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Prevalence of *Clostridium botulinum* Type A, B, E and F Isolated From Directly Sold Honey in Lithuania

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ABSTRACT

The aim of the study was the analysis of Lithuanian honeys for the presence of the spores of *Clostridium botulinum*. The analyses were performed on multifloral, honeydew, nectar-honeydew, rape, buckwheat, heather and linden honey bought in direct sale. Prior to culture on solid media the samples were prepared by supernatant filtration method and cultured in parallel in Cooked Meat Medium (CMM) and Tripticase Peptone Glucose Yeast (TPGY) enrichment broths. Bacteria of typical phenotypic features were obtained by culture method from 48 honey samples. Identification of *Clostridium botulinum* types A, B, E and F was performed with the use of multiplex-PCR method. The prevalence analysis showed 30 (60%) samples contaminated with *Clostrdium botulinum* spores. The major serotype detected was type A: 20 (66.6%) positive honey samples; while type B was found in 7 samples (23.3%), type E in 4 samples (13.3%) and type F in 3 (10%) positive honey samples. Contamination with more than one type of *C. botulinum* spores was observed in 3 honey samples.

Keywords: Botulism, Contamination, *C. botulinum* spores, Cooked meat medium, Multiplex-PCR, TPGY.

INTRODUCTION

Honey is one of natural products widely considered as clean and healthy. Its physico-chemical properties related to low water activity, high sugar concentration, as well as the presence of hydrogen peroxide, flavonoids and phenolic acids prevent the growth or even survival of bacteria. However, many researches have pointed at different sources of microorganisms in honey, such as pollen, the digestive tract of honey bees, dead bees, soil, water, air, and nectar (Nakano *et al*., 1994; Bianco *et al*., 2009; Gale *et al*., 2009; Gomes *et al*., 2011; Estevinho *et al*., 2012). There are also hygiene of processing, handling, and storage of honey that contribute to its contamination with the spores of different microorganisms that can be isolated (Loncaric *et al*., 2011; Różańska, 2011; Madras-Majewska *et al*., 2014). The anaerobes, including *C. botulinum,* are reported as the predominant microflora (Różańska, 2011). *C. botulinum* is a diverse group of Gram-positive spore-forming organisms that produce Botulinum NeuroToxins (BoNTs) during their growth. BoNTs are the most potent toxins known, and when entering human or animal tissues and subsequently blood circulation, they block neurotransmitter release from nerve endings, causing a neuroparalytic condition known as botulism. Unlike food-borne botulism, the other forms of human botulism are actually infections where the toxigenesis

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occurs *in vivo* (Sobel, 2005). The most common of these is infant botulism, where botulinal spores germinate, grow, and produce toxin in the gastrointestinal lumens of small babies, in whom the normal gut microflora is poorly developed (Lindström and Korkeala, 2006). Children aged between 2 weeks and 1 year are most susceptible.

C. botulinum, which is a hazard to human health, as a source of infant botulism was shown to be a common finding throughout the whole honey production chain (Nevas *et al*., 2006). Moreover, it is impossible to trace this bacteria exactly to their origin (Loncaric *et al*., 2011) or to produce honey totally free from their spores (Nevas *et al*., 2006). The average Lithuanian apiary consists of 12.1 hives and the consumption of honey in Lithuania is 0.1 kg per inhabitant (Pidek and Pohorecka, 2004). The consumers perceive honey bought directly from the beekeepers as a safe product with healing properties, often used not only in preventing but also in treatment of various diseases. The survey of Vilnius residents showed that almost half of the organic food consumers buy this kind of food at farmers' markets and food fairs (Stukas *et al*., 2010). In these terms, following strict sanitary rules and hygiene procedures along the whole process of honey harvesting and extraction significantly reduces the risk of its unwished bacterial contamination (Nevas *et al*., 2006). Thus, the aim of our study was to evaluate the prevalence of *C. botulinum* spores in Lithuanian honey from apiaries delivering their product through direct sale.

MATERALS AND METHODS

Honey Samples

A total of 50 honeys samples were used including 21 multifloral honeys, 13 linden honeys, 6 nectar-honeydew honeys, 4 honeydew honeys, 3 rape honeys, 2 buckwheat honeys, and 1 heather honey purchased directly from the beekeepers in the open-air markets and apiaries in Northeastern and Southern Lithuania. The classification of honey origin was based on the beekeepers declaration consistent with the time of harvest, location of hives, and basic sensory features (color, consistency, odor and taste) according to the Polish Norm "Honey" (PN-88 A-77626, 1988). All honey samples were collected from May to September 2014.

Culture in Liquid and Solid Media

The samples were prepared and processed according to the supernatant filtration method described by Küplülü *et al*. (2006) with reference to the procedure published by Koluman *et al*. (2013). In order to process qualitative testing, two enrichment media were used i.e. Cooked Meat Medium (CMM, Himedia) and Tripticase Peptone Glucose Yeast broth (TPGY, Himedia). The solutions of 10 g honey sample, 90 mL sterile distilled water and 1% Tween® 80 (Sigma-Aldrich) were stirred until they became homogenous, then, heated in a water bath at 65°C for 30 minutes to inactivate non–spore forms and centrifuged for 30 minutes at 9,000×g. The spores were captured from supernatant by filtration through 0.45 µm filter. The filters were inoculated into 9 mL of CMM and 9 mL of TPGY and covered with sterile paraffin oil. The inoculated broths were incubated under anaerobic conditions for 7-10 days. CMM broth was incubated at 35°C for isolation of proteolytic strain of *C. botulinum*, and TPGY broth was incubated at 26°C to isolate non-proteolytic strains of *C. botulinum*. After 7 days of incubation, each culture was examined for turbidity and gas production. Cultures that showed no significant growth within 7 days were re-incubated for an additional 3 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. A droplet of material taken from the tubes was used to prepare

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bacterioscopical slide subjected to Gram staining. Once the presence of bacilli was confirmed, the isolation stage was performed. The material was applied with a loop on the surface of Anaerobic Egg Yolk Agar (EYA, Himedia) and incubated under anaerobic conditions at 35°C for 48 hours. Typical iridesce colonies with precipitation zones were re-streaked on AEY for aerobic and anaerobic parallel culture at 35°C for 48 hours. The cultures grown in anaerobic conditions were used for PCR assay.

DNA Isolation

Typical colonies obtained on the AEY plates were transferred to 1.5 mL microtubes with TPGY broth. The specimens were centrifuged at 12,000×g for 10 minutes. The sediment obtained was suspended in Tris buffer and DNA isolation was performed with the use of Genomic-Mini Kit (A and A Biotechnology), according to the producer's instruction.

PCR Reaction

Diluted samples were used in multiplex PCR assay that was carried out on 5 µL of DNA template in a final mixture of 50 µL containing 2.5 µL 10X PCR buffer, 1.5 µL MgCl2 (50 mM), 0.2 µL d NTP (10 mM), 0.8 µL of each forward and reverse primers (final concentration 0.1 mM) for bont/A, bont/E genes and bont/B, bont/F genes, respectively, 0.2 µL Taq DNA polymerase (50 μL^{-1}). The PCR primers are given in Table 1. Reaction cycle for PCR consisted in an initial denaturation at 95°C for 15 minutes, 30 repetitions of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds and extension at 72°C for 85 seconds. Final extension was at 72°C for 3 minutes. The amplified PCR products were visualized in 2% LSI agarose gel in TAE buffer stained with ethidium bromide of 5 µg mL^{-1} concentrations. Electrophoresis was performed with the use of horizontal electrophoresis system Sub-Cell®GT Cell Bio-Rad at 100 V for 60 minutes. Visualization of DNA fragments after electrophoresis was performed on the UView Transiluminator (Bio-Rad).The weight of amplification products obtained was compared with 100 bp molecular weight marker. The strains classified as A, B, E, and F were additionally compared with reference *C. botulinum* strains from NCTC (National Collection of Type Cultures): NCTC 887, NCTC 3815, NCTC 8266 and NCTC 10281, respectively. DNA of the reference *C. botulinum* strains was used as a positive control for multiplex PCR reaction

Table 1. PCR primers used in the study according to standard ISO/TS 17919:2013.

Toxin type	Primer	Sequences (5' – 3')	PCR product size (bp)	References
A	IOA_f	*GGG CCT AGA GGT AGC GTA RTG* [a]	101	Fenicia *et al.* (2007)
	IOA_r	*TCT TYA TTT CCA GAA GCA TAT TTT* [b]		
B	$CBMLB_f$	*CAG GAG AAG TGG AGC GAA AA*	205	Lindstrom *et al.* (2001)
	$CBMLB_r$	*CTT GCG CCT TTG TTT TCT TG*		
E	$CBMLE_f$	*CCA AGA TTT TCA TCC GCC TA*	389	Lindstrom *et al.* (2001)
	$CBMLE_r$	*GCT ATT GAT CCA AAA CGG TGA*		
F	$CBMLF_f$	*CGG CTT CAT TAG AGA ACG GA*	543	Lindstrom *et al.* (2001)
	$CBMLF_r$	*TAA CTC CCC TAG CCC CGT AT*		

[a] R= A or G. [b] Y= C or T.

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presented in the study.

Statistical Analysis

Statistical analyses were performed using Statistical software 9PL. The Chi-square (χ^2) test was used to estimate the differences in the levels of spores isolation based on the type of honey. The Probability (P) less than or equal to 0.05 was considered as statistically significant.

RESULTS

Fifty honey samples were subjected to microbiological analyses. Gas production and turbidity was not noted in 3 samples of multifloral honey after 7 days of incubation. Additional 3 days of incubation caused the signs described in one more sample. All positive samples subjected to Gram staining showed purple rods, sporulating forms and spores visible as empty spaces inside the purple stain. Culture on AEY in anaerobic conditions resulted in obtaining typical white opaque diffuse zone and iridescent sheen colonies in 48 samples subjected to the following molecular testing. The presence of *C. botulinum* was confirmed by mPCR assay in 30 (60%) samples of the 50 honey samples analyzed from the beginning of the study. *C. botulinum* spores were found in 9 (42.9%) of the 21 multifloral honey samples, 9 (69.2%) of the 13 linden honey samples, 5 (83.3%) of the 6 nectar-honeydew honey samples, 3 (75%) of the 4 honeydew honey samples, 1 (33.3%) of the 3 rape honey samples, 2 (100%) of the 2 buckwheat honey samples, and 1 (100%) of the one heather honey sample. Three samples, i.e. 2 of multifloral and 1 of linden honey, were originally contaminated with more than one type of *C. botulinum* spores (Table 2). No amplification was shown by 18 samples qualified for molecular tests based on the results of microbiological analyses and PCR analyses did not confirm the presence of *C. botulinum*.

BoNT type A strains were the most numerous. They were noted in total in 20

Table 2. Prevalence of *C. botulinum* types A, B, E and F in honey sold directly in Lithuania as detected by mPCR.

Types of honey	Number of samples analyzed	Number of mPCR positive samples	Number of *C. botulinum* isolates	Detected types of *Clostridium botulinum*			
				Type A	Type B	Type E	Type F
Multifloral honey	21	9 (42.9%)	12	6 (50%)	3 (25%)	1 (8.3%)	2 (16.7%)
Honeydew honey	4	3 (75%)	3	2 (66.7%)	1 (33.3%)	-	-
Nectar – honeydew honey	6	5 (83.3%)	5	2 (40%)	1 (20%)	1 (20%)	1 (20%)
Rape honey	3	1 (33.3%)	1	1 (100%)	-	-	-
Buckwheat honey	2	2 (100%)	2	2 (100%)	-	-	-
Heather honey	1	1 (100%)	1	-	-	1 (100%)	-
Linden honey	13	9 (69.2%)	10	7 (70%)	2 (20%)	1 (10%)	-
In total	50	30 (60%)	34	20 (58.8%)	7 (20.6%)	4 (11.8%)	3 (8.8%)

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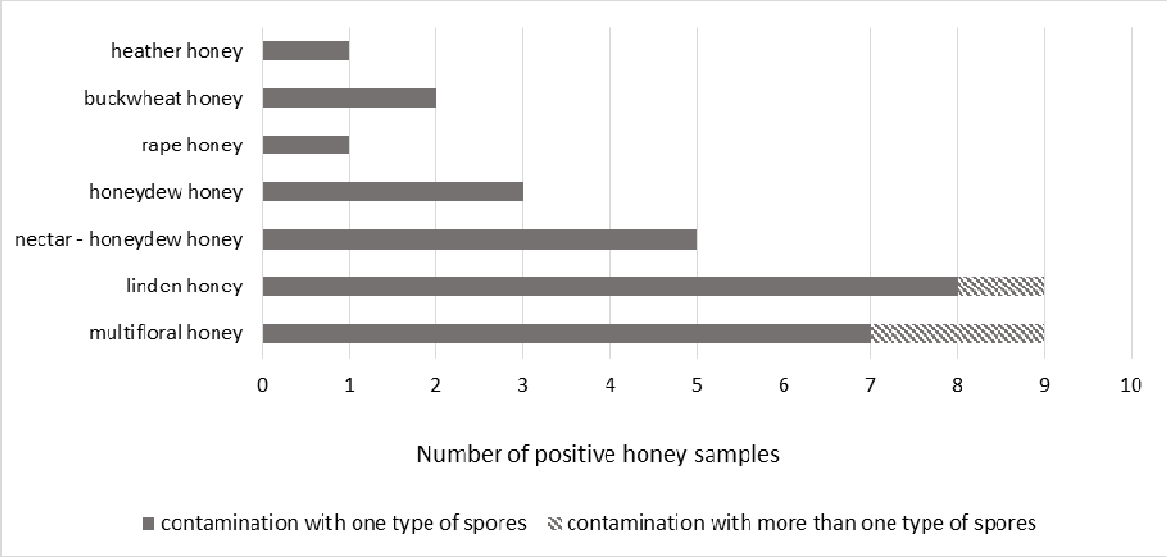

Figure 1. Number of honey samples positive for *C. botulinum* spores as detected by mPCR.

honey samples, type B was detected in 7 samples, type E in 4 samples, and type F in 3 samples.

The spores of *C. botulinum* type A were found in the majority of types of positive honey samples analyzed, except from heather honey. They were present in 40% (nectar-honeydew honey) to 100% (rape and buckwheat honey) of the isolates obtained. Statistical analysis did not show significant differences in the prevalence of spores type A in the honey types analyzed. The spores of *C. botulinum* type B were found in multifloral, linden, nectar-honeydew, and honeydew honey samples. No significant differences ($P > 0.05$) were present in their prevalence in these four types of honey, however, a trend towards higher prevalence of type B spores (33.3%) was observed in honeydew honey. Type E of *C. botulinum* spore was detected in four types of honey: multifloral, linden, nectar-honeydew, and heather honey. Taking into account the number of all positive samples within the honey type, there were no significant differences in the prevalence of type E spores between nectar-honeydew, multifloral, and linden honey samples. However, statistically significant differences ($P \leq 0.05$) were shown in heather honey towards multifloral and linden honey, since type E of *C. botulinum* was the only one isolate obtained. The spores of *C. botulinum* type F were found in multifloral and nectar-honeydewe honey samples. No statistically significant differences were observed between its prevalence in these two honey types.

Multifloral and nectar-honeydew honey showed the presence of all four BoNTs (A, B, E and F). Multicontamination was noted in 2 (9.5%) of the 21 samples of multifloral honey and in 1 (7.7%) sample of linden honey (Figure 1). One sample of multifloral honey showed the presence of A, E and F type spores and one sample showed the presence of A and B type spores. Multicontaminated samples of linden honey showed the presence of A and E type spores of *C. botulinum.*

DISCUSSION

It is estimated that 85% of honey in the EU is sold directly to consumers. The retailers, including beekeepers, play an important role in the distribution of honey to consumers (CBI market survey, 2009). It is relevant mainly in terms of honey being

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consumed primarily for nutritional benefits and as a preventive treatment. Honey is also a common component of a diet of elderly and children (Roman *et al*., 2013), who can suffer from botulism (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 to 100 spores (Arnon, 1979). The above is of particular significance due to the fact that the presence of *C. botulinum* spores in honey is a risk of infection not only to infants but also to patients with anatomical or functional bowel abnormalities or to patients using antimicrobials (Sobel, 2005) and those with immune deficiencies. It is also noteworthy that the link between infant botulism and sudden infant death is practically unknown (Böhnel *et al*., 2001). Annual honey production in Lithuania is approximately 1,300 tones (CBI market survey, 2009). The center of Communicable diseases and AIDS that reports epidemiological situation in Lithuania registers 0-3 cases of botulism annually with no distinction of infant botulism. Our results show that the level of contamination of Lithuanian honey from small apiaries with *C. botulinum* spores is very high, which indicates potent contamination of bee pasture or poor hygienic level of honey collecting. The fragmentation of Lithuanian agriculture and frequent location of apiaries in the vicinity of livestock farming, where land is more contaminated with spores of *C. botulinum* are another important factor that contribute to the presence of the spores of *C. botulinum* in the investigated honeys. Sandy loams as well as sands that can easily be carried over long distances represent the majority of soils in Lithuania (Hamnett, 2000). The phenomenon of correlation between localization of the farms and *C. botulinum* contamination of soil was confirmed by studies conducted in Denmark (Huss, 1980) and in central part of Argentina (Luquez *et al*., 2005). Compared to the research carried out by other authors (Nevas *et al*., 2002; Nevas *et al*., 2005) that confirmed contamination of honey with *C. botulinum* spores at the level of 7-11% or even 26% in Danish honey, the results obtained show very high contamination of Lithuanian directly sold honey with *C. botulinum* spores, that reaches the level of 60%. Analysis of Lithuanian honey samples showed the presence of *C. botulinum* types A, B, E and F that is consistent with the results obtained while analyzing honey from Scandinavian countries (Nevas *et al*., 2005). BoNT types A and B were the most numerous, like in honey from Turkey (Koluman *et al*., 2013; Gücükoğlu *et al*., 2014), Italy (De Medici *et al*., 2009), or Fennoscandia (Nevas *et al*., 2002).

Notably, small apiaries represent the majority of all apiaries in Lithuania, which is synonymous with low profitability of production and lack of sufficient funds for investments, especially in small-scale amateur apiaries, run by people with different practical experience. Moreover, honeybee breeding does not improve as rapidly as the breeding of other livestock (Tahmasbi *et al*., 2015). In this regard, production hygiene-dependent factors have a significant influence on the contamination, and thus the number and frequency of *C. botulinum* spores in honey could possibly be diminished by increasing hygienic level in honey production (Nevas *et al*., 2006).

CONCLUSIONS

Honey coming from direct sale in Lithuania in the year 2014 was highly contaminated with spores of *C. botulinum* types A, B, E and F. Statistically significant differences in the types of Lithuanian honey were not found in regard to its contamination with BoNT types of bacteria analyzed. Production in small apiaries that sell honey directly to the consumers in Lithuania is not equal to the process that

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ensures highest quality final product with respect to the content of *C. botulinum.*

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شیوع *Clostridium botulinum* تیپ A، B، E، و F جدا سازی شده از عسل های فروش مستقیم در لیتوانی

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چکیده

هدف پژوهش حاضر تجزیه عسل های لیتوانی برای بررسی حضور هاگ *Clostridium botulinum* بود. تجزیه روی عسل گل های رز بالارونده (multifloral)، خربزه عسلی (honeydew)، شهد عسلک (nectar-honeydew)، کلزا (rape)، گندم سیاه(buckwheat) ، خلنگزار(heather) و عسل زیرفون (linden honey) که مستقیما (از تولید کننده) خریداری شده بود

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انجام شد. قبل از کشت نمونه ها روی یک بستر جامد، نمونه ها با روش فیلتراسیون مایع رویی آماده شد و در محیط گوشت پخته(CMM) و محیط کشت مایع غنی شده پپتون تریپتیکاز مخمر قند (TPGY)به طور موازی کشت شد. با استفاده از روش کاشت، باکتری های دارای ویژگی های فنوتیپی نمونه وار از میان ۴۸ نمونه عسل به دست آمد. شناسایی *Clostridium botulinum* تیپ A،B،E، و F با کاربرد روش multiplex-PCRانجام شد. تجزیه و تحلیل شیوع باکتری نشان داد که ۳۰ نمونه(۶۰٪ نمونه ها) به هاگ *Clostridium botulinum*آلوده بود. بیشترین "سروتیپ" شناسایی شده از تیپ A بود: ۲۰ نمونه عسل (معادل ۶۶/۶٪) آلوده بود، در حالیکه حضور تیپ B در ۷ نمونه(۲۳/۳٪)، تیپ E در ۴ نمونه (۱۳/۳٪) و تیپ F در ۳ نمونه(۱۰٪) مثبت بود. همچنین، در سه نمونه عسل، آلودگی با بیش از یک تیپ هاگ *C. botulinum*مشاهده شد.

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