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The investigation of the presence of *Clostridium botulinum* spores in honey in Serbia

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Abstract

The presence of *Clostridium botulinum* spores in 59 honey samples originating from different regions of the Republic of Serbia was studied. In addition to microbiological methods, after enrichment, centrifugation and membrane filtration, molecular methods (PCR methods) were utilized. The number of spores in PCR positive samples was estimated by the most probable number (MPN) method. PCR confirmed *C. botulinum* spores in 5 (8.47%) honey samples. MPN of spores varied from 20/kg to 204/kg honey. PCR was more sensitive than cultural methods. Natural honey contamination with *C. botulinum* spores is low-level and not homogeneous, and therefore, PCR methods require multiple sub-sampling.

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Keywords: Honey; *Clostridium botulinum*; microbiological methods; PCR; MPN

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1. Introduction

During the last fifty years it has been confirmed that *C. botulinum* is one of the most pathogenic bacteria since it produces lethal botulinum neurotoxin (BoNT)[1,2]. To this day there are a few known forms of the disease: alimentary, inhalation, infantile, intestinal, iatrogenic botulism and botulism caused by infected wound[3]. Botulism mortality rate is quite low. However, botulism can be incorrectly diagnosed and mistaken for various different diagnostic conditions like sepsis, different neurological disorders and sudden infant death syndrome CDC[4,5,6].

As a result of increased prevalence in the environment, the spores of these bacteria can contaminate honey[7,8].

To our knowledge, the presence of *C. botulinum* in honey in the Republic of Serbia has not yet been determined. The aim of this study was to determine the presence and toxic diversity of *C. botulinum* spores in honey of different botanical origins.

2. Materials and methods

Fifty-nine honey samples and control samples originated from Republic of Serbia were included in the mentioned examination and submitted to the Veterinary Specialized Institute, Kraljevo. For the purpose of contamination of negative honey samples, reference strain of *C. botulinum* NCTC 7272 was used in order to verify the methods. For performing positive control and applying PCR methods, genomic fragments *C. botulinum*: *bont*A, *bont*B, *bont*E and *bont*F were used.

The preparation of honey samples for investigating the presence of *C. botulinum* by using conventional microbiological methods and PCR method was conducted by enrichment, centrifugation and membrane filtration[9]. Seeding on Zeissler blood and egg yolk agar (EYA) was performed from prepared honey samples after which cultural, microscopic and biochemical identification of isolated bacteria cultures was completed[3,10,11,12]. The conventional method, ISO 15213:2003 was applied to investigate the presence of *C. botulinum* in uncontaminated and deliberately contaminated honey samples.

The PCR method of detecting *C. botulinum* was used to detect DNA of *C. botulinum* in prepared honey samples, described by Lindström and Lindström's collaborators[9]. Twenty replicates were processed from each honey sample.

The most probable number method (MPN) was utilized to determine the *C. botulinum* number of spores in honey samples[13].

3. Results and discussion

C. botulinum NCTC 7272 was detected in artificially inoculated and uninoculated honey samples as shown in Table 1.

Table 1. Detection of *C. botulinum* NCTC 7272 in artificially inoculated and uninoculated honey samples.

The level of contamination/(CFU/g)	ISO 15213:2003 The number of positive samples/number of examined samples	PCR The number of positive samples/number of examined samples
0/0	0/20	0/20
I/0.1-1	0/20	20/20
II/1-5	0/20	20/20
III/5-10	3/20	20/20
IV/10-50	7/20	20/20

C. botulinum was not detected in 59 honey samples using conventional microbiological techniques (every sample was processed in 20 replicates/subunits). However, when PCR was used, *C. botulinum* spores were detected in five of these samples (23 subunits).

The results of honey samples testing in which the presence, number and *C. botulinum* spore type was detected by

PCR method and MPN technique, are shown in Table 2.

Table 2. The number of positive subunits and the type of detected *C. botulinum* spores, in tested honey samples.

Number of samples/sample label	Number of examined subunits/number of positive subunits (*C. botulinum* type)	MPN/kg
4/3888	20/5 (2A, 3E)	116
7/4630	20/6 (6B)	144
20/6344	20/8 (8E)	204
23/5034-5	20/3 (1A, 1B, 1E)	64
29/5480-4	20/1 (A, E)	20

Determining the cause of botulism in the honey samples has always been problematic because of its biological and biochemical characteristics, the level and dispersion method and the nature of matrix[14]. The results of our study confirm this claim.

High honey viscosity due to high sugar level and the small amount of water results in uneven distribution of *C. botulinum* spores which significantly hinders preparation, processing and the use of honey samples by applying both conventional and molecular methods in laboratory diagnosis[8,10,11,14,15]. All this can lead to false negative results both using microbiological and molecular testing methods, especially in honey with low contamination levels which is confirmed by the studies of other authors[5,8,16]. PCR has proven itself as a significantly more sensitive method for detection of *C. botulinum* spores in honey samples than cultural methods; however, PCR can produce false negative results.

Using PCR, the presence of *C. botulinum* spores was detected in all deliberately contaminated honey samples, including in the sample with very low level contamination. The PCR method also proved to be more sensitive during the analysis of 59 honey samples from the Republic of Serbia. The presence of *C. botulinum* spores was found in five of these honey samples using PCR, but was not found using traditional cultural method.

The prevalence of *C. botulinum* in the Serbian honey samples was 8.47%. *C. botulinum* spores type A, B and E were detected and the number of *C. botulinum* spores in the mentioned testing was from 20-204/kg of honey (Table 2).

By determining the number of spores and by using MPN method, uneven distribution of spores was confirmed, data not shown. This was also claimed by other authors[5,14]. Taking this into account and considering honey samples with low and uneven level of natural *C. botulinum* spore contamination, there is a possibility that a part of a sample which does not contain spores can be taken for testing. That is why it is necessary to include larger number of subunits/replicates when it comes to honey in comparison to other food items.

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