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Clostridium perfringens spores in Polish honey samples

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Abstract

Introduction: The aim of this study was examination of honey samples collected from apiaries situated in all Polish provinces for occurrence of *Clostridium* spp., especially *C. perfringens*. **Material and Methods:** The study was carried out on 240 honey samples (15 samples/province). Estimation of *Clostridium* titre, its cultures and *C. perfringens* isolate characterisation were performed according to the standard PN-R-64791:1994. A multiplex PCR method for detection of genes coding *cpa* (α toxin), *cpb* (β), *cpb2* (β2), *etx* (ε), *iap* (ι), and *cpe* (enterotoxin) toxins was used. **Results:** *Clostridium* spp. was noticed in 56% (136/240) of samples, and its titres ranged between 0.1 g and 0.001 g. *Clostridium perfringens* occurrence was evidenced in 27.5% (66/240) of samples. All isolates were classified to toxinotype A. **Conclusions:** Evidence of a high number of positive samples with occurrence of *Clostridium* spp. indicates a potential risk to consumers' health. The infective number of *Clostridium* spp. is unknown; however, the obtained results have shown that a risk assessment on the entire honey harvesting process should be made in order to ensure microbiological safety. Moreover, a detailed study should be undertaken on the antibiotic resistance of *C. perfringens* isolates from honey samples.

Keywords: *Clostridium*, honey, toxins, Poland.

Introduction

The natural properties of honey are considered to be influential on its microorganism content. The primary source of honey contamination by microorganisms is the initial occurrence of moulds, yeasts, and bacterial spores originating from sources when the nectar is being harvested, stored, and matured. These primary sources are pollen and the digestive tract of the honey bee. A high concentration of sugars and substances like gluconic acid and hydrogen peroxide suppress the growth of vegetative forms of bacteria (2, 12, 17).

Clostridium spp. are bacteria able to produce spores resistant to unfavourable conditions and may survive despite the high concentration of sugars and occurrence of antibacterial substances. The hygienic and epidemiological aspects of infection by this genus, and especially its pathogenic species like *C. botulinum*, *C. perfringens*, and *C. difficile*, imply it could pose a risk to human health (2, 8, 12).

Clostridium perfringens is a spore-forming, Gram-positive anaerobe residing in soil, water, and the gastrointestinal tract of various animals and humans. This ubiquitous microorganism is able to produce about 20 different protein toxins (3, 13). Five toxinotypes (from A to E) of these bacteria are distinguished by their dissimilar abilities to produce one or more of four main toxins marked as α, β, ε, and ι (10, 16). The majority of human infections are related to type A toxinotype. Toxinotype A is considered an opportunistic pathogen and is usually found among saprophytic microflora of humans and animals. *Clostridium perfringens* possesses characteristic metabolic and morphological features which enable its detection more easily than *C. botulinum* or *C. sporogenes* strains. Not possessing flagella, *C. perfringens* is not able to move. Strains of this pathogen have the ability to grow fast and dynamically at 37°C, supplanting the other clostridia of weaker growth (*e.g. C. botulinum*) (15). In humans, it causes diarrhoea and abdominal cramps, usually within 24 h. People infected with *C. perfringens* usually do not suffer from fever or vomiting. Transmission of illness among people is unexpected (4, 7).

The aim of this study was examination of honey samples collected from apiaries situated in all Polish provinces for occurrence of *Clostridium* spp., especially

C. perfringens. This is the first study on *C. perfringens* occurrence in honey samples collected from Polish apiaries.

Material and Methods

Material. The study was carried out on 240 honey samples from all 16 provinces in Poland (15 samples per province). Honey was collected directly from apiaries after the extraction process.

Clostridium perfringens strains ATCC 13124 (type A able to produce α toxin) and A544/84 (type A from the collection of the Department of Hygiene of Animal Feedingstuffs, able to produce α and β2 toxins) were used as the positive control.

Culture methods. The direct addition method (DA) was used as previously described (6, 9). A 10 g sample was diluted in 90 mL of sterile distilled water with 1% Tween 80 and stirred until the solution became homogenous. Subsequently, other serial decimal dilutions were prepared in order to estimate the anaerobe titre. The titre was expressed as the lowest mass of honey sample in which the anaerobic bacteria are detectable (the last decimal dilution with a positive result). A 1 ml volume of each dilution was transferred to tryptone peptone glucose yeast extract broth (TPGY) constituted of casein enzymic hydrolysate 50 g/L, peptic digest of animal tissue 5 g/L, yeast extract 20 g/L, dextrose 4 g/L, and sodium thioglycollate 1 g/L, with final pH 7.0 ±0.2 at 25°C. The inocula were pasteurised for 15 min at 70 ±2°C, and then the prepared samples were incubated under anaerobic conditions at 37 ±1°C for seven days. After incubation, a few drops from each liquid culture were inoculated onto Willis–Hobbs and fastidious anaerobe agar (FAA) medium plates. The first of these media comprised peptic digest of animal tissue 10 g/L, meat extract 10 g/L, sodium chloride 5 g/L, lactose 12 g/L, neutral red 0.032 g/L, and agar 10 g/L, with final pH 7.0 ± 0.2 at 25°C and the second peptone 23 g/L, sodium chloride 5 g/L, soluble starch 1 g/L, sodium bicarbonate 0.4 g/L, glucose 1 g/L, sodium pyruvate 1 g/L, L-cysteine $HCl \times H_2O$ 0.5 g/L, sodium pyrophosphate 0.25 g/L, L-arginine 1 g/L, sodium succinate 0.5 g/L, Hemin 0.01 g/L, vitamin K 0.001 g/L, and agar 12 g/L, with final pH 7.2 ±0.2 at 25°C. The inoculated plates were incubated at 37 ±1°C for 48 h. Anaerobic conditions were obtained by using anaerobic jars (Thermo Scientific, USA) with anaerobic atmosphere generating sachets (AnaeroGen, Thermo Scientific, USA).

Clostridium spp. was detected after obtaining the characteristic growth on Willis–Hobbs and FAA agar media in regard to aerobic control. Characteristic colonies (yellow on Willis–Hobbs and cream on FAA) were subjected to Gram staining. The evaluation of *Clostridium* spp. morphology was conducted according to the protocol described in the Polish Standard PN-R-64791:1994 (11).

C. perfringens-suspected strains isolated from both media were evaluated, taking into account surface shape, size, lecithin and gelatin hydrolysis, and ability to ferment lactose. These criteria are set down in the Polish Standard PN-R-64791:1994 (11).

DNA preparation. The DNA of *C. perfringens* was extracted from characteristic colonies on Willis–Hobbs agar, according to the method described by Kukier and Kwiatek (5). To perform a reliable multiplex PCR with a heat-lysed bacterial suspension, material from an overnight culture on Willis–Hobbs agar was resuspended with a plastic disposable inoculating loop in 2 mL of PBS to obtain a 3.5 McFarland turbidity, and then bacterial suspensions in 1.5 mL disposable Eppendorf tubes were incubated at 95ºC for 15 min. Next, the tubes were subjected to heat lysis at 95ºC for 15 min, placed on ice for 5 min, and centrifuged at 11,000 × g for 8 min in an Eppendorf Mini Spin Plus centrifuge (Merck, Germany).

PCR methods for *C. perfringens* detection. The isolates were examined for the presence of *cpa* (α toxin), *cpb* (β), *cpb*2 (β2), *etx* (ε), *iap* (ι), and *cpe* (enterotoxin) toxin genes by multiplex PCR (mPCR) according to the method of Baums *et al.* (1). The reaction was performed in a volume of 50 µL with 2.5 mM $MgCl_2$, 0.25 mmol of each deoxyribonucleotide triphosphate (dNTP), and 2.5 U of Taq polymerase (Thermo Fisher Scientific, USA) as reagents and with the following primers according to Baums *et al.* (1): 200 nM CPA5L, 200 nM CPA5R, 138 nM CPBL, 138 nM CPBR, 67 nM CPEL, 67 nM CPER, 46 nM CPETXL, 46 nM CPETXR, 83 nM CPIL, 83 nM CPIR, 117 nM CPB2L, and 117 nM CPB2R (Table 1).

Table 1. Sequences of primers used in mPCR for *C. perfringens* detection

Toxin gene	Primer	Sequence	Length of products	Literature
cpa	CPA5L	AGTCTACGCTTGGGATGGAA	900	1
	CPA5R	TTTCCTGGGTTGTCCATTTC		
cpb	CPBL	TCCTTTCTTGAGGGAGGATAAA	611	
	CPBR	TGAACCTCCTATTTTGTATCCCA		
cpe	CPEL	GGGGAACCCTCAGTAGTTTCA	506	
	CPER	ACCAGCTGGATTTGAGTTTAATG		
etx	CPETXL	TGGGAACTTCGATACAAGCA	396	
	CPETXR	TTAACTCATCTCCCATAACTGCAC		
iap	CPIL	AAACGCATTAAAGCTCACACC	293	
	CPIR	CTGCATAACCTGGAATGGCT		
cpb2	CPB2L	CAAGCAATTGGGGGAGTTTA	200	
	CPB2R	GCAGAATCAGGATTTTGACCA		

Gel electrophoresis. Gel electrophoresis was conducted on 2% agarose gel stained with SimplySafe (EURx, Poland) and run in 1 × TBE buffer (Thermo Fisher Scientific, USA) for 1.5 h under 100 V. The reaction mixture in a 10 μL volume and 2 μL of loading buffer 6 × DNA Loading Dye (Thermo Fisher Scientific, USA) were loaded into each well. The molecular weight of the obtained products was compared with a GeneRuler™ 100 bp DNA Ladder Mix molecular weight marker (Thermo Fisher Scientific, USA). PCR products were analysed under a Chemi-Smart 3000 UV light transilluminator (Vilbe-Lourmat, France).

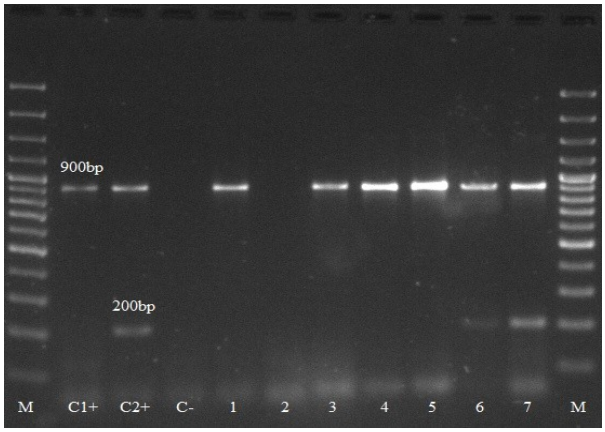

Fig. 1. Example of positive products characteristic for *C. perfringens* type A
C1+ – positive control (DNA from *C. perfringens* ATCC 13124 able to produce α toxin); C2+ – positive control (DNA from *C. perfringens* A544/84 able to produce α and β2 toxins); C− – negative control; 1, 3, 4, 5 – PCR products characteristic for *C. perfringens* type A able to produce α toxin; 6, 7 – PCR products characteristic for *C. perfringens* type A able to produce α and β2 toxins

Table 2. *Clostridium* spp. and *C. perfringens* occurrence in honey samples from Polish apiaries

Province	Number of samples	*Clostridium* spp.	*Clostridium perfringens*
Pomeranian	15	5	2
Lubuskie	15	3	2
Kuyavian-Pomeranian	15	5	4
Łódź	15	8	6
Greater Poland	15	9	7
Lublin	15	4	1
Lower Silesian	15	2	1
Podlaskie	15	7	4
West Pomeranian	15	4	2
Warmian-Masurian	15	11	5
Subcarpathian	15	11	7
Holy Cross	15	15	8
Opole	15	9	4
Lesser Poland	15	14	6
Silesian	15	14	4
Masovian	15	15	3
Total	240	136	66 A(43) Aβ2(23)
Total %	100%	56.7%	27.5% A (17.9%) Aβ2 (9.6%)

Results

***Clostridium* spp. occurrence.** *Clostridium* spp. was noticed in 56.7% (136/240) of honey samples. In 44.1% (97/220) of samples, the titre was evaluated at the level of 0.1 g, whilst in 11.7% (28/240) of samples titre fell to 0.01 g, and only in 4.6% (11/240) of samples did it decrease to 0.001 g.

***Clostridium perfringens* occurrence and differentiation of toxin types.** mPCR analysis showed *Clostridium perfringens* occurrence in 27.5% (66/240) of samples. All 66 isolates were classified to toxinotype A, and 23 of them possessed the genes determining β2 toxin production (Fig. 1). The highest level of *Clostridium* spp. prevalence was noticed in the Holy Cross and Masovian provinces (15/15), and specifically concerning *C. perfringens*, its highest level was noticed in the Holy Cross (8/15), Subcarpathian (7/15), and Greater Poland (7/15) provinces (Table 2).

Discussion

The obtained results showed the high number of samples contaminated with *Clostridium* spp. and *C. perfringens* strains. In literature, occurrence of *Clostridia* is presented mainly in regard to C. *botulinum* and infant botulism cases (6, 9). Other data published with a focus on the hygienic aspects of honey extraction and *C. perfringens* occurrence are very rare.

Contamination with *Clostridia* spores was reported by Mustafina *et al.* (8) who noticed their presence in 39.1% (43/110) of examined samples collected in Kazakhstan. Różańska and Osek (14) observed that honey samples from Poland were contaminated by *Clostridia* spores at a mean level of 22.9% (25/109). Pucciarelli *et al.* (12) reported that the incidence of these microorganisms in yateí (*Tetragonisca angustula*) honey samples from Argentina reached 64% (18/28). Erkan *et al.* (2) noted occurrence of mesophilic anaerobic bacteria at the level of 44% (22/50) in honey collected from Şirnak province in Turkey. The contamination level of *Clostridia* spores could be dependent on the harvesting region of honey and hygienic aspects of the entire harvesting process. A high number of spores found in honey might be due to the growth of the organism in dead bees in the hive (12).

The hygienic aspects and contamination route in honey production in regard to *C. botulinum* occurrence were described by Nevas *et al.* (9). These authors listed the potential sources of contamination during the honey harvesting process. The same factors could be influential on *Clostridia* occurrence in general. According to Nevas *et al.* (9), the presence of *C. botulinum* spores in honey is mostly determined by extractor size, the apiarist 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 evidence of *C. perfringens* in honey has been very rarely reported in literature. Tomassetti *et al.* (18) 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 the Latium region. Our study indicated that the *C. perfringens* occurrence was noted in 66 (27.5%) samples. *Clostridium perfringens* is known as a causative agent of a spectrum of human and animal diseases. It is considered the one of the most common aetiological factors of foodborne illnesses in Europe, USA, and Japan (4, 17). All *C. perfringens* isolates from the examined honey samples were classified to type A. The genes which determine the ability to produce α toxin were noticed in all the isolates. Some of them showed the occurrence of genes determining β2 toxin production. The α toxin is most studied major toxin of *C. perfringens*, and it was the first bacterial toxin established to possess enzymatic activity. It has necrotic, cytolytic, and haemolytic activity, it can lyse platelets and leukocytes, and is able to damage fibroblasts and muscle cell membranes. Expression of genes determining α toxin production is down-regulated in the normal healthy gut; however, it is upregulated to initiate enteric disease in response to an environmental signal. The toxin designated β2 is a pore forming toxin which is involved in necrotic enteritis of pigs and horses, in haemorrhagic enteritis of cattle, in diarrhoea cases of dogs, and (along with enterotoxin) in diarrhoea cases in humans (19). Moreover, antibiotic-resistant *C. perfringens* strains are becoming one of the major health problems. Teuber (17) indicated that intensive use of antibiotics creates a resistance problem in foodborne pathogens, including *C. perfringens*. Evidence of a high number of samples positive for *Clostridia* reveals a food which potentially could affect consumer health. The infective number of *Clostridia* is unknown; however, the obtained results have shown that risk assessment on the entire honey harvesting process should be provided in order to ensure the microbiological safety. A detailed study should be undertaken on the antibiotic resistance of *C. pefringens* isolates from honey samples.

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