	Pommes Risolées	+	-	-	-
CH_29	Carrots and peas	+	-	-	-
CH_30	Tomato sauce	+	-	-	-
CH_31	Okra and vegetables	+	-	+ (*cytK2*)	-
CH_32	Nasi Goreng & chicken	+	-	+ (*cytK2*)	-
CH_33	Carrots and peas	-	-	-	-
CH_36	Pumpkin soup	+	-	+ (*cytK2*)	-
CH_37	Mixed vegetables	+	-	-	+

Table 2. Distribution of toxin genes found in the *B. cereus* isolates based on their food origin.

Toxin genes	Meat dishes, n=3 (%)	Starchy side dishes or pasta, n=6 (%)	Vegetables, n=5 (%)	Sauces, n=2 (%)	Soup, n=1 (%)	Teas, n=4 (%)
nhe	3 (100)	6 (100)	4 (80)	2 (100)	1 (100)	4 (100)
hbl	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
cytK2	3 (100)	1 (17)	2 (40)	0 (0)	1 (100)	1 (25)
ces	0 (0)	0 (0)	1 (20)	0 (0)	0 (0)	0 (0)

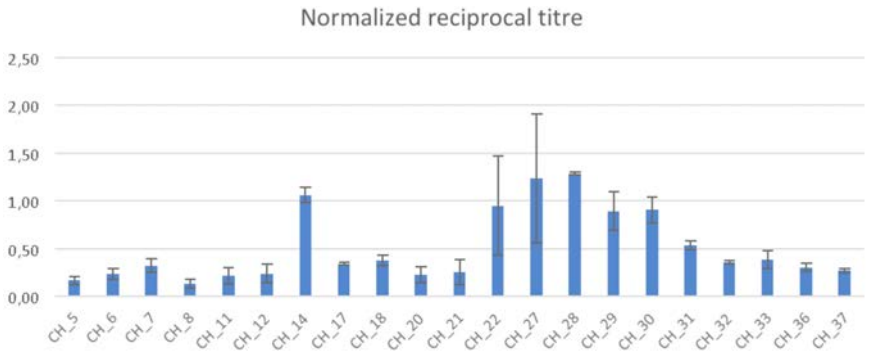

Figure 1. Reciprocal titres displayed by the 21 *B. cereus* isolates. A reciprocal titre of 1.00 equals a cytotoxicity level identical to the cytotoxicity of the highly toxic reference strain.

present with a frequency of 40% to 97% (Guinebretière *et al.,* 2010).

Several studies have stated that cereulide-producing strains are often associated with rice and pasta (Dierick *et al*., 2005; Naranjo *et al.*, 2011). Although starch-degrading properties are linked to the production of cereulide (Choma *et al*., 2000), the cereulide-producing strain detected in this study was isolated out of vegetables. Such findings have also been made by Messelhäusser *et al.* isolating emetic strains out of a wide variety of food products (Messelhäusser *et al.*, 2014). Cereulide is a heat- and acid-stable toxin able to survive cooking processes. The potential for cereulide formation cannot be solely predicted from the presence of the *ces*-gene, but also depends on external parameters such as the food matrix (Rajkovic *et al*., 2006). Kreuzberger has shown that 50% of all detected cereulide-producing strains in catering facilities of the German army were isolated from the staff's hands (Kreuzberger, 2007). Interestingly, these isolates were associated with higher Nhe toxin titres compared to isolates collected from equipment surfaces. This finding underlines the importance of personal hygiene and shows the potential risk of cross-contamination with cereulide-positive strains, which are in general rarely isolated from the environment (Messelhäusser *et al.*, 2014).

nhe is implicated as the most dominant diarrhoeal toxin, and correlation of cytotoxicity with Nhe concentration in supernatants has been described (Moravek *et al.*, 2006). However, the cytotoxic potential of the strain lacking the *nhe* gene (CH_33) in our study did not differ from the other isolates. Most isolates displayed middle or low cytotoxicity, which is consistent with the study from Kreuzberger (Kreuzberger, 2007). There, 16% of all strains were classified as high-level toxin-producers, which is in accordance with the present study showing that 14% of all isolates were more toxic than the reference strain. The isolate present with the highest bacterial counts also exhibited high level cytotoxicity (strain CH_27).

Conclusions

As shown in this study, the level of cytotoxicity is highly variable between isolates, ranging from barely toxic to highly toxic. Besides temperature control of heated foods, it is important to avoid contamination of kitchen surfaces with soil and dust to reduce the risk of foodborne illness caused by *B. cereus*. Meticulous hygienic procedures are essential to reduce cross-contamination. Therefore, cleaning and disinfection of equipments with agents exhibiting sporicidal activity is of highly importance (Ernst *et al*., 2006).

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