Original article

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Clostridium botulinum spores in Polish honey samples

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The aim of this study was examination of 240 multifloral honey samples collected from Polish apiaries for *C. botulinum* occurrence. Honey was collected directly from apiaries after the extraction process. Samples were inoculated by using the dilution and centrifugation (DC) method. Suspected isolates were examined by using mouse bioassay, PCR and real-time PCR methods. *Clostridium botulinum* type A and B strains were detected in 5 (2%) of 240 examined honey samples. Strains were also detected which were phenotypically similar to *C. botulinum* but which did not exhibit the ability to produce botulinum toxins and did not show presence of botulinum cluster (*ntnh* and *bont* genes) or expression of *ntnh* gene. The methods used in the examination, especially expression analysis of *ntnh* gene, enabled specific analysis of suspected strains and could be used routinely in environmental isolate analyses of *C. botulinum* occurrence.

Keywords: *Clostridium botulinum*, honey, neurotoxins, Polish apiaries.

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 BoNTs (botulinum neurotoxins) and into eight different serotypes (A–G and X) [3, 6, 11, 12, 28]. BoNT/X, discovered in 2017, is the first BoNT serotype identified by sequencing and bioinformatics approaches [28]. *Clostridium botulinum* strains, being divided into 4 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 the potent neurotoxins formed during the growth of *C. botulinum* [12]. Infant botulism is a common form, to which children between 2 weeks and 1 year old are most susceptible [19]. It differs from foodborne botulism which proceeds from the ingestion of the ready-formed neurotoxin, because in infant botulism immature infantile intestinal flora allow ingested spores to germinate, multiply and produce BoNTs in the intestinal lumen. The first infant botulism case was recognized in the USA in 1976 [21]. 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 (CDC) 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, colour, or smell from uncontaminated honey [1].

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

Material and Methods

Samples

The study was carried out on 240 honey samples (Table 1) from 16 provinces (15 samples per province) in Poland (1 sample = 1 apiary). Honey was collected between 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).

Culture process

The Direct Centrifugation method (DC) previously described [13, 19, 20] was used for culturing *C. botulinum*. A mass of 10g of each honey was diluted in 90mL of sterile distilled water with 1% Tween 80 and stirred until the solution became homogenous. Subsequently, the centrifugation was conducted for 30 min at 9000 x g in a 4K15 centrifuge (Sigma, Germany). The precipitates were transferred into 90mL of Tryptone Peptone Glucose Yeast Extract Broth (TPGY: 50g/L casein enzymic hydrolysate, 5g/L peptic digest of animal tissue, 20g/L yeast extract, 4g/L dextrose, and 1g/L sodium thioglycolate with final pH of 7.0 ± 0.2 at 25°C). The inocula were pasteurized for 15 min at 70 ± 2°C and next incubated under anaerobic conditions at 30 ± 1°C for 7d. After incubation, 1mL of each liquid culture was transferred to tubes with 10mL of TPGY broth. Simultaneously, a few drops from each liquid culture were inoculated onto plates with Willis–Hobbs medium (10g/L peptic digest of animal tissue, 10g/L meat extract, 5g/L sodium chloride, 12g/L lactose, 0.032g/L neutral red, and 10g/L agar, with final pH of 7.0 ± 0.2 at 25°C) and FAA medium (fastidious anaerobe agar: 23g/L peptone, 5g/L sodium chloride, 1g/L soluble starch, 0.4g/L sodium bicarbonate, 1g/L glucose, 1g/L sodium pyruvate, 0.5g/L L-cysteine $HClxH_2O$, 0.25g/L sodium pyrophosphate, 1g/L L-arginine, 0.5g/L sodium succinate, 0.01g/L haemin, 0.001g/L vitamin K, 12g/L agar, with final pH of 7.2 ± 0.2 at 25°C). The inoculated tubes and plates were incubated at 30 ± 1°C for 48h.

Strains mooted to be *Clostridium botulinum* isolated from Willis–Hobbs and FAA were evaluated taking into account surface, shape, size, and lipolytic and proteolytic features of the cultures. In order to evaluate BoNT production, the colonies suspected of belonging to *C. botulinum* species were inoculated into tubes with 10mL of TPGY broth and incubated anaerobically for 48h at 30 ± 1°C.

Nucleic acids preparation

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

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

cDNA synthesis: cDNA was synthesised with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, part of Thermo Fisher Scientific, USA). Before it was subjected to real-time PCR, cDNA was diluted 10 times, to a concentration of about 100ng/reaction mixture.

Molecular methods

Amplification and Sequencing of 16S rDNA: For identification of *16S rDNA* from unidentified anaerobic strains suspected of belonging to *C. botulinum* species, primers according to Vaneechoutte et al. [25] were used (Table 2). Reactions were performed in the volume of 25µL with these reagent constituents: 5µL of DNA matrix, 2.5µL 10xTaq buffer with KCl (Fermentas, Lithuania), 4mM $MgCl_2$, 200µM dNTP, and 1.25U/25µL 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 s, annealing at 55°C for 45 s, extension at 72°C for 1 min, and final extension at 72°C for 10 min.

The length of products yielded was about 1500bp. Sequencing of obtained amplicons was entrusted to Genomed (Poland). The obtained FASTA files were analysed using the BLAST (NCBI) algorithm, and the results were compared and assigned to the sequences from the NCBI with the highest score and identity.

Real-time PCR for ntnh and 16S rRNA housekeeping gene: The set of 7 primers and TaqMan probe (Table 3) were used for detection of the *ntnh* gene according to Raphael et al. [22]. In the case of expression analysis, the set of 2 primers and TaqMan probe designed (Table 4) for *C. botulinum* sequences available from the NCBI databases was used additionally to detect the *16S rRNA* housekeeping gene. The reactions for both genes were conducted with reagents comprising: 5µL DNA or cDNA, 4µL LightCycler TaqMan Master (Roche Diagnostics, Germany), 0.7µM of each primer, and 0.24µM TaqMan probe.Real-time PCR was performed using a LightCycler 2.0 thermocycler on a temperature profile of 10 min at 95ºC as initial denaturation, 45 cycles of denaturation at 95°C for 15s, annealing at 42°C for 15s, and elongation at 55°C for 1 min.

Normalisation of the expression changes was performed according to Shmittgen and Livak's [23] protocol, on the assumption that reaction efficiency is close to 100%. The efficiency (E) was calculated on the basis of the slope value (m) obtained from the relationship between Ct and dilutions of cDNA, according to following formula:

$E=10^{(-1/m)}$

Calculation of ntnh gene expression: Relative expression was calculated according to the comparative method described by Smittgen and Livak [23]. The calculations were prepared applying the following formulas:

Fold change = $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct$ = [(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 from positive honey were used as sample B. All samples were tested 3 times and for further analyses the mean value was used:

ΔΔCt = [(Ct *ntnh* – Ct *16S rRNA*) *C. botulinum* NCTC 887-(Ct *ntnh*-Ct *16S rRNA*) *C. botulinum* isolated from honey]

Multiplex PCR for bont/A, bont/B, bont/E, bont/F genes: *bont/A, B, E,* and *F* genes were detected according to the method described by DeMedici et al. [5], utilising 4 pairs of primers (Table 5). The reaction was prepared in a volume of 25µL with this set of reagents: 5µL of DNA, 0.3µM of each primer, 2.5µL of 10xTaq buffer with KCl (Thermo Scientific, USA), 4mM of $MgCl_2$, 200µM of dNTP, and 1.25U/25µL of Taq polymerase (Thermo Scientific, USA). Detection of products was carried out on agarose gel.

Gel electrophoresis was conducted on 2% agarose gel stained with SimplySafe (EURx, Poland) and run in 1 × TBE buffer (Thermo Scientific, USA) for 1.5h at 100V. The reaction mixture in a 10µL volume and 2µL of loading buffer 6 × DNA Loading Dye (Thermo Scientific, USA) were loaded into each well. The molecular weight of the obtained products was compared with the GeneRuler 100bp DNA Ladder Mix (Thermo Scientific, USA) molecular weight marker. Finally, PCR products were analysed 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 mooted *C. botulinum* isolates. The single experiment involved three laboratory mice and followed FDA procedure (2). After centrifugation of liquid culture in TPGY broth, the supernatant was divided into three 0.2mL portions. One of them was heated at 100 ± 2 ºC for 10min and administered intraperitoneally to one mouse. The other two were administered intraperitoneally into two

mice, one of which had previously been seroneutralised by equine monovalent antitoxin to botulinum neurotoxin 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 strains with characteristic phenotypic features for *C. botulinum* species were isolated from the examined samples. The obtained colonies were covered by the characteristic "pearl layer" and precipitation zones indicated lipolytic properties. The proteolytic activity of all isolates (bright zones surrounding the colonies) on agar media was also noticed (Table 6).

Sequencing analysis of 16S rDNA

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

Real-time PCR for *ntnh*

The *ntnh* gene was noticed in only 5 samples: 16, 24, 139, 143, and 144. Positive results were noticed 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 occurrence of *bont/A* and *bont/B* strains was detected in 5 (2%) of 240 examined honey samples. Two of the detected strains were qualified to toxinotype A and three to toxinotype B (Table 6).

Expression analysis of *ntnh* gene

The efficiency of the real-time PCR was calculated at 92.89% for *ntnh* gene and at 96.38% for the *16S rRNA* housekeeping gene. Relative expression was assessed for *C. botulinum* isolates and presented as a fold change in relation to *ntnh* 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 *C. botulinum* isolate was from sample 139 and was calculated at 181.01 fold changes in relation to NCTC 887 reference strain. The lowest expression was noticed for an isolate from sample 144 and equalled −1.86 fold changes in relation to NCTC 887 (Fig. 1). Positive samples were collected from Lubusz, Greater Poland, and Warmia-Masuria provinces.

MBA results

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

Discussion

Since 1976, there have been over 1500 cases of infant botulism reported in more than 15 countries worldwide [16]. Among all the various potential sources of spores (soil, dust etc.), honey is the only dietary source that has been linked to the disease through both laboratory and epidemiological studies [1, 17, 18].

The dose of *C. botulinum* spores which causes infection in human infants is undetermined. In a review by Austin [2] the author states that a minimum dose for infant botulism has not been established. Arnon et al. [1] estimated 10 to 100 spores as being able to cause infection. The lowest minimum number of cells needed to cause disease was obtained in a sample of honey from Canada, which contained 1 spore/g. 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/g could pose a potential risk of infant botulism [7].

The occurrence of *C. botulinum* noticed in Polish apiaries (2%) is lower or similar to *C. botulinum* presence reported in samples from most other countries. Nevas et al. [20] described contamination of 26% (29/112) of Danish samples by *C. botulinum* spores (1 sample positive for toxin type A and 28 for B). The same authors [20] also reported that *C. botulinum* occurrence in Finnish honey samples was detected in 11% (20/190, 8 samples showing the type A toxin and 12 showing type B). In Norway the percentage of positive samples was determined to be 10 (12/112, 7 of type B, 4 of E, and 1 of F) [20]. The lowest level of contamination in Nordic countries was detected in Swedish samples where it was 2% (1 occurrence of toxin type E) [20]. In Japan *C. botulinum* occurrence is reported twice in the literature data: according to Nakano and Sakaguchi [18] the percentage of positive samples reached 31% (11/36), whereas Nakano et al. [17] observed occurrence of the pathogen in 8.5% of examined samples. In Taiwan *C. botulinum* presence was disclosed in 1% (2/152) of examined samples [10]. In Turkey, Küplülü et al. [13] described *C. botulinum* spore detection in 12.5% of honey samples. Whereas Gücükoğlu et al. [10] reported 2% (4/150) prevalence of *C. botulinum* spores in honey collected from the Turkish Black Sea region. Midura [15] reported occurrence in 10% (9/90) of examined samples from the USA. In Kazakhstan, Mustafina et al. [16] noticed *C. botulinum* occurrence in only 0.91% (1/110) of honey samples. Polish honey was examined by two independent scientific teams and a sample contamination level of 8.5% (6/70) from the Lublin and Subcarpathia provinces was described by Grenda et al. [8] whilst Wojtacka et al. [27] detected spores from small apiaries from an undetermined area in Poland at the level of 22% (22/102). Wojtacka et al. [26] also described a high prevalence of *C. botulinum* spores in Lithuanian honey sold directly from the apiary at the level of 60% (30/48), which is the highest level described to date in the literature. The procedure described by Wojtacka et al. [26, 27]

comprised only one multiplex PCR method, which in our opinion is inadequate to survey the *C. botulinum* occurrence in honey samples. The literature data 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 on agarose gel [9]. The level of naturally-occurring *C. botulinum* spore contamination is estimated in the range of 10–1000 spores/g of honey [14]. The detection of *C. botulinum* spores is a complicated task because of the high heterogenicity of this pathogen and horizontal gene transfer during isolation process. The genus *Clostridium* comprises strains which show similar biochemical features to *C. botulinum* but are not able to produce botulinum toxins. Because of their heterogenicity *C. botulinum* strains are classified into four metabolic groups and the other microorganisms which are not considered able to produce botulinum toxins are also related to these groups (excluding some toxinogenic strains of C. *butyricum* and *C. beijerincki*, 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, *C. novyi* to group III, and *C. subterminale* and *C. schirmacherense* are related to group IV. In the honey samples we detected strains phenotypically similar to *C. botulinum* which 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* species [3, 4]. This method enabled discrimination of suspected isolates only at the level of genus. Among 6 strains with the highest score and identity to *C. botulinum*, only 5 were classified to this species 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 and hygienic aspects of the entire process of the honey harvest. According to Nevas et al. [20], 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 of hand-washing facilities in the extraction room, and the presence of *C. botulinum* in soil samples. The variability of phenotypic features among

the strains of this pathogen, silent *bont* genes, and the high probability of toxin gene loss through passages beset 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 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. Expression analysis is also easier, because it is limited to only one gene. The presented set of methods could be used as a tool for supporting the laboratory diagnosis of botulism, serving as an alternative to MBA in *C. botulinum* detection.

The number of Clostridia spores sufficient for infection is undetermined, however even 1 cfu 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 made 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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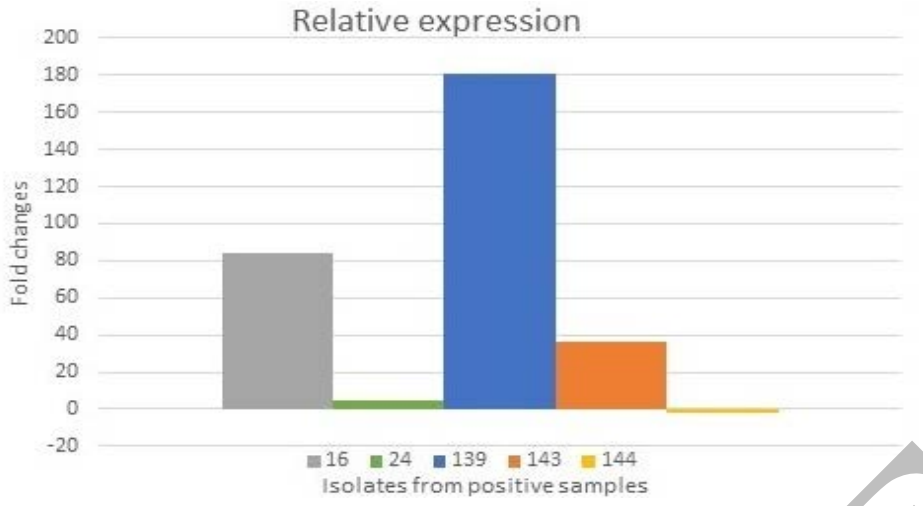

Fig. 1. Relative expression of *ntnh* gene from *C. botulinum* isolates. Fold changes obtained after expression analysis of *ntnh* gene for isolates from samples no. 16, 24, 139, 143, 144 in relation to *C. botulinum* NCTC 887 reference strain. The highest expression level for *C. botulinum* isolate was from sample 139 and was calculated at 181.01 fold changes. The lowest expression was noticed for an isolate from sample 144 and equalled −1.86 fold changes.

1 **Table 1.** Origin and numbers of samples collected from subsequent Polish provinces

Province	Samples numbers
Pomeranian	1-15
Lubusz	16-30
Kuyavian-Pomeranian	31-45
Łódź	46-60
Greater Poland	61-75
Lublin	76-90
Lower Silesian	91-105
Podlaskie	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

2

3

Table 2. Sequences of primers used in amplification of conservative *16S rDNA* region

Primers	Sequence	Literature
16S fw	5' -TGGCTCAGATTGAACGCTGGCGGC-3'	25
16S rev	5'-TACCTTGTTACGACTTCACCCCA-3'	

Table 3. Sequences of primers and molecular probe for real-time PCR used in *ntnh* gene detection

Primers	Sequence	References
F1	5'GATTTAAGTGAAAATTTATTTAATATAT'3	
F2	5'CCACTAAATGATTTAAATGAA'3	
F3	5'TGATGAAATACCTAATAGTATGTTAAAT'3	
CD2F	5'GACATATCAGATAGTTTATTGGGA'3	
R1	5'TTTAGCCATACAAATTAAATC'3	22
R2	5'ACTAGCCATACAAATTAGATC'3	
R3	5'TATTAAACTTTCTTGAGCTA'3	
Probe	Sequence	
NTNH410	5'FAM-ATCAATGGTGGACACAATATTATAGTCA-BHQ'	

FAM – 6 – Carboxyfluorescein, BHQ – Black Hole Quencher, A –LNA (Locked Nucleic Acid)

Table 4. Primers and molecular probe for Housekeeping *16S rRNA* gene detection in real-time PCR

Primers	Sequences	Reference numbers of sequences from GeneBank
HK fw	5'TTACCTGGACTTGACATC'3	L23477; NC_009495; NC_010674; NC_015425; NC_015425; NC_009698
HK rev	5'GGTCTTGCNTCTTATTGT'3	
Probe	Sequence	
HK probe	5'FAM-CAGGTGGTGCATGGTTGT BHQ'3	

N – A/C/G/T, FAM – 6 – Carboxyfluorescein, BHQ – Black Hole Quencher

Table 5. Primers in mPCR method for detection of *bont/A, B, E, F* genes

Toxotype	Primer	Sequence	Length of PCR product
A	IA_03_fw	5'GGGCCTAGAGGTAGCGTARTG'3	101 bp
	IA_03_rev	5'TCTTYATTTCCAGAAGCATATTTT' 3	
B	CBMLB1	5'CAGGAGAAGTGGAGCGAAAA'3	205 bp
	CBMLB2	5'CTTGCGCCTTTGTTTTCTTG '3	
E	CBMLE1	5'CCAAGATTTTCATCCGCCTA'3	389 bp
	CBMLE2	5'GCTATTGATCCAAAACGGTGA'3	
F	CBMLF1	5'CGGCTTCATTAGAGAACGGA'3	543 bp
	CBMLF2	5'TAACTCCCCTAGCCCCGTAT '3	

R = A, G; Y = C, T

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

Samples number*	MBA	Real-time PCR (*ntnh* gene presence)	mPCR (*bont/a* gene presence)	mPCR (*bont/b* gene presence)	mPCR (*bont/e* gene presence)	mPCR (*bont/f* gene presence)
16	+	+	-	+	-	-
24	+	+	+	-	-	-
62	-	-	-	-	-	-
66	-	-	-	-	-	-
70	-	-	-	-	-	-
139	+	+	-	+	-	-
143	+	+	-	+	-	-
144	+	+	+	-	-	-

*Only samples from which characteristic colonies suspected of belongings to *Clostridium botulinum* species were isolated.

Table 7. Sequencing analysis of isolates phenotyphically similar to *C. botulinum* species

Sample number	Name of the most similar sequence and ID	% of identity
16	*Clostridium botulinum* 16S ribosomal RNA gene, partial sequence Sequence ID: KM017077.1	99%
24	*Clostridium botulinum* strain mfb_cift 16S ribosomal RNA gene, partial sequence Sequence ID: MF039482.1	98%
62	*Clostridium botulinum* strain mfb_cift 16S ribosomal RNA gene, partial sequence Sequence ID: MF039482.1	98%
66	*Clostridium sporogenes* strain cift_MFB_fs116 16S ribosomal RNA gene, partial sequence Sequence ID: MF062498.1	98%
70	Uncultured *Clostridium sp.* clone VE46H03 16S ribosomal RNA gene, partial sequence Sequence ID: GQ179698.1	99%
139	*Clostridium botulinum* strain cift_MFB_55cb 16S ribosomal RNA gene, partial sequence Sequence ID: MF062271.1	92%
143	*Clostridium botulinum* strain cift_MFB_55cb 16S ribosomal RNA gene, partial sequence Sequence ID: MF062271.1	91%
144	*Clostridium botulinum* strain cift_MFB_54cb 16S ribosomal RNA gene, partial sequence Sequence ID: MF062272.1	99%

Table 8. Relative expression of honey isolates

Sample number	Relative expression (fold changes)
16	84,44
24	4,92
139	181,01
143	36,75
144	-1,86