

Honey Sold Directly by Producers in the Silesian Region of Poland as a Source of *Clostridium botulinum* Types A, B, E, and F

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Abstract

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The level of contamination of honey with *Clostridium botulinum* spores is considered as an indicator of the adequacy of hygienic practices during collection, extraction, and subsequent processing. A total of 39 honey samples purchased directly from beekeepers at outdoor markets and from small amateur apiaries in Silesia were analysed for *Clostridium botulinum* spores. The samples were prepared using a dilution centrifugation method and cultured in parallel in cooked meat medium (CMM) and tripticase peptone glucose yeast (TPGY) enrichment broths. Identification of *C. botulinum* toxin types A, B, E, and F was performed with the use of a multiplex PCR method. The analysis showed six (15.4%) samples to be contaminated with *C. botulinum* spores. The major serotypes detected were type A – in two (5.1%) and type B – in two (5.1%) honey samples, respectively. Types E and F were found in 1 (2.6%) and 1 (2.6%) positive honey sample analysed, respectively.

Keywords: anaerobic bacteria; bee product; PCR; spores

The quality, integrity, purity, and nutritional value of honeys is receiving growing attention on an international level (ESTEVINHO *et al.* 2012). The same is also true for the unwelcome contamination of these products with bacteria (LONCARIC *et al.* 2011; RÓŻAŃSKA 2011; MADRAS-MAJEWSKA *et al.* 2014) and especially with *Clostridium botulinum*, which, as a cause of infant botulism, represents a hazard to human health. This bacterium is a common finding throughout the entire honey production chain (NEVAS *et al.* 2006) as it can be attached to soil particles and be spread by wind over long distances, deposited on

plants or even enter hives directly. Moreover, bees can carry soil particles on their body, for instance, in their pollen baskets or even in collected nectar or honeydew (KĘDZIA & HOŁDERNA-KĘDZIA 2010). In some cases, heavy contamination of honey with *C. botulinum* spores may be caused by dead bees in which *C. botulinum* has proliferated (NAKANO *et al.* 1994) and that have fallen on the bottom board. This occurs mainly in colonies that consist of so-called non-hygienic bees with low rates of debris and bee cadaver removal (SPIVAK & DOWNEY 1998; PALACIO *et al.* 2000).

In this context, different factors, e.g., climate change, changes in human activity such as land use (e.g., deforestation), transport or movement of animals, intensity of animal production, and changes to habitat may directly or indirectly pose a risk of contaminating honey with *C. botulinum*. Thus, the prevalence of botulism as well as other diseases caused by spore-forming bacteria may increase due to wet weather conditions after flooding or increased exposure to dust as a result of poorer quality grass and over-grazing (GALE *et al.* 2009). These circumstances make it impossible to either precisely trace these bacteria to their origin (LONCARIC *et al.* 2011), or to produce honey that is totally free of their spores (NEVAS *et al.* 2006). Nevertheless, a strict adherence to sanitary rules and hygienic procedures throughout the whole process of honey harvesting and extraction may significantly reduce the risk of unwanted bacterial contamination. Thus, the aim of our study was to evaluate the prevalence of *C. botulinum* spores in honey sold directly by producers in Poland.

MATERIAL AND METHODS

Honey samples. A total of 39 honey samples including 19 multifloral honeys, four linden honeys, four rape honeys, four buckwheat honeys, three nectar-honeydew honeys, two puffball honeys, one acacia honey, one goldenrod honey, one honeydew honey, and one heather honey purchased directly from Polish beekeepers in outdoor Silesian markets were used. The classification of honey origin was based on the beekeeper's declaration concerning the time of harvest, location of hives, and basic sensory features (colour, consistency, odour, and taste) according to the Polish Honey Norm PN-88 A-77626. All honey samples were collected from May to September 2015.

Culture in liquid and solid media. The samples were prepared and processed according to the method described by KÚPLŮLŮ *et al.* (2006) with reference to the scheme published by KOLUMAN *et al.* (2013). In order to carry out qualitative testing, two enrichment media were used, i.e., cooked meat medium (CMM; Himedia, India) and tripticase peptone glucose yeast extract broth (TPGY; Himedia, India). Solutions consisting of 10 g honey sample, 90 ml sterile distilled water, and 1% Tween[®] 80 (Sigma-Aldrich, USA) were stirred until they became homogenous, heated in a water bath at 65°C for 30 min to inactivate non-spore forms, and then centrifuged for 30 min at 9000 g.

The precipitates were added to 9 ml of CMM or 9 ml of TPGY and covered with sterile paraffin oil. The inoculated broths were incubated in an anaerobic chamber in an atmosphere of 90% N₂, 5% CO₂, and 5% H₂ for 7–10 days. CMM broth was incubated at 35°C for isolation of proteolytic strains of *C. botulinum* and TPGY broth was incubated at 26°C for isolation of non-proteolytic strains of *C. botulinum*. After seven days of incubation, each culture was examined for turbidity and gas production. Cultures that showed no significant growth within seven days were re-incubated for an additional three days. After 10 days of incubation, cultures with no significant signs of bacterial growth were classified as negative. All cultures showing turbidity and gas production were used for further analyses. The material taken from the tubes was used to prepare bacterioscopic slides that were subjected to Gram staining. Once the presence of bacilli was confirmed, isolation was performed. The material was inoculated with a loop onto the surface of anaerobic egg yolk agar (EYA; Himedia, India) and incubated under anaerobic conditions at 35°C for 48 hours. Typical iridescent colonies with precipitation zones were re-streaked on EYA for parallel aerobic and anaerobic culture at 35°C for 48 hours. The cultures grown in anaerobic conditions were used for PCR assays.

DNA isolation. Due to animal welfare considerations, a PCR assay was preferred over a mouse bioassay to identify the A, B, E, or F toxin types of *C. botulinum* strains. DNA was isolated from typical colonies obtained on the EYA plates and transferred to 1.5 ml microtubes with TPGY broth. The specimens were centrifuged at 12 000 g for 10 minutes. The obtained sediment was suspended in Tris buffer and DNA isolation was performed with the use of the Genomic Mini Kit (A&A Biotechnology, Poland), according to the producer's instructions.

PCR reaction. Multiplex PCR (mPCR) was carried out in a mixture containing 5 µl 10 × PCR buffer, 10 µl (5 mM) MgCl₂, 5 µl dNTPs (final concentration 200 mM), 0.5 µl of each forward and reverse primer (final concentration 0.1 mM) for bont/A, bont/E genes, and bont/B, bont/F genes, respectively, 2 µl Taq DNA polymerase, 5 µl template DNA and DNase- and RNase-free deionised water to a final volume of 50 µl in a thermocycler (Mastercycler, Eppendorf, Germany). The PCR primers are given in Table 1. The reaction cycle for PCR consisted of initial denaturation at 95°C for 15 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 60°C

Table 1. PCR primers used in the study

| Toxin type | Primer | Sequences (5'→3') | PCR product size (bp) | References |
|------------|--------------------|--|-----------------------|--------------------------------|
| A | IOA _f | GGG CCT AGA GGT AGC GTA RTG ^a | 101 | FENICIA <i>et al.</i> (2007) |
| | IOA _r | TCT TYA TTT CCA GAA GCA TAT TTT ^b | | |
| B | CBMLB _f | CAG GAG AAG TGG AGC GAA AA | 205 | LINDSTROM <i>et al.</i> (2001) |
| | CBMLB _r | CTT GCG CCT TTG TTT TCT TG | | |
| E | CBMLE _f | CCA AGA TTT TCA TCC GCC TA | 389 | LINDSTROM <i>et al.</i> (2001) |
| | CBMLE _r | GCT ATT GAT CCA AAA CGG TGA | | |
| F | CBMLF _f | CGG CTT CAT TAG AGA ACG GA | 543 | LINDSTROM <i>et al.</i> (2001) |
| | CBMLF _r | TAA CTC CCC TAG CCC CGT AT | | |

^aR = A or G; ^bY = C or T

for 30 s and extension at 72°C for 85 seconds. Final extension was at 72°C for 3 minutes. The amplified PCR products were visualised in 2% LSI agarose gel in TAE buffer stained with ethidium bromide at a concentration of 5 µg/ml. Electrophoresis was performed with the use of the Sub-Cell[®]GT Cell Bio-Rad horizontal electrophoresis system at 100 V for 60 minutes. Visualisation of DNA fragments after electrophoresis was performed on a UVView Transilluminator (Bio-Rad, UK). The size of obtained amplification products was compared with a 100 bp molecular weight marker. Strains classified as A, B, E, and F were additionally compared with the following *C. botulinum* reference strains from NCTC (National Collection of Type Cultures): NCTC 887, NCTC 3815, NCTC 8266, and NCTC 10281. DNA of the *C. botulinum* reference strains was used as a positive control for the mPCR reaction.

Statistical analysis. Statistical analyses were performed using Statistica software 9PL. The chi-squared (χ^2) test was used to estimate the differences in the spore prevalence rates based on the type of honey. A probability (*P*) less than or equal to 0.05 was considered as statistically significant.

RESULTS AND DISCUSSION

Out of a total of 39 honey samples, gas production and turbidity were noted in nine samples of multifloral honey, four samples of linden honey, four samples of rape honey, one sample of nectar-honeydew honey, one sample of puffball honey, and one sample of honeydew honey after seven days of incubation. After an additional three days of incubation, these signs were also observed in four more samples of multifloral honey and one more sample

of nectar-honeydew honey. There were no signs of gas production and turbidity in 13 (33.3%) of the samples analysed. All positive samples subjected to Gram staining showed purple rods, sporulating forms, and spores visible as empty spaces inside the purple stain. Culture on EYA in anaerobic conditions resulted in a typical white opaque diffuse zone and colonies with the appearance of an iridescent sheen in 26 samples, which were subjected to further molecular analyses in order to enable identification of *C. botulinum* toxin types. The presence of *C. botulinum* was confirmed by the mPCR assay in six (15.4%) samples out of the total of 39 honey samples analysed in this study. The number and distribution of Botulinum neurotoxin (BoNT) types in honey samples positive for *C. botulinum* spores as detected by microbiological culture and mPCR are given in Table 2. The identification of *C. botulinum* toxin types revealed BoNT type A and B strains to be the most common. These were noted in a total of four honey samples, whereas types E and F were detected in one sample each. There was no significant difference in the prevalence of any of the BoNT spore types between the different samples ($P > 0.05$). The spores of *C. botulinum* type B were found in two types of positive honey samples, linden (25%) and nectar-honeydew (33.3%) honey. There was no significant difference in prevalence between these two types of honey ($P > 0.05$). The spores of *C. botulinum* type A were found only in multifloral honey samples, which accounted for 10.5% of all samples of this type of honey that were analysed. *C. botulinum* spores of types E and F were detected in puffball honey and in nectar-honeydew honey, respectively, representing 2.6% of all the samples analysed in both cases. Taking into account the number of positive samples of each honey type, there

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Table 2. Contamination of Silesian honey sold directly by producers with *C. botulinum* types A, B, E, and F as detected by mPCR

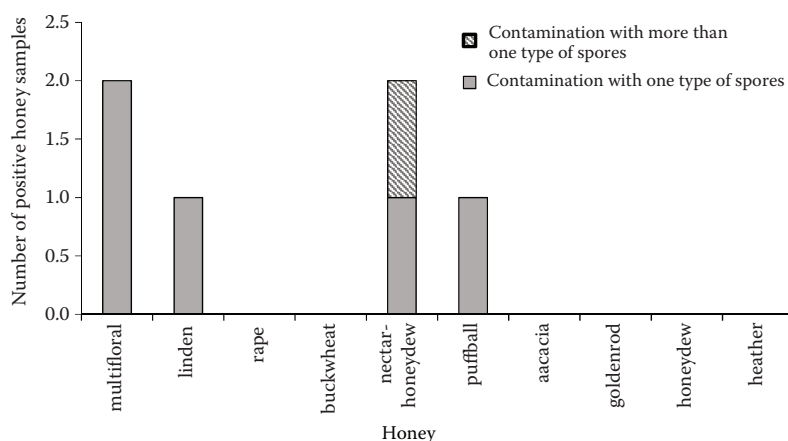
| Honey type | Number of samples | Number of microbiological culture positive samples | Number of mPCR positive samples | Number (%) of detected BoNT types | | | |
|-----------------|-------------------|--|---------------------------------|-----------------------------------|-----------|----------|-----------|
| | | | | A | B | E | F |
| Multifloral | 19 | 13 (68.4%) | 2 (10.5%) | 2 (10.5%) | – | – | – |
| Linden | 4 | 4 (100%) | 1 (25%) | – | 1 (25%) | – | – |
| Rape | 4 | 4 (100%) | 0 (0%) | – | – | – | – |
| Buckwheat | 3 | 0 (0%) | 0 (0%) | – | – | – | – |
| Nectar-honeydew | 3 | 2 (66.7%) | 2 (66.7%) | – | 1 (33.3%) | – | 1 (33.3%) |
| Puffball | 2 | 2 (100%) | 1 (50%) | – | – | 1 (50%) | – |
| Acacia | 1 | 0 (0%) | 0 (0%) | – | – | – | – |
| Goldenrod | 1 | 0 (0%) | 0 (0%) | – | – | – | – |
| Honeydew | 1 | 1 (100%) | 0 (0%) | – | – | – | – |
| Heather | 1 | 0 (0%) | 0 (0%) | – | – | – | – |
| Total | 39 | 26 (66.7%) | 6 (15.4%) | 2 (5.1%) | 2 (5.1%) | 1 (2.6%) | 1 (2.6%) |

were no significant differences in the prevalence of any BoNT spore types detected between any of the 10 types of honey. Nectar-honeydew honey was the only type of honey that showed multi-contamination with different BoNT types, whereas rape, acacia, goldenrod, honeydew, and heather honey samples ($n = 11$) were totally free from *C. botulinum* spores (Figure 1).

Clostridium spp. and *Bacillus* spp. spores were first analysed in Polish honey by KĘDZIA *et al.* (1996). They noted that *Clostridium* spp. spores were twice as likely to be found in honey compared to *Bacillus* spores. Honey is consumed primarily for nutritional benefits and as a preventive treatment, and for these reasons it is a common component of the diet of children and the elderly (ROMAN *et al.* 2013). In this way, children can contract botulism from the consumption of contaminated honey (ARNON 1998).

The minimum infective dose of *C. botulinum* spores for human infants is not known, but on the basis of exposure to spore-containing honey, it has been estimated to be as low as 10–100 spores (ARNON *et al.* 1979). However, there are no official estimates of infant botulism in Poland and the link between infant botulism and sudden infant death is not clear (BÖHNEL *et al.* 2001). The above is of particular significance due to the fact that the presence of *C. botulinum* spores in honey may pose a risk of infection not only to infants, but also to people with anatomical or functional bowel abnormalities or to patients using antimicrobials (SOBEL 2005).

Compared to studies carried out by other Authors (NEVAS *et al.* 2002) that confirmed contamination of honey with *C. botulinum* spores at a level of 7–11%, the results obtained in this study show that Silesian honey from small amateur apiaries is relatively highly

Figure 1. Occurrence of *Clostridium* spp. spores in different types of honey

contaminated with *C. botulinum* spores, at levels reaching 15.4%. However, a much higher contamination level of 26% was found in a Danish study carried out on honey originating from apiaries of different sizes and on a similar number of samples (NEVAS *et al.* 2005). Analysis of honey samples from the south-western region of Poland showed the presence of four types of *C. botulinum*, which is in line with results obtained in Scandinavian countries and Turkey (NEVAS *et al.* 2005; KOLUMAN *et al.* 2013).

Our results show that although the guidelines concerning adequate lighting, access to sources of running water, and appropriate rooms during the whole production process are very strict, the level of contamination of Polish Silesian honey from small apiaries with *C. botulinum* spores remains relatively high. Significantly, apiaries of up to 20 colonies, mainly amateur, represent over 60% of all apiaries in Silesia, which is indicative of low profitability and a lack of sufficient funds for investment. In this regard, in Poland, the proportion of professional beekeepers in relation to non-professionals is very small – about 0.59% – while the EU average is 3.2%, and in some countries, such as Greece and Spain, it is as high as 36% and 25%, respectively (SEMKIW & OCHAL 2012). The high rate of contamination may also be due to the contamination of bee pastures since small apiaries are often run by farmers and consequently are located close to small farms with different animal species.

Silesian agriculture is a very good example of the fragmentation of Polish agriculture and of the frequent location of apiaries in the vicinity of livestock farming where land is more likely to be contaminated with spores of *C. botulinum*. This is an additional important factor contributing to the presence of *C. botulinum* spores in the investigated honeys. The correlation between the localisation of farms and *C. botulinum* contamination of soil was confirmed by studies conducted in Denmark (HUSS 1980) and in the central part of Argentina (LUQUEZ *et al.* 2005); a higher prevalence of *C. botulinum* spores was observed in non-virgin soils compared to virgin soils. Moreover, the prevalence of *C. botulinum* spores found in unwrapped linden flowers that are several feet over the soil surface was lower (BIANCO *et al.* 2009) than that detected in small medical plants (SATORRES *et al.* 1999), which indicates that environmental conditions leading to the production of dust may contribute to the contamination of honey with *C. botulinum* spores.

CONCLUSION

Contamination of honey harvested and then directly sold in Silesia, Poland, in the year 2015 with spores of *C. botulinum* types A, B, E, and F was estimated at 15.4%. There were no differences between the types of Polish honey with regard to contamination with different BoNT types. The presence of *C. botulinum* spores in honey originating from amateur apiaries indicates a relatively high contamination of bee pastures.

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