Contamination of honey produced in the Republic of Kazakhstan with \textit{Clostridium botulinum}

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Received: March 9, 2015 \hspace{1cm} Accepted: June 3, 2015

Abstract

The paper presents the first results of a study on the contamination of honey produced in the Republic of Kazakhstan with \textit{C. botulinum} spores known to pose a potential infection threat to infants. During microbiological analysis, culturing methods with TPGY, Willis-Hobbs agar, FAA agar connected with PCR, sequencing, and a mouse bioassay were used. The \textit{C. botulinum} contamination rate of honey was relatively low as determined, at 0.91%. Nonetheless, the potential danger of the bacteria to children's health should not be neglected.

Keywords: honey, \textit{C. botulinum}, microbiological contamination, microbiological analysis.

Introduction

Honey is a unique product, which owing to its composition, taste, and other properties may provide a healthy alternative to sugar or sugar products, and therefore it is a valued food used throughout the world, including in children's nutrition. However, due to the risk of infant botulism, honey should be given to children only under special restrictions.

The first infant botulism case was recognised in the USA in 1976. Subsequently, numerous studies have associated the occurrence of infant botulism with the consumption of honey. Moreover, samples of honey in the United States have tested positive for \textit{C. botulinum} spores and toxins. Given such evidence, the Centers for Disease Control and Prevention have issued a special recommendation that honey should not be given to children under the age of 12 months (25). In California, 29.2\% (12/41) of hospitalised botulism patients had been fed honey prior to the onset of constipation; worldwide, honey exposure occurred in 34.7\% (28/75) of hospitalised cases. Of all food items tested, only honey contained \textit{C. botulinum} organisms (1). Spores of the pathogen are widespread in the environment and can get into honey from dust, pollen, and herbs (20), or accumulate in beeswax (6). When introduced into a child's body, \textit{C. botulinum} multiplies in the intestines and releases neurotoxins which can cause serious health problems or even death (14, 21, 29). Twenty-six countries representing five continents have reported the occurrence of infant botulism among their inhabitants (12) - the Arabian Gulf States (30), the Netherlands (26), Denmark (11), Norway (27), Italy (9), Japan (29), the United States (23), and Spain (28). The largest numbers of cases have been reported from the United States, Argentina, Australia, Canada, Italy, and Japan (12). Most countries have not yet reported any cases of infant botulism but considering the common prevalence of clostridia spores in the environment, it is rather a question of cases having remained undiagnosed (12, 29). What is significant is that honey contaminated with \textit{C. botulinum} spores does not differ in taste, colour, or smell from uncontaminated honey. None of the currently available tools is capable of detecting the
infection. Moreover, even heat treatment does not always kill the bacteria (2). Considering the above and the lack of reference data in world literature, we have performed a study on the contamination of honey produced in the Republic of Kazakhstan with C. botulinum spores.

Material and Methods

Samples and strains. The analyses were performed on 110 honey samples from Kazakhstan. The samples were collected from the food reference points of different regions: Eastern, Northern, Central, and Southern Kazakhstan, and this differentiation was based on the features of climate and flora of the country. However, it should be said that the eastern district of the Republic is the main producer of honey. In addition, this region is a developed industrial area. In the course of analyses, the following reference C. botulinum strains from NCTC (National Collection of Type Cultures, UK) were used as positive 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), and the following C. botulinum type A strains were obtained from the Military Institute of Hygiene and Epidemiology in Warsaw, Poland: AZK3 and A72.

Culture and DNA extraction. Five grammes of each honey sample was inoculated into the bottles with 45 mL of TPGY (Tryptone Peptone Glucose Yeast Extract) broth, pasteurised at 70°C for 15 min, and incubated under anaerobic conditions at 30°C for 5 d. Subsequently, several drops from each liquid culture were inoculated onto Willis-Hobbs agar and FAA (fastidious anaerobe agar) medium plates. The inoculated plates were incubated at 30°C for 48 h. Shape, size, and lypolytic and proteolytic features of the cultures were evaluated. DNA was isolated from 1 mL 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 isolated DNA was examined for C. botulinum and its toxinotype genes using PCR. The level of contamination by C. botulinum spores was estimated with a 95% confidence interval, according to the FDA protocol for the most probable number (MPN) method (4).

Biochemical characterisation. Biochemical features of the strains were estimated by Rapid ID 32A and API 20A (bioMérieux, France). Bacterial suspensions were prepared using a Densimat densitometer (bioMérieux, France). The Rapid ID 32A and API 20A test strips were read visually and by mini API autoreader, (bioMérieux, France), with database version 1.3.1.

Mouse bioassay. The single experiment conducted involved three laboratory mice and followed FDA procedure (21). After centrifugation of liquid culture in TPGY broth, the supernatant was divided into three 0.2 mL portions. One of them was heated at 100°C for 10 min 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) (HPA, UK). Five experiments were performed.

PCR methods. A set of seven primers: F1, F2, F3, CD2F, R1, R2, and R3, and TaqMan probe NTNH410 for real-time PCR were used according to the previously described protocol (18). This screening method was used to detect nth gene, common to all C. botulinum strains. The final volume of reaction mixture was 20 μL and contained: 5 μL of DNA template; 4 μL of LightCycler TaqMan Master (Roche); 0.24 μM of NTNH410 probe, and 0.7 μM of each primer. Thermocycling was performed in the LightCycler 2.0 (Roche, Switzerland). Following a 10 min activation step at 95°C, reactions were subjected to 40 cycles at 95°C for 15 s, at 42°C for 15 s, and at 55°C for 60 s. Fluorescence data was collected after the third step of each cycle (55°C).

For determination of A, B, E, and F C. botulinum toxinootypes, a set of eight primers was used, according to the European Committee for Standardisation (CEN) (1): IA_03_fw, IA_03_rev, CBMLB1, CBMLB2, CBMLE1, CBMLE2, CBMLF1, and CBMLF2. This set allowed analyses to be conducted using mPCR (multiplex PCR) which enables the detection of genes determining the production of BoNTs in particular toxinootypes. The final volume of reaction mixture was 25 μL and contained: 5 μL of DNA template; 0.3 μM of each primer; 4 mM of MgCl2 (Fermentas, Lithuania); 2.5 μL of 10xTaq buffer with KCl (Fermentas); 200 μM of dNTP mixture (Fermentas), and 1.25 U of Taq DNA polymerase (Fermentas). Thermocycling was performed on a T1 thermocycler (Biometra, Germany). Following an initial denaturation step at 95°C for 60 s, the reaction was subjected to 27 cycles at 95°C for 30 s, at 53°C for 30 s, and at 72°C for 3 min. The final extension was carried out at 72°C for 3 min. Detection of PCR products was performed on agarose gel.

For determination of C and D C. botulinum toxinootypes, a set of 4 primers was used: CS-11, CS-22, DS-11, and DS-22, according to the method used by Takeshi et al. (23). This set enabled the detection of the gene region—which determines the production of the light chain (Lc) in BoNT. The final volume of reaction mixture was 25 μL and contained: 5 μL of DNA template; 2.5 μL of 10xTaq buffer (Fermentas); 0.3 μM of each primer; 200 μM of dNTP mixture (Fermentas); 4 mM of MgCl2 (Fermentas), and 1.25 U of Taq DNA polymerase (Fermentas). Thermocycling was
performed with a T1 thermocycler (Biometra). Following an initial denaturation step at 95°C for 60 s, the reaction was subjected to 30 cycles at 95°C for 30 s, at 55°C for 30 s, and at 72°C for 3 min. The final extension was carried out at 72°C for 3 min. Detection of PCR products was performed on agarose gel.

**Gel electrophoresis.** The gel electrophoresis was conducted on 2% agarose gel stained with ethidium bromide and run in 1 × TBE buffer (Fermentas) for 1.5 h under 100 V. Reaction mixture in a 10 μL volume and 2 μL of loading buffer 6 × DNA Loading Dye (Fermentas) were loaded into each well. The molecular weight of the obtained products was determined by comparison with a molecular weight marker and the GeneRuler™ 100bp DNA Ladder Mix (Fermentas) was selected for this purpose. After electrophoresis, PCR products were analysed under a UV light transiluminator (Vilbe-Lourmat, France).

**Sequencing.** The sequence analysis of positive PCR products for *C. botulinum* toxinoype A was performed by Genomed (Poland). The analysis was conducted with the same set of primers as used for PCR. The interpretation of the obtained results was achieved using the BLAST algorithm (Basic Local Alignment Search Tool), in order to find a homological sequence available in GenBank (3).

**Results**

The occurrence of *C. botulinum* type A strain in 1 (0.91%) of 110 examined honey samples was demonstrated in a mouse bioassay, real-time PCR, and PCR. The presence of *Clostridium* sp. was noted in 43 (39%) examined samples.

The isolated *C. botulinum* strain showed species-typical culture characteristics in that the obtained colonies were covered by the characteristic “pearl layer” and precipitation zones indicated lipolytic properties. Proteolytic activity of the isolate in the form of bright zones surrounding the colonies on Willis-Hobbs and FAA agar media was also noticed (Fig. 1). The contamination of honey samples with *C. botulinum* spores was estimated at the level of 1.8 (0.09; 6.8) spores/g.

According to the Rapid ID 32A test, the examined *C. botulinum* strain was able to produce arginine dihydrolase. The interpretation of the obtained results by the mini API autoreader indicated “low discrimination”. The API 20A test showed that the isolated strain was able to ferment D-glucose, to induce gelatin hydrolysis, and to ferment D-trehalose. The interpretation of the mini API reader indicated “probable identification”. This test was unable to differentiate *C. botulinum* from *C. sporogenes*.

The experiment on laboratory mice showed the presence of toxin type A produced by the isolated strain. The seroneutralisation test showed that neutralising BoNT/A was possible with an antitoxin dose of 2 IU, whereas the neutralisation of toxin produced by *C. botulinum* NCTC 887, AZK3, and A72 strains was possible after application of a 1 IU dose of antitoxin.

The real-time PCR screening analysis demonstrated the occurrence of the *nth* gene in the analysed strain (Fig. 2). According to the results of PCR analysis with detection on agarose gel, the examined strain showed the presence of the *bont/A* gene (Fig. 3).

The sequence analysis of PCR product showed 98% homology to the *bont/A* gene sequence deposited in NCBI GenBank (Figs 4, 5).

Fig. 1. Characteristic growth of *C. botulinum* type A isolated from honey sample on Willis-Hobbs (A) and FAA (B) media
Fig. 2. Amplification curves of real-time PCR screening analysis.

Fig. 3. Positive results of PCR with detection on agarose gel. M - molecular mass marker; 1, 2 - positive PCR products indicating *Clostridium botulinum* type A in honey sample.

*Clostridium botulinum* strain CDC54068 botulinum neurotoxin (bont/A) gene, complete cds
Sequence ID: gb|JX12707.1|Length: 3891 Number of Matches: 1

Score = 106 bits(57), Expect = 3e-20, Identities = 60/61(98%), Gaps = 1/61(1%), Strand = Fw/Fw

Query  
10   ATTC-AGTTTTGTTACGGGGGACAAAAATTATTTATTTAAAAAATATGCTTTGGAAATAAAG  68

Sbjct  
3452 ATTCAAGTTTTGTTACGGGGGACAAAAATTATTTATTTAAAAAATATGCTTTGGAAATAAAG  3511

Query  
69   A  69

Sbjct  
3512 A  3512

Fig. 4. Sequencing result for primer IA03fw.
Discussion

When infant botulism was acknowledged in the medical world as a new disease entity and honey consumption was recognised as one of the causative factors, scientists from different countries focused on the contamination of honey with *C. botulinum*. The data they provided estimated the *C. botulinum* contamination rate of honey at 10% in California (5), 1.1% in Argentina (8), 7.06% in Brazil (21), 26% in Denmark, 7% and 16% respectively for home and imported honey samples in Finland (16), 10% in Norway, 2% in Sweden (17), and 2.6% in Turkey (10).

The present research estimated the contamination rate at 0.91% (1/110). Although such a contamination rate is relatively low, the potential danger and consequences of the causative agent for the child’s body should not be neglected. The most typical age for infant botulism occurrence is between 2 weeks and 6 months, and the average age is estimated at 10 or 12 weeks, however, the onset of botulism symptoms has been reported at the age of several days or even tens of hours (15).

The prevalence of *C. botulinum* spores in honey may be considered to reflect the overall existence of botulinal spores in the environment. This pathogen’s prevalence in honey could be associated with its occurrence in soil. Data on correlation between soil and honey contamination were reported in Denmark and Sweden (17). The significantly higher prevalence in honey might be explained by the behaviour of honeybees (*Apis mellifera*), since they tend to prefer a dirty source of water to a clean one, which may result from its odour and salt content. Therefore, water contaminated with manure might attract honeybees. Faecal pollution from farms and agricultural areas could pose a potential risk of contamination by *C. botulinum*. Nordic data on the prevalence of *C. botulinum* in faecal samples from slaughtered pigs indicated that 62% of swine carried the pathogen and it was reported that manure is also used as a fertiliser spread over the fields (7, 16, 17). As it is likely that honeybees collect water from areas contaminated with animal faeces, the transfer of *C. botulinum* spores through water could account for the high prevalence of the bacterium in honey (17). The honey sample contaminated with *C. botulinum* was taken from the Glubokovsky region of East Kazakhstan, located 27 km from the regional centre. This is an agricultural area with different communication routes such as railway tracks, arterial roads and also the navigable Irtysh River. The region’s primary economic activity is exploiting natural resources as building materials.

Considering the environmental conditions in the area from which the examined honey samples were collected, we assume that the contamination could have occurred by ingestion of contaminated dust particles from the environment, as well as in the processing of honey.

The described set of molecular methods based on PCR techniques enabled specific detection of all strains which belong to the *C. botulinum* species. As of present, no selective media have become available which enable the growth of this pathogen and inhibit the other anaerobic microflora competing with this species for nutrition. The biochemical properties are not sufficient criteria for unequivocal identification of *C. botulinum*, which has been confirmed by our and other authors’ results (13).

The isolation of this pathogen is also suppressed by the occurrence of anaerobic *C. botulinum*-like bacteria which do not have the ability to produce BoNTs. The loss of toxigenicity among A, B, E, and F toxin types has been described. The toxigenicity of C and D toxotypes is determined by specific pseudolysogenic bacteriophages. These facts are the main cause of difficulties in *C. botulinum* detection and isolation (19).

Conflict of Interests Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

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