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Short communication

# Isolation of *Bacillus cytotoxicus* from various commercial potato products

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ABSTRACT

*Bacillus* (*B.*) *cytotoxicus* is a newly described thermotolerant member of the *Bacillus cereus* group. This potential foodborne pathogen had so far only been isolated from vegetable products, including mashed potatoes. Here we report the detection of *B. cytotoxicus* in a variety of potato products taken on retail level or from catering establishments (n = 151). Identification of isolates as *B. cytotoxicus* was performed after enrichment at 50 °C, followed by differentiation using Fourier transform-infrared spectroscopy and detection of the specific *cytK*-1 gene by PCR.

Thirty-five percent of all samples were positive for *B. cytotoxicus*. Highest prevalence was found in dehydrated potato products (44/62 = 71%) such as powder for mashed potatoes and products made thereof. *B. cytotoxicus* was not detected in products that were evidently made directly from potatoes (n = 24) but in one sample of raw potatoes (n = 10; 10%).

The high prevalence of this thermotolerant pathogen in potato products could pose a risk for consumers, especially if prepared foods are held at improper holding temperatures.



## 1. Introduction

Members of the *Bacillus* (*B.*) *cereus* group are generally known to cause two types of foodborne diseases: the diarrhoeal and the emetic type. In 1998, a new thermotolerant *B. cereus*-like strain (NVH391-98) was isolated from a food related outbreak in which three people died of necrotic enteritis (Lund et al., 2000). Extensive biochemical and genetic characterization of strain NVH391-98 and a small number of similar strains that have been reportedly isolated to date led to the proposal and recent designation of the new species *Bacillus cytotoxicus* (Lapidus et al., 2008; Guinebretiére et al., 2013). *B. cytotoxicus* does not produce the emetic toxin cereulide, and features an unusual operon for the *nhe* genes, encoding the components of the non-haemolytic enterotoxin (Nhe). Furthermore, its pathogenicity is mainly attributed to the greater cytotoxic activity of the pore-forming cytotoxin K-1 (CytK-1) compared to CytK-2, as can be found in *B. cereus* strains (Fagerlund et al., 2004, 2007). CytK-1 is the product of the *cytK*-1 gene (Fagerlund et al., 2004; Guinebretiére et al., 2006) and is thought to cause lysis of epithelial cells in the small intestine, resulting in diarrhoea (Lund et al., 2000; Hardy et al., 2001). However, while the presence of the *cytK*-1 gene is a reliable marker for *B. cytotoxicus*, not all strains feature a cytotoxic activity (Fagerlund et al., 2007, 2010). Nevertheless, Guinebretiére et al. (2010) still estimated the food poisoning potential for *B. cytotoxicus* (phylogenetic group VII) to be high.

While the natural habitat of *B. cytotoxicus* has yet to be determined, the majority of strains described so far were isolated from vegetable products (Lund et al., 2000; Guinebretiére et al., 2006; Rau et al., 2009; Guinebretiére et al., 2013).

Bacilli are common in agricultural soils and therefore can be expected to be part of the natural vegetable microflora. Due to their spore-forming capability, they are highly resistant to processing steps like heating or drying. Consistently *Bacillus* spp. were not only found to be the dominant aerobic mesophilic bacteria in pasteurised and packed commercial purees of various vegetables such as broccoli, carrot, leek, potato, split pea and zucchini (courgette) (Carlin et al., 2000; Guinebretiére et al., 2001), but was also identified as the major foodborne pathogen in various dehydrated potato products like granules, flakes or powder (reviewed by Doan and Davidson, 2000; Turner et al., 2006).

To investigate the occurrence of thermotolerant *B. cytotoxicus* in potato products, a selection of food samples (n = 151) containing potatoes or dehydrated potatoes were analysed by an enrichment procedure at 50 °C, followed by differentiation of *B. cytotoxicus* by Fourier transform-infrared spectroscopy (FT-IR) and detection of the specific *cytK*-1 gene by PCR.

## 2. Material and methods

### 2.1. Sample material

A total of 151 food samples representing typical products with potatoes or dehydrated potatoes as an ingredient were analysed (Table 1). All samples were acquired in South-West Germany over the course of one year except for the raw potatoes which were sampled in May and October. Products from categories A and C to J (Table 1) were

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**Table 1**
Samples tested for presence of *Bacillus cytotoxicus*.

| | Product group | Samples tested total (no.) | Samples tested positive (no.) | Samples tested positive (%) |
|---|---|---|---|---|
| A | Mashed potato powder/flakes/granules | 17 | 15 | 88 |
| B | Mashed potatoes, cooked | 9 | 9 | 100 |
| C | Other potato powders/flakes/granules[a] | 12 | 8 | 67 |
| D | Products made from potato powders/flakes/granules[b] | 24 | 12 | 50 |
| E | Raw potatoes | 10 | 1 | 10 |
| F | Pre-heated products directly made from potato[c] | 21 | 0 | 0 |
| G | Products with uncertain origin of potato ingredient[d] | 19 | 1 | 5 |
| H | Potato chips (crisps) | 13 | 2 | 15 |
| I | Ready-to-feed baby food with potato as an ingredient | 11 | 0 | 0 |
| J | Others[e] | 15 | 5 | 33 |
| | Total | 151 | 53 | 35 |

[a] E.g. for potato dumplings.
[b] E.g. potato dumplings and dough.
[c] E.g. fried potatoes and potato salad.
[d] E.g. French fries, either made directly from potato or from potato powder.
[e] E.g. potato bread and flour and instant soups.

sampled as being sold on retail level. These samples were taken at various establishments and from various brands to avoid a bias towards one producer. If samples of the same product were taken twice it was tried to analyse different lots. Cooked products (Table 1: category B) were sampled at hospital or nursing home kitchens (n = 7) or catering service establishments (n = 2), respectively.

Samples included 29 dehydrated storable pre-products, such as powder/flakes/granules for mashed potatoes or various potato dumplings (product categories A and C in Table 1; e.g. gnocchi, Schupfnudeln) and 9 samples of cooked mashed potato (B), 24 samples of foods made from dehydrated potato-material (potato dumplings or dough; D), 10 samples of raw potatoes (E), 21 samples of precooked/preheated foods evidently not made from dehydrated potato-material (F; e.g. potato salad or fried potatoes), 13 samples of potato chips (crisps) (H; 7 from sliced potatoes, 6 from dehydrated potatoes) and 15 samples of various other products with potato as an ingredient (J; e.g. instant soups, baking mixes with potato). For 19 samples, it was not obvious if they had been made from dehydrated pre-products or directly from potatoes (G, French fries, hash browns, potato pancakes).

As products for a sensitive consumer group, 11 ready-to-feed baby purees containing potato as an ingredient were integrated in the investigation (I).

### 2.2. Isolation of *B. cytotoxicus*

The results of pre-tests had indicated that the level of *B. cytotoxicus* contamination in potato products is typically rather low (<200 cfu/g; data not shown). Hence an initial qualitative approach was used in this study.

Ten grams of food were incubated in 90 ml of casamino acids–glucose–yeast extract medium (CGY; Merck, Darmstadt, Germany; Beecher and Wong, 1994) at 50 °C. Two loopfuls of the overnight culture were streaked onto sheep blood agar plates (blood agar base CM0055; Oxoid, Wesel, Germany; containing 5% defibrinated sheep blood, Oxoid) and mannitol–egg yolk–polymyxin (MYP) agar plates (Heipha, Heidelberg, Germany; Mossel et al., 1967), respectively. After overnight incubation at 50 °C (blood agar) and 37 °C (MYP) respectively, plates were checked for *B. cereus*-like growth: rough and dry colonies with haemolysis on blood agar; rough and dry colonies with a bright pink background surrounded by an egg yolk precipitate on MYP agar. Typically *B. cytotoxicus* will show the same growth characteristics but will grow in smaller colonies than *B. cereus* on MYP at 37 °C.

Samples that were tested positive after enrichment were subsequently analysed quantitatively if enough sample material was available. Ten grams of sample were diluted 1:10 in buffered saline; 100 µl of this dilution were plated on MYP agar and incubated at 50 °C overnight, resulting in a detection limit of 100 colony forming units per gram of food (100 cfu/g) for the quantitative analysis.

### 2.3. Identification of *B. cytotoxicus* by FT-IR plus ANN

Fourier transform infrared spectroscopy (FT-IR) in combination with artificial neural network based data analysis (ANN) was used for further differentiation of foodborne *Bacillus* isolates as described by Rau et al. (2009).

### 2.4. Isolation of DNA

DNA was isolated from pure cultures by thermal lysis: colony material from agar plates was resuspended in 100 µl $H_2O$ and incubated for 10 min at 99 °C. After centrifugation at 14,000 rpm for 30 s at 4 °C, the supernatant was used as a template for PCR assays.

### 2.5. Amplification and sequencing of the gene for 16S rRNA

The gene for 16S rRNA was partially amplified using primers 27f and 1522rN as described by Johnson (1994). Amplification and sequencing of both strands of the amplified 16S rDNA fragment were performed as described previously (Rau et al., 2009).

### 2.6. Amplification of the genes for cytotoxin K-1 and cytotoxin K-2

Amplification of *cytK*-1 and *cytK*-2 was performed using primers CK1F, CK1R, CK2F and CK2R according to Guinebretiére et al. (2006).

## 3. Results and discussion

Using enrichment in CGY at 50 °C with subsequent differentiation of isolates by FT-IR and confirmation by PCR analysis of the *cytK*-1 gene, thermotolerant *B. cytotoxicus* was isolated and identified with high specificity. Growth of *B. cereus* or *Bacillus thuringiensis* was successfully suppressed under the chosen conditions. Five isolates that were not unambiguously identified as *B. cytotoxicus* by FT-IR analysis were confirmed by sequencing of their partial gene for 16S rRNA, which resulted in sequences that showed highest similarities to known 16S rDNA sequences of *B. cytotoxicus*.

*B. cytotoxicus* could be isolated after enrichment from 53 (35%) of all samples tested (Table 1). Thermotolerant *B. cytotoxicus* were common in dehydrated material, powder or granules for potato products like mashed potatoes or dumplings (product categories A and C in Table 1; prevalence 79%, 23/29), as well as in the respective rehydrated,