Clostridium botulinum spores in Polish honey samples

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The aim of this study was an examination of 240 multifloral honey samples collected from Polish apiaries to determine Clostridium botulinum occurrence. Honey was collected from apiaries directly after the extraction process. Samples were inoculated by using the dilution and centrifugation method. Suspected isolates were examined by using mouse bioassay, polymerase chain reaction (PCR), and real-time PCR methods. C. botulinum type A and B strains were detected in 5 of 240 examined honey samples (2.1%). Bacterial strains were also detected that were phenotypically similar to C. botulinum but that did not exhibit the ability to produce botulinum toxins and did not show the presence of the botulinum cluster (ntnh and bont genes) or expression of the nth gene. The methods used in the examination, especially the expression analysis of nth gene, enabled specific analysis of suspected strains and could be used routinely in environmental isolate analyses of C. botulinum occurrence.

Keywords: Clostridium botulinum, Polish apiaries, honey, neurotoxins

Introduction

Clostridium botulinum is a bacterium commonly found in soil and aquatic environments. This species is divided into four physiological groups (I–IV) which produce botulinum neurotoxins (BoNTs) and into eight different serotypes (A–G and X) [3,6,11,12,28]. BoNT/X, isolated in 2017, is the first BoNT serotype identified by applying sequencing and bioinformatics approaches [28]. C. botulinum strains, being divided into four genetically diverse metabolic groups, have inter-group heterogeneity, which causes problems in the detection of this microorganism.

Foodborne botulism is a severe type of food poisoning caused by the ingestion of food containing potent neurotoxins formed during the growth of C. botulinum [12]. Infant botulism is a common form, to which children between 2 weeks old and 1 year old are most susceptible [19]. It differs from foodborne botulism which proceeds after ingestion of the ready-formed neurotoxin, while in infant botulism the immature infantile intestinal flora allows ingested spores to germinate, multiply, and produce BoNTs in the intestinal lumen. The first infant botulism case was reported in the USA in 1976 [20]. Subsequently, numerous studies have associated the occurrence of infant botulism with the consumption of honey [15,19,24]. Given such evidence, the Centers for Disease Control and Prevention have issued a special recommendation that honey should not be given to infants under the age of 1 year [24]. It is significant that honey contaminated with C. botulinum spores does not differ in taste, color, or smell from uncontaminated honey [1].

The aim of this study was to examine honey samples collected from Polish apiaries situated in all 16 provinces of Poland for the presence of C. botulinum. This is the first representative study on the occurrence of C. botulinum in honey collected from the entire area of Poland.

Materials and Methods

Samples

The study was carried out on 240 honey samples (1 sample = 1 apiary) from 16 provinces (15 samples per province) in Poland (Table 1). Honey was collected in 2015 and 2016, directly from apiaries after the extraction process. The analyses were conducted using C. botulinum reference stains from the National Collection of Type Cultures (NCTC) collection as controls: NCTC 887 (toxinotype A), NCTC 3815 (toxinotype B), NCTC 8548 (toxinotype C), NCTC 8265 (toxinotype D), NCTC 8266 (toxinotype E), and NCTC 10281 (toxinotype F).
Table 1. Province of origin and numbers of samples collected from each Polish province

| Province               | Sample No. |
|------------------------|------------|
| Pomeranian             | 1–15       |
| Lubusz                 | 16–30      |
| Kuyavian-Pomeranian    | 31–45      |
| Łódź                   | 46–60      |
| Greater Poland         | 61–75      |
| Lublin                 | 76–90      |
| Lower Silesian         | 91–105     |
| Podlasie               | 106–120    |
| West Pomeranian        | 121–135    |
| Warmia-Masuria         | 136–150    |
| Subcarpathian          | 151–165    |
| Holy Cross             | 166–180    |
| Opole                  | 181–195    |
| Lesser Poland          | 196–210    |
| Silesian               | 211–225    |
| Masovian               | 226–240    |

Culture process

The direct centrifugation method, previously described [13,19], was used for culturing C. botulinum. A mass of 10 g of each honey sample was diluted in 90 mL of sterile distilled water with 1% Tween 80, and the mixture stirred until the solution became homogeneous. Subsequently, centrifugation was conducted for 30 min at 9,000 × g in a 4K15 centrifuge (Sigma, Germany). The precipitates were transferred into tubes with 10 mL of TPGY broth and incubated anaerobically for 48 h at 30 ± 1°C.

Nucleic acids preparation

DNA preparation: DNA was isolated from 1 mL of liquid culture and from several characteristic colonies obtained on agar plates by using the commercial Genomic Mini AX Bacteria kit (A&A Biotechnology, Poland) according to the manufacturer’s instructions. The DNA isolated from suspected C. botulinum strains was subjected to amplification of the 16S rDNA gene according to the method described by Vaneechoutte et al. [25]. The isolated DNA from liquid culture and the suspected strains were examined to detect ntnh and bont genes by using polymerase chain reaction (PCR) and real-time PCR techniques.

RNA preparation: RNA was extracted from isolates suspected of being C. botulinum. Total RNA was prepared by using the commercial Total RNA Mini kit (A&A Biotechnology). At least 500 ng of total RNA were subjected to cDNA synthesis.

cDNA synthesis: cDNA was synthesized with a High Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific, USA). Before being subjected to real-time PCR, the cDNA was diluted 10 times to achieve a concentration of approximately 100 ng per reaction mixture.

Molecular methods

Amplification and Sequencing of 16S rDNA: For identification of 16S rDNA from unidentified anaerobic strains suspected of being C. botulinum, primers previously described by Vaneechoutte et al. [25] were used (Table 2). Reactions were performed in the volume of 25 μL with the following reagent constituents: 5 μL of DNA matrix, 2.5 μL 10× Taq buffer with KC1 (Fermentas, Lithuania), 4 mM MgCl2, 200 μM dNTP, and 1.25 U per 25 μL Taq polymerase. The reaction was staged as follows: initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C for 45 sec, annealing at 55°C for 45 sec, extension at 72°C for 1 min, and a final extension at 72°C for 10 min.

The length of the obtained product was about 1500 bp. Sequencing of the obtained amplicons was entrusted to Genomed (Poland). The obtained FASTA files were analyzed...
by using the BLAST (National Center for Biotechnology Information, NCBI) algorithm, and the results were compared and assigned to the sequences from the NCBI that had the highest score and identity.

**Real-time PCR for nth and 16S rRNA housekeeping gene:** A set of 7 primers and a TaqMan probe (Table 3) were used for detection of the nth gene according to the method described by Raphael and Andreadis [21]. For expression analysis, a set of 2 primers and a TaqMan probe designed (Table 4) for C. botulinum sequences, and available from the NCBI database, was used additionally to detect the 16S rRNA housekeeping gene. The reactions for both genes were conducted with reagents comprising: 5 μL DNA or cDNA, 4 μL LightCycler TaqMan Master (Roche Diagnostics, Germany), 0.7 μM of each primer, and 0.24 μM TaqMan probe. Real-time PCR was performed by using a LightCycler 2.0 thermocycler with a temperature profile of 10 min at 95°C for initial denaturation, 45 cycles of denaturation at 95°C for 15 sec, annealing at 42°C for 15 sec, and elongation at 55°C for 1 min.

Normalization of the expression changes was performed according to Schmittgen and Livak’s protocol [22], on the assumption that reaction efficiency was close to 100%. The efficiency (E) was calculated on the basis of the slope value (m) obtained from the relationship between the cycle threshold (Ct) and dilutions of cDNA, according to the formula: E = 10^(-1/m).

**Calculation of nth gene expression:** Relative expression was calculated according to the comparative method described by Schmittgen and Livak [22]. The calculations were undertaken by applying the following formula: fold change = 2^(-ΔΔCt), where ΔΔCt = [(Ct gene of interest − Ct internal control)sample A − (Ct gene of interest − Ct internal control)sample B]. The reference C. botulinum NCTC 887 strain (toxin type A) served as sample A and the isolates obtained from positive honey were used as sample B as shown in the following:

\[ \Delta \Delta Ct = [(C_{nth} - Ct_{16S rRNA})_{C. botulinum}^{NCTC 887} - (C_{nth} - Ct_{16S rRNA})_{C. botulinum}^{isolated from honey}] \]

All samples were tested three times, and, for further analyses, the mean value was used.

**Multiplex PCR for bont/A, bont/B, bont/E, and bont/F genes:** The bont/A, B, E, and F genes were detected according to the multiplex PCR (mPCR) method described by De Medici et al. [5] and by utilizing four pairs of primers (Table 5). The reaction was prepared in a volume of 25 μL with the following set of reagents: 5 μL of DNA, 0.3 μM of each primer, 2.5 μL of 10× Taq buffer with KCl (Thermo Fisher Scientific), 4 mM of MgCl2, 200 μM of dNTP, and 1.25 U per 25 μL of Taq polymerase (Thermo Fisher Scientific). Detection of products was carried out on agarose gel.

Gel electrophoresis was conducted on 2% agarose gel stained with SimplySafe (EURx, Poland) and was run in 1× TBE buffer (Thermo Fisher Scientific) for 1.5 h at 100 V. The reaction mixture in a 10 μL volume with 2 μL of loading buffer, 6× DNA

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### Table 3. Sequences of primers and the molecular probe used for real-time polymerase chain reaction for nth gene detection

| Primer | Sequence | Reference |
|--------|----------|-----------|
| F1     | 5′ GATTTAAGTGAAAATTTATTTAATATAT 3′ |          |
| F2     | 5′ CCACTAAATATTTAATATGAA 3′ |          |
| F3     | 5′ TGATGAAAATCTAAATGTATGTAATATAT 3′ |          |
| CD2F   | 5′ GACATACAGATGATTTATGGGA 3′ | [21]     |
| R1     | 5′ TTAGGCTTACAAATATGATGGA 3′ |          |
| R2     | 5′ ACTAGCCATACAAATATGATGGA 3′ |          |
| R3     | 5′ TATTAAACTTCTCTGAGCTA 3′ |          |
| Probe  | Sequence |          |
| NTNH410 | 5′ FAM-ATCAATGGGGACGATATATTACGTA-BHQ 3′ |          |

All locked nucleic acids are marked with underlines (A;T). FAM, carboxyfluorescein; BHQ, black hole quencher.

### Table 4. Primers and molecular probe for housekeeping 16S rRNA gene detection in real-time polymerase chain reaction

| Primer | Sequence | Reference No. of sequences from GenBank |
|--------|----------|----------------------------------------|
| HK fw  | 5′ TTACCTGGAATGGACATC 3′ | L23477, NC_009495, NC_010674, NC_015425, NC_015425, NC_009698 |
| HK rev | 5′ GGTCTTGCGCTCTTTATGTT 3′ |          |
| Probe  | Sequence |          |
| HK probe | 5′ FAM-CAGGGTGTCATGGTGTGATGTA-BHQ 3′ |          |

N = A/G/T; FAM, carboxyfluorescein; BHQ, black hole quencher.
Table 5. Primers used in multiplex PCR method for detection of bont/A, B, E, F genes

| Toxotype | Primer            | Sequence              | Length of PCR product (bp) |
|----------|-------------------|-----------------------|---------------------------|
| A        | IA_03_fw          | 5′ GGCCCTAGAGGTAGCGTARTG 3′ | 101                       |
|          | IA_03_rev         | 5′ TCTTYYTATTCGAGATATCTTT 3′ |                           |
| B        | CBMLB1            | 5′ CAGGAGAAGTGGAGCCAAA 3′  | 205                       |
|          | CBMLB2            | 5′ CTGGGCGCTTCTTCTTGTG 3′  |                           |
| E        | CBMLE1            | 5′ CCAAGATTTTCATCCGCTTA 3′  | 389                       |
|          | CBMLE2            | 5′ GCTATTTCAAGAAGCTGA 3′   |                           |
| F        | CBMLF1            | 5′ CGGCTTCATTAGAGAACCGA 3′  | 543                       |
|          | CBMLF2            | 5′ TAACTCCCCATTGCCCGTAT 3′  |                           |

R = A and G; Y = C and T. PCR, polymerase chain reaction.

Table 6. Summary of culture, MBA, real-time PCR, and mPCR results obtained for suspected *Clostridium botulinum* isolates from examined honey samples

| Sample No. | MBA | Real-time PCR (ntrH gene presence) | mPCR |
|------------|-----|-----------------------------------|------|
|            |     | (bont/a gene presence)           | (bont/b gene presence) | (bont/e gene presence) | (bont/f gene presence) |
| 16         | +   | +                                 | –    | +                          | –                           |
| 24         | +   | +                                 | –    | –                          | –                           |
| 62         | –   | –                                 | –    | –                          | –                           |
| 66         | –   | –                                 | –    | –                          | –                           |
| 70         | +   | –                                 | –    | –                          | –                           |
| 139        | +   | +                                 | –    | –                          | –                           |
| 143        | +   | +                                 | –    | +                          | –                           |
| 144        | +   | +                                 | –    | –                          | –                           |

Only samples from which characteristic colonies suspected of belonging to *C. botulinum* were isolated. MBA, mouse bioassay; PCR, polymerase chain reaction; mPCR, multiplex PCR.

Loading Dye (Thermo Fisher Scientific), were loaded into each well. The molecular weights of the obtained products were compared with the GeneRuler 100 bp DNA Ladder Mix (Thermo Fisher Scientific) molecular weight marker. Finally, PCR products were analyzed under a Chemi-Smart 3000 UV light transilluminator (Vilber-Lourmat, France).

Mouse bioassay (MBA)

In order to verify positive PCR results, an MBA was performed for isolates considered to be *C. botulinum*. The single experiment involved three laboratory mice and followed procedure described by Solomon and Lilly [23]. After centrifugation of liquid culture in TPGY broth, the supernatant was divided into three 0.2 mL portions. One portion was heated at 100 ± 2°C for 10 min and, after cooling, administered intraperitoneally to one mouse. The other two portions were administered intraperitoneally into two mice, one of which had previously been seroneutralized by treatment with equine monovalent antitoxin to BoNT A (BoNT/A) and B (BoNT/B) (HPA, UK). All experiments on animals were conducted in an approved laboratory unit after obtaining permission from the II Local Ethical Committee in Lublin (Poland) (permission No. 5/2015).

Results

Culture characteristics

Eight bacterial strains with phenotypic features characteristic of *C. botulinum* were isolated from the examined samples. The obtained colonies exhibited the characteristic “pearl layer” and their precipitation zones indicated lipolytic properties. Proteolytic activity of all isolates (bright zones surrounding the colonies) on agar media was also observed (Table 6).

Sequencing analysis of 16S rDNA

Analysis of 16S rDNA sequences revealed that 6 isolates among the 8 isolates phenotypically similar to *C. botulinum* had the highest scores and identities with *C. botulinum* in the BLAST search. These strains were isolated from samples.
isolated from Lubusz, Greater Poland, and the Warmia-Masuria provinces. The percentage identity of all 8 examined isolates with phenotypic features characteristic of *C. botulinum* ranged from 91% to 99% (Table 7).

**Real-time PCR for nthh**

The *nthh* gene was detected in only 5 samples (16, 24, 139, 143, and 144). Positive results were obtained for DNA extracted from liquid culture and from isolates (Table 6).

**Multiplex PCR for detection of bont/A, bont/B, bont/E, and bont F genes**

The occurrences of *bont/A* and *bont/B* strains were detected in 5 of the 240 examined honey samples (2.1%). Two of the detected strains were qualified as toxinotype A and three as toxinotype B (Table 6).

**Expression analysis of nthh gene**

The efficiency of the real-time PCR was calculated at 92.89% for the *nthh* gene and at 96.38% for the 16S rRNA housekeeping gene. Relative expression was assessed for the *C. botulinum* isolates and is presented as a fold change relative to *nthh* gene expression from the *C. botulinum* NCTC 887 reference strain.

Relative expression values are reported in Table 8.

After data analysis, the highest expression level for a *C. botulinum* isolate was from sample 139 and was calculated at indicating a 181.01-fold change relative to the NCTC 887 reference strain. The lowest expression was calculated for an isolate from sample 144 and was equal to a −1.86-fold change relative to NCTC 887 (Fig. 1). Positive samples were collected from Lubusz, Greater Poland, and the Warmia-Masuria provinces.

**MBA results**

The MBA test proved the specificity of results that were obtained in the PCR and expression analyses. Positive results were obtained for only 5 of the samples included above.

**Discussion**

Since 1976, there have been over 1,500 cases of infant botulism reported in more than 15 countries worldwide [16].

Table 8. Relative expression of honey isolates

| Sample No. | Relative expression (fold change) |
|------------|----------------------------------|
| 16         | 84.44                            |
| 24         | 4.92                             |
| 139        | 181.01                           |
| 143        | 36.75                            |
| 144        | −1.86                            |

![Fig. 1.](https://example.com/fig1.png)
Among the various potential sources of *C. botulinum* spores (soil, dust, etc.), honey is the only dietary source that has been linked to botulism through both laboratory and epidemiological studies [1,17,18].

The dose of *C. botulinum* spores that can cause infection in human infants is undetermined. In a review by Austin [2], the author stated that a minimum dose for infant botulism has not been established. Arnon et al. [1] estimated that 10 to 100 *C. botulinum* spores are able to cause an infection. The lowest minimum number of cells needed to cause botulism was obtained in a sample of honey from Canada, which contained 1 spore per gram of honey. These figures were calculated from honey samples involved in actual infant botulism cases [15] and are based on the exposure of human infants to spore-containing honey. Therefore even 1 spore per gram can pose a potential risk of infant botulism [7].

The occurrence of *C. botulinum* noticed in Polish apiaries (2%) is lower or similar to the *C. botulinum* presence reported in samples from most other countries. Nevas et al. [19] described contamination of 25.9% (29/112) of Danish samples by *C. botulinum* spores (1 sample positive for toxin type A and 28 for type B). Nevas et al. [19] also reported that *C. botulinum* occurrence in Finnish honey samples was 10.5% (20/190; 8 samples showing type A toxin and 12 showing type B). In Norway, the percentage of positive samples was determined to be 10.7% (12/112; 7 of type B, 4 of type E, and 1 of type F) [19]. The lowest level of contamination in Nordic countries was observed in Swedish samples where it was 2% (1 occurrence of toxin type E) [19]. In Japan, *C. botulinum* occurrence has been reported twice; according to Nakano and Sakaguchi [18], the percentage of positive samples was 30.6% (11/36), whereas Nakano et al. [17] observed the occurrence of *C. botulinum* in 8.5% of the examined samples. In Taiwan, *C. botulinum* presence was detected in 1.3% (2/152) of examined samples [10]. In Turkey, Küplülü et al. [13] described *C. botulinum* spore detection in 12.5% of honey samples. Whereas Güçüköşghu et al. [10] reported a 2.7% (4/150) prevalence of *C. botulinum* spores in honey collected from the Turkish Black Sea region. Midura [15] reported *C. botulinum* occurrence in 10.0% (9/90) of examined samples from the USA. In Kazakhstan, Mustafina et al. [16] noted *C. botulinum* occurrence in only 0.9% (1/110) of honey samples. Polish honey was examined by two independent scientific teams and a sample contamination level of 8.6% (6/70) from the Lublin and Subcarpathia provinces of Poland was described by Grenda et al. [8], whereas Wojtacka et al. [27] detected *C. botulinum* spores in 21.6% (22/102) of samples from small apiaries in an undetermined area in Poland. Wojtacka et al. [26] described a high prevalence of *C. botulinum* spores in Lithuanian honey sold directly from the apiary, 62.5% (30/48), which is the highest level described to date in related literature. The procedure described by Wojtacka et al. [26,27] comprised only one mPCR method, which, in our opinion, is inadequate to survey *C. botulinum* occurrence in honey samples. The data in relevant literature and our own experience indicate the possibility of false-positive results caused by silent genes or frequently noticed non-specific PCR products, especially with detection performed on agarose gel [9]. The level of naturally occurring *C. botulinum* spore contamination is estimated to be in the range of 10 to 1,000 spores per gram of honey [14].

The detection of *C. botulinum* spores is a complicated task because of the high heterogeneity of this pathogen and horizontal gene transfer during the isolation process. The genus *Clostridium* comprises strains that show similar biochemical features to *C. botulinum* but are not able to produce botulinum toxins. Because of their heterogeneity, *C. botulinum* strains are classified into four metabolic groups, and other microorganisms that are not considered able to produce botulinum toxins are also related to these groups (excluding some toxigenic strains of *Clostridium butyricum* and *Clostridium beijerinckii* with the former having been reported as the causative agent in some infant botulism cases). *Clostridium sporogenes* is related to group I, *C. beijerinckii* and *C. butyricum* to group II, *Clostridium novyi* to group III, and *Clostridium subterminale* and *Clostridium schwimerenese* are related to group IV. In the honey samples of this study, we detected strains phenotypically similar to *C. botulinum* that did not show the presence of *ntnh* or *bont* genes. On the basis of 16S rDNA analysis, these strains were most related to *C. botulinum* and *C. sporogenes* [3,4]. This method used in this study enabled discrimination of suspected isolates only to the genus level. Among the 6 strains with the highest score and a *C. botulinum* identity, only 5 were classified to *C. botulinum* on the basis of real-time PCR, mPCR, and MBA analyses. The contamination level of *C. botulinum* spores could be dependent on the harvesting region of the honey samples and on hygienic aspects of the entire honey harvest process. According to Nevas et al. [19], the most influential factors on the presence of *C. botulinum* spores are: extractor size, wearing the same footwear outdoors and in the extraction room, the availability of hand-washing facilities in the extraction room, and the presence of *C. botulinum* in soil samples. The variability in phenotypic features among the strains of this pathogen, the silent *bont* genes, and the high probability of toxin gene loss in culture process (through the subsequent culture passages) beset *C. botulinum* detection with difficulties.

The set of methods used in this study enabled specific detection of *C. botulinum* and we recommend using them in routine analyses of honey samples for the occurrence of this pathogen. Simultaneous detection of *ntnh* and *bont* genes enables false positive results caused by non-specific products to be avoided. The common presence of the *ntnh* gene in all *C. botulinum* toxin types simplifies screening of suspected strains. In addition, expression analysis is easier because it is limited to

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only one gene. The presented set of methods could be used as a set of tools for supporting the laboratory diagnosis of botulism, thereby serving as an alternative to performing MBA for *C. botulinum* detection.

The number of Clostridia spores sufficient for infection is undetermined; however, even a single colony forming unit of *C. botulinum* could cause botulism symptoms in an infant. The obtained results have shown that risk assessment of the entire honey harvesting process should be undertaken in order to ensure the microbiological safety of the product, especially for infants and people with weakened immune systems.

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**Conflict of Interest**

The authors declare no conflicts of interest.

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