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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