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Prevalence of *C. botulinum* and *C. perfringens* spores in food products available on Polish market

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Abstract

Introduction: The aim of this study was to evaluate the prevalence of *Clostridium botulinum* and *Clostridium perfringens* in food samples purchased from Polish producers. **Material and Methods:** The analyses were performed on 260 food samples collected in Lublin and Subcarpathian regions: 56 of smoked meat, 21 of pork meat, 20 of dairy products, 26 of vegetable and fruit preserves, 40 of ready-to-eat meals, 27 of fish preserves, and 70 of honey collected directly from apiaries. **Results:** *C. botulinum* strains were isolated from 2.3% (6/260) of samples and the isolates were classified as toxin types A (4/260) and B (2/260). *C. perfringens* strains were isolated from 14% (37/260) of samples. All the isolates were classified as toxin type A, 28 of them were able also to produce α toxin and 9 – β2 toxin. **Conclusion:** On the basis of the obtained results it could be suggested that risk assessment, especially regarding the entire honey harvesting process, should be provided in order to ensure the microbiological safety of the products to be consumed by infants and people with a weakened immune system.

Keywords: food, *Clostridium botulinum*, *Clostridium perfringens*, Poland.

Introduction

Clostridium is a genus of Gram positive, spore-forming bacteria, which are able to grow under anaerobic conditions. Generally, the majority of these bacterial species are considered as saprophytic; however, some of them could cause severe health failures to the host organism (1, 3, 9, 23).

Spore-forming, anaerobic rods, which are able to produce botulinum toxins, are classified as the *C. botulinum* species. However, other species belonging to the genus *Clostridium*, such as *C. argentinense* (reported in literature as *C. botulinum* type G) and some strains of *C. baratii* and *C. butyricum*, can produce these toxins. The toxins are considered as the most dangerous biological substances in the environment (1, 9, 10).

Botulism is caused by immunologically distinct toxins (types A-G, and potentially H; data on the latter toxin type are under verification (3, 6)), produced by *C. botulinum*, or rarely *C. butyricum* (E) or *C. baratii* (F) (1, 4, 9, 10). Human botulism cases are caused mainly by A, B, and E toxin types. Five clinical forms of human botulism are recognised. Three of them are associated with consumption of food contaminated with toxins or *C. botulinum* spores. The most frequent form is classic or foodborne botulism and it represents a typical form of intoxication, where botulinum toxin is ingested and transported through blood to the synapses of neurons. Infant botulism is characterised by ingestion, multiplication, and colonisation of *C. botulinum* spores in the intestinal tract. Intestinal microbiota and composition of bile acids of infant's digestive tract do not represent a sufficient biological barrier to inhibit the multiplication and colonisation with *C. botulinum* (4); 15% of cases are associated with the presence of honey in infants' nutrition (19). Another form of intoxication is hidden botulism, with the symptoms of this form similar to infant botulism. It is also determined as adult variant of infant form and is caused by abnormalities of the digestive tract of adults, possibly due to previous antibiotic therapy or gastroenterological surgery (4).

The lethal dose of botulinum toxin for humans ranges from 0.2 μg/kg to 2 μg/kg of body weight (24),

but the exact infective number of *C. botulinum* spores is unknown.

Clostridium perfringens is a Gram-positive, spore-forming, anaerobic bacterium residing in soil, water, and gastrointestinal tract of various animals and humans. This ubiquitous microorganism is able to produce more than 15 different protein toxins with diverse modes of action. Five toxin types of this bacteria are distinguished, from A to E, on the basis of its ability to produce one or combination of four main toxins which are marked α, β, ε, and ι (7). The majority of human infections are caused by type A, despite the fact that this toxin type is considered an opportunistic pathogen, usually found as a natural saprophytic inhabitant of native microbiota of humans and animals. Occurrence of B, C, D, and E toxin types is always connected with disease symptoms. *Clostridium perfringens* could be transferred to food with soil, dust, through intestinal tract of slaughtered animals, and contamination due to inadequate hygiene during technological processing in abattoirs. In humans, it could cause diarrhoea and abdominal cramps within 24 h. The illness usually begins suddenly and lasts for less than 24 h. People infected with *C. perfringens* usually do not show fever or vomiting. The infection does not spread from person to person (2, 7).

The aim of this study was to evaluate the prevalence of *C. botulinum* and *C. perfringens* in food samples purchased from Polish producers. The samples of the products were analysed with a traditional culturing method, mouse bioassay, and molecular biology methods based on real-time PCR, PCR, and sequence analysis of PCR products.

Material and Methods

Positive controls. The following *C. botulinum* reference strains from NTCT collection were used as positive controls: NCTC 887 (type A), NCTC 3815 (type B), NCTC 8548 (type C), NCTC 8265 (type D), NCTC 8266 (type E), NCTC 10281 (type F) and *C. perfringens* strains*:* ATCC 13124 (type A able to produce toxin α) and A544/84 (isolate type A from the collection of the Department of Hygiene of Animal Feedingstuffs, able to produce toxins α and β2).

Samples. The analyses were carried out on 260 food samples: 56 of smoked meat, 21 of pork meat, 20 of dairy products, 26 of vegetable and fruit preserves, 40 of ready-to-eat meals, 27 of fish preserves, and 70 of honey collected directly from apiaries. The samples were collected in Lublin and Subcarpathian regions in 2011–2015. All products selected for the experiment were purchased from Polish producers.

Inoculation and culturing. Each sample (10 g) was inoculated directly to TPGY broth. Subsequently, inocula were subjected to pasteurisation process and incubated under anaerobic conditions for 72 h at 30°C. Anaerobic conditions were obtained using Oxoid system (Thermo Scientific, UK). Subsequently, 2–3 drops of liquid culture were inoculated onto the surface of Willis-Hobbs agar and incubated at 30°C for 48 h. After incubation on Willis-Hobbs agar, the phenotypic features characteristic for *C. botulinum* and *C. perfringens* were evaluated according to the protocol described in PN-R-64791:1994 (21). At the same time, 1 mL of liquid culture was transferred into tube with fresh TPGY broth, and incubated for 18 h at 30°C under anaerobic conditions. After incubation, the obtained culture was subjected to DNA extraction.

Extraction of DNA. The DNA was extracted from liquid culture and suspected colonies with the use of GenomicMini AX Bacteria commercial kit (A&A Biotechnology, Poland) according to the protocol described by the producer.

PCR methods. The real-time PCR screening method was used for *C. botulinum* detection, based on *ntnh* gene detection according to Raphael *et al.* (22) with modifications by Grenda *et al.* (8). Real-time PCR results were verified using multiplex PCR method for detection of specific BoNT genes determining production of A, B, E, and F toxins according to De Medici *et al.* (5). All PCR methods used in this study were previously validated on artificially contaminated food and feed matrices (8).

Sequence analysis. Because of difficulties with isolation and discrimination of *C. botulinum*, positive results of PCR were confirmed by sequencing. The analysis was performed with the same set of primers which was used in multiplex PCR. The obtained results of sequencing were analysed with the BLAST (Basic Local Aligment Search Tool) algorithm in order to detect the homology of PCR product sequences with those from GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

DNA extraction for *C. perfringens* detection. DNA extraction from the strains suspected of belonging to *C. perfringens* species was conducted according to the protocol described by Baums *et al.* (2).

Multiplex PCR method. Detection and determination of *C. perfringens* toxin types were conducted according to the method described by Baums *et al.* (2). This method enables detection of *cpa*, *cpb*, *cpe*, and *cpi* genes determining the production of main toxins: α, β, ε, and ι, and *cpb2* and *cpe* genes determining the production of additional toxins: β2 and CPE.

Gel electrophoresis. Gel electrophoresis was conducted in 2% agarose gel stained with ethidium bromide and run in 1 × TBE buffer (Fermentas, Lithuania) for 1.5 h under 100 V. Reaction mixture in a 10 µL volume and 2 µL of loading buffer 6 × DNA Loading Dye (Fermentas, Lithuania) was loaded into each well. The molecular weight of the obtained products was compared with a molecular weight marker – GeneRuler™ 100 bp DNA Ladder Mix

(Fermentas, Lithuania). PCR products were analysed under a UV light transluminator (Vilbe-Lourmat, France).

Mouse bioassay. This test was performed for isolates suspected of *C. botulinum* occurrence. The single experiment was carried out on three laboratory mice, following FDA protocol (1). TPGY cultures of suspected strains were centrifuged and the supernatant was divided into three 0.2 mL portions. One of them was heated at 100ºC for 10 min and administered intraperitoneally to one mouse. The other two were administered intraperitoneally to two other mice, one of which had previously been seroneutralised by equine monovalent antitoxin to botulinum neurotoxins A (BoNT/A) and B (BoNT/B) (HPA, UK).

Biochemical tests. The biochemical properties of suspected strains were evaluated with API 20A and Rapid ID 32A tests for anaerobic bacteria. The analyses were performed according to the producer's protocol.

Results

C. botulinum strains were isolated from 2.3% (6/260) of samples and the isolates were classified as toxin types A (4/260) and B (2/260). Positive results were only observed for honey samples and their number equalled 8.5% (6/70). Only proteolytic *C. botulinum* strains were isolated (Table 1). Also, the number of strains phenotypically similar to *C. botulinum* species (*C. botulinum*-like strains) was the highest for this matrix. The exact discrimination among *Clostridium* strains was not possible with API 20A test. This test failed to differentiate strains of metabolic group I to which proteolytic strains of *C. botulinum* and *C. sporogenes* are classified. Suspected strains were classified as *C. botulinum/ C. sporogenes*. Strains phenotypically similar to *C. botulinum* species were classified to *C. botulinum* (2/8) and to *C. sporogenes* (6/8) according to the Rapid ID 32A test. Both the latter and API 20A tests are invalid in *C. botulinum* detection. The sequencing of PCR products characteristic for *C. botulinum* species showed high level of homology to BoNT sequences deposited in GenBank. Mouse bioassay confirmed all positive results obtained from PCR analyses for *C. botulinum* species (Table 2). Example of positive PCR results for *C. botulinum* type B isolates is shown in Fig. 1.

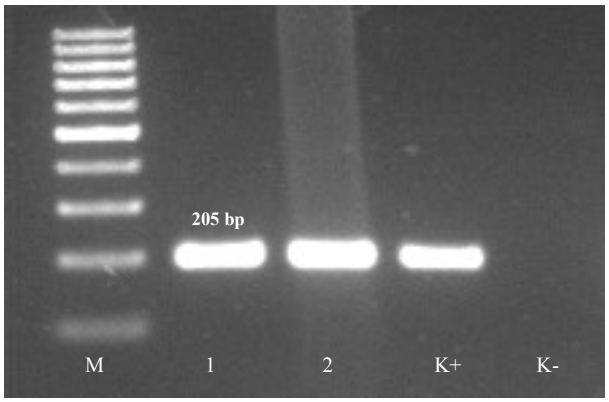

Fig. 1. Example of positive PCR products characteristic for *C. botulinum* toxin type B (205 bp).
M – molecular mass marker; 1, 2 – products characteristic for *C. botulinum* type B (205 bp); K+ – positive control (DNA from *C. botulinum* NCTC 3815 able to produce BoNT/B); K- – negative control

C. perfringens strains were isolated from 14% (37/260) of examined samples. All isolates were classified as toxin type A; additionally, 28 of them were able to produce α and 9 – β2 toxins (Table 1). According to the results of API 20A and Rapid ID 32A tests, all isolates were classified as *C. perfringens* species. An example of positive PCR results for *C. perfringens* type A isolates is shown in Fig. 2.

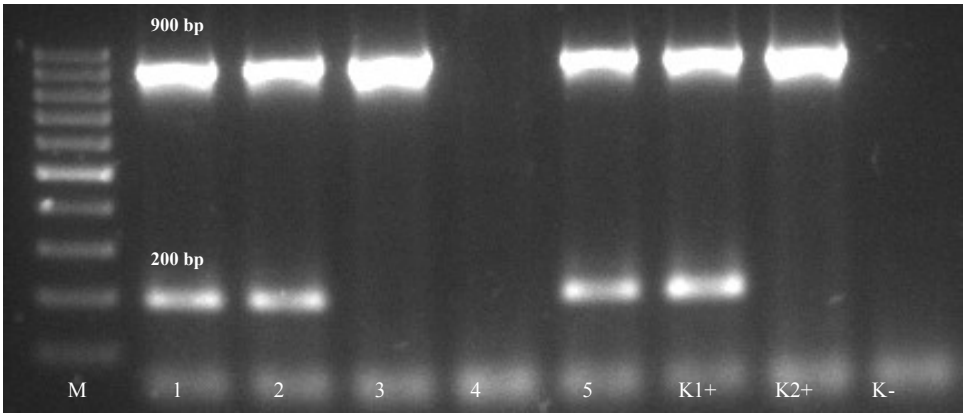

Fig. 2. Example of positive PCR products characteristic for *C. perfringens* type A able to produce toxins α (900 bp) and β2 (200 bp).
M – molecular mass marker; 1, 2, 5 – products characteristic for *C. perfringens* type A able to produce toxins α and β2; 3 –product characteristic for *C. perfringens* type A able to produce toxin α; K1+ – positive control (DNA from *C. perfringens* A544/84 able to produce toxins α and β2) K2+ – positive control (DNA from *C. perfringens* ATCC 13124 able to produce toxin α); K- – negative control

Table 1. Occurrence of *Clostridium* strains in examined food samples

Sample	Number of samples	*C. perfringens*	*C. botulinum*	*C. botulinum*-like strains
Smoked meat	56	-	-	-
Meat	21	4	-	-
Dairy products	20	-	-	-
Vegetable and fruit preserves	26	2	-	-
Ready-to-eat meals	40	4	-	1
Fish preserves	27	1	-	-
Honey	70	26	4A, 2B	7
Total	260	37 (14%)	6 (2.3%)	8 (3%)

Table 2. Homology of PCR products characteristic for *C. botulinum* isolates type A and B to sequences from GenBank

Strain number	Homology (%)	GenBank number of homologous sequences
APH1	92%	FJ981688
APH2	85%	FJ981688
APH3	88%	EU341306
APH4	97%	FJ981688
BPH1	93%	NC_012654
BPH2	95%	NC_012654

APH1; APH2; APH3, APH4 – toxin type A; BPH1; BPH2 – toxin type B

Discussion

Botulism cases in Poland have been reported since 1952. The frequency of the disease was very high in the 1960s and 1970s (*e.g.* in 1964 – 201 cases were reported), as well as in the 1980s, when due to the politically driven food crisis the production of home-made preserves was popular, albeit not always under sufficiently hygienic conditions. For example, in 1982 – 784 cases were reported in Poland, whereas the reports from other European countries showed a significantly lower number of botulism cases: Italy – 412 cases, Germany – 177, Spain – 92 (12). Nowadays, according to the reports of the National Institute of Public Health – National Institute of Hygiene, approximately 30 botulism cases are reported in Poland annually (29 cases in 2014 and 29 in 2015) (http://wwwold.pzh.gov.pl/oldpage/epimeld/index_p.html).

The natural contamination level of food products with *C. botulinum* spores is generally low and ranges from 10 to 1,000 spores/g (14). The obtained results showed difficulties in *C. botulinum* detection caused by low level of contamination, which could be higher than the limit of detection of methods used in the experiment and by the isolation of non-toxigenic strains or high contamination level, especially of honey samples by the other *Clostridium* strains. The location of neurotoxin genes cluster on plasmids and the loss of toxigenic properties during the culturing process are possible (4). This limitation was clearly visible for the isolates which have shown the biochemical features characteristic for *C. botulinum* strains. Biochemical tests are not sufficient for differentiation between *C. botulinum* and suspected strains belonging to this species. The high occurrence of *C. perfringens* could cause the inhibitory effect in *C. botulinum* isolation and detection, because of faster growth and lower nutritional demands of the first one. Combase model (http://www.combase.cc) shows that the number of *C. perfringens* can double during approximately 50 min at 30°C (pH 5, aw = 0.997), whilst the number of *C. botulinum* cells does the same after approximately 154 min. Furthermore, the results of biochemical testing seem to correlate with the results described by Linström *et al.* (13) who noticed that *C. botulinum* and botulinum-like strains show high biochemical similarity. They reported that Rapid ID 32A test did not offer proper identification, because α-β-glucosidase positive strains of *C. botulinum* were classified as *C. sporogenes* and API 20A did not allow for the proper identification of the two species mentioned above (13).

Honey seems to be one of the food products most contaminated with *C. botulinum*. We observed 8.5% prevalence of *C. botulinum* in this matrix. This result correlated with reports from other authors. Nevas *et al.* (20) described contamination of 26% (29/112) Danish samples by *C. botulinum* spores. The lowest level of contamination was detected in Swedish samples – 2% (1/50) (19). In Japan, two reports have been prepared on *C. botulinum* occurrence and according to Nakano and Sakaguchi (18) the percentage of positive samples reached 31% (11/36), whereas Nakano *et al.* (17) found the occurrence of this pathogen in 8.5% (4/48) of examined samples. In Turkey, Küplülü *et al.* (12) described that *C. botulinum* spores were detected in

12.5% of honey samples. Midura (15) reported the contamination of 10% (9/90) of examined samples in the USA. However, in Khazakhstan, Mustafina *et al.* (16) noticed the occurrence of *C. botulinum* only in 0.91% (1/120) of honey samples.

C. perfringens possesses characteristic metabolic and morphological features which enable easier detection than in the case of *C. botulinum* strains. The strains of this opportunistic pathogen are capable of fast and dynamic growth at 30°C in contrast to the other *Clostridia* (*e.g. C. botulinum*) (14). The number of examined samples with *C. perfringens* was significantly higher than those contaminated with *C. botulinum*.

The occurrence of *C. perfringens* in honey has been very rarely reported in literature. Tomassetti *et al.* (25) described the presence of this microorganism in 16.2% (6/37) of jar honey and in 11.3% (6/53) of comb honey obtained from 37 farms of Latium Region.

The exact number of infective number of *Clostridium* spores remains undetermined; however, even 1 cfu of *C. botulinum* could cause botulism symptoms in infants (15). On the basis of the occurrence of *C. botulinum* spores in examined samples, we could suggest that risk assessment should be implemented, especially on the entire honey harvesting process.

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Animal Rights Statement: All experiments on animals were conducted in approved laboratory unit with the approval of the II Local Ethics Committee in Lublin (Poland).

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