Characterization of *Clostridium botulinum* spores and its toxin in honey

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Summary

Botulism is a serious paralytic disease caused by *Clostridium botulinum* toxin in foods. There are seven recognized serotypes of *botulinum* neurotoxins among which the principal prevalent types in humans include A, B and E. Infant botulism results from intestinal colonization and toxin production by *C. botulinum* spores in babies less than 1 year old. Honey is the most important food discriminated as the cause of infant botulism. In this study, a total of 100 honey samples were collected from retail markets in Shiraz, Iran and analysed for the presence of *C. botulinum* using a multiplex PCR assay. Three pairs of primers were designed and optimized to identify A, B and E strains in the specimens. Positive samples were further examined to find out whether they carried the toxin gene; toxin products were also checked by mouse bioassay. The results showed that out of 100 honey samples tested, 2% were found positive for type B which carried the neurotoxin B gene confirmed by sequencing. All the injected mice died, whereas no symptoms were observed in the control groups. The honey collected from Shiraz retail markets was contaminated with the spore of the bacteria and can thus potentially cause infant botulism. Bioassay is described as a standard method; however, molecular based techniques also easily, quickly and reliably detect *C. botulinum*, its toxins and spores in food stuffs and is strongly advised for use in the food microbial lab.

Key words: *Clostridium botulinum*, Multiplex PCR, Honey

Introduction

Botulism is considered as a serious paralytic condition caused by *Clostridium botulinum*. Although seven kinds of immunologically distinct types (A to G) are recognized, human botulism is mainly caused by types A, B, E and F. The intoxication is most frequently caused by the ingestion of inappropriately processed or stored food. The toxin interferes with cholinergic autonomic neuromuscular plaques at presynaptic terminals (Lindström and Korkeala, 2006). Depending on the modes of action, botulism is clinically categorized into four groups: food borne, infant, wound, iatrogenic botulism and adult infectious botulism (Sobel, 2005; Peck, 2009).

Spores of the bacteria are widely distributed in the environment. However, children under a year old are more vulnerable to the germination of spores in the gastrointestinal (GI) tract with subsequent multiplication and toxin production (Nevas *et al*., 2006). Moreover, due to the fact that non-proteolytic *C. botulinum* spore loads are very small and un-observable for most food materials, using a modular process risk model and molecular based technique to identify *C. botulinum* is highly recommended (Malakar *et al*., 2011).

Honey is the only incriminated food stuff to be associated with infant botulism and clinicians must consider infantile botulism in the differential diagnosis of weakness in infancy (Smith *et al*., 2010).

The taste and structure of honey would inevitably degrade by an efficient heat process to destroy the spores and thus the risk of contaminated honey consumption in babies is high (Nevas *et al*., 2006).

Laboratory diagnosis of the intoxication has been previously reviewed by Lindström and Korkeala (2006). While a rapid detection of botulism is a prerequisite to patient recovery, more epidemiological studies are required regarding the various forms of the disease. This includes the isolation of spores and toxin from different sources, serodiagnosis of the genotypes and molecular assays that characterize the intoxication. Among different techniques, the mouse bioassay has remained the standard test for the detection of neurotoxins, whilst it is problematic due to its time consuming and laborious procedure (Dario *et al*., 2009). However, the identification of neurotoxin gene fragments by PCR is a rapid alternative method. More recently, a multiplex PCR assay has successfully identified the spores in various food stuffs including honey (Ahmed *et al*., 2011).

The aim of present study was to identify genotype *C. botulinum* present in honey samples and to conduct an epidemiological survey in order to give a better understanding of the risk of honey that induces infant botulism.

Materials and Methods

Experimental design

A total of 100 honey samples were collected from retail markets in Shiraz, Iran. 10 g of honey was diluted into 50 ml of distilled water and kept at 65°C for 10 min,

in order to inactivate non-spore forming bacteria. The samples were then centrifuged at 9000 × g for 30 min. the supernatants were then discarded before a thermal shock at 80°C for 10 min. (Kuplulu *et al*., 2006). Enrichment of the samples was carried on sediments obtained from centrifugation in a cooked meat medium (Merck, Germany) followed by incubation at 30°C for 7 days in anearobically used type A gas packs (Merck, Germany). One ml of the enriched samples was subsequently collected in 1.5 ml microtubes to extract DNA and further PCR assay.

PCR assay

DNA extraction was performed using a Cinnapur DNA kit (CinnaGen, Iran). The specimens were centrifuged at 12000 rpm for 10 min. The supernatant was discarded and the pellet was vortexed and transferred into a 1.5 ml microtube. 200 µl of lysis buffer and 40 µl of proteinase K were added and incubated at 65°C for 15 min. The DNA was further purified and re-suspended in 30 µl elution buffer according to the manufacturer's instruction, and kept at -20°C for further use. The concentration of DNA was subsequently estimated by absorbance at 260 nm and the purity of the DNA was checked by taking the ratio of O.D. reading at 260 and 280 nm using a spectrophotometer.

Three pairs of primers were used as previously described: species specific 205 bp, fragments 782 and 389 bp, which were subjected to serotypes B, A and E, respectively (Saadati *et al*., 1994) (Table 1).

Table 1: Primers used for specification of *BoNT/A*, *BoNT/B* and *BoNT/E* amplicons corresponding to types A, B and E of *C. botulinum*

Primers	Oligonucleotid sequence (5' 3')	Product size
Af	GCTACGGAGGCAGCTATGTT	782 bp
Ar	CGTATTTGGAAAGCTGAAAAGG	
Bf	CAGGAGAAGTGGAGCGAAAA	205 bp
Br	CTTGCGCCTTTGTTTTCTTG	
Ef	CCAAGATTTTCATCCGCCTA	389 bp
Er	GCTATTGATCCAAAACGGTG	

Multiplex PCR was carried out on 1 µl of the DNA template in a final reaction mixture of 25 µl containing 2.5 µl 10 × PCR buffer, 1.5 µl $MgCl_2$ (50 mM), 0.2 µl dNTP (10 mM), 0.8 µM of each forward and reverse primers, 0.2 µl Taq DNA polymerase (5 U/µl) (CinnaGen, Iran). PCR cycling was performed in a gradient thermocycler (Eppendorf, Germany) with an initial denaturation step of 94°C for 5 min followed by 25 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 30 sec and extension at 72°C for 30 sec. The final extension was then carried out at 72°C for 5 min (BIOR XP, China). The amplified products were subsequently electrophoresed in 1.5% agarose gel, stained with ethidium bromide and photographed under UV light. A species specific fragment of 205, 782 and 389 bp (Fig. 1), corresponding to the botulism neurotoxin B, A, and E were then amplified. 1280 bp toxin B was further amplified (Saadati *et al*., 1994) (Fig. 2).

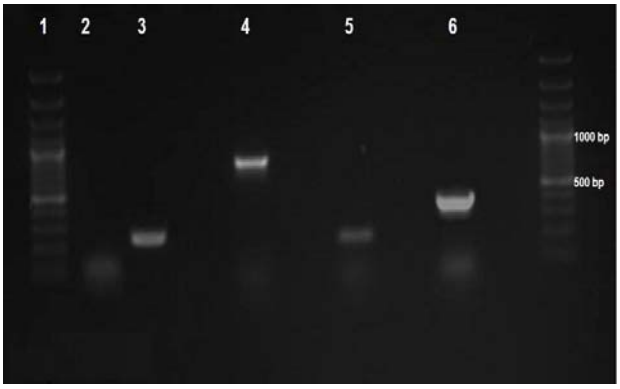

Fig. 1: Representing agarose gel electrophoresis for detection of different types of *C. botulinum* using mPCR assay. Lane 1: 100 bp ladder, Lane 2: Negative control, Lane 3: 205 bp positive samples, Lane 4: 782 bp positive control, Lane 5: 205 bp positive control, and Lane 6: 389 bp positive control

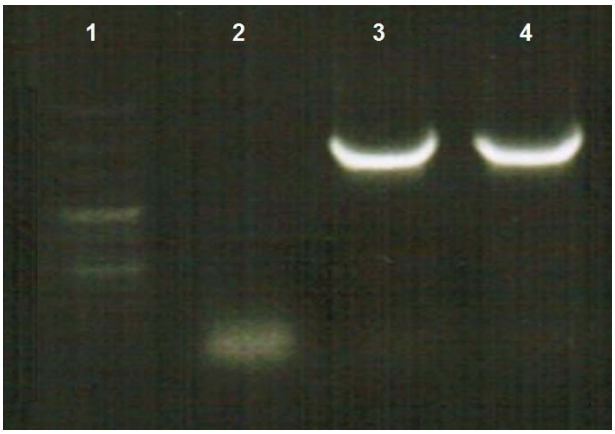

Fig. 2: Representing agarose gel electrophoresis for detection of the purified neurotoxin producing gene. Lane 1: 100 bp ladder, Lane 2: Negative control, Lane 3 and Lane 4: 1280 bp positive samples control

Mouse bioassay

Each PCR positive sample was prepared using the dilution centrifuge method (Kuplulu *et al*., 2006) *Clostridium botulinum* in honey obtained from positive samples by PCR was enriched and incubated at 30°C for 7 days in cooked meat anaerobically followed by subculturing onto blood agar (Oxoid, UK) incubated anaerobically at 35°C for 5 days. Suspected colonies confirmed with gram staining and PCR method (Kuplulu *et al*., 2006) were diluted in 1 ml distilled water, cultured on cooked meat (9 ml) and incubated anaerobically at 30°C for 7 days. The suspension was then added to a 10 ml PBS (pH ~ 6), (Kuplulu *et al*., 2006) centrifuged at 8000 × g for 5 min. The supernatant was then collected and trypsinized as follows: 1.8 ml of the supernatant was added to 0.2 ml (1: 250, at a final concentration of 0.1%) trypsine (Difco, UK) (Boehnel and Lube, 2000). 0.5 ml of the trypsinized samples filtered through 0.45 µm was injected to three mice intra-peritoneally for each positive sample. Three control mice were also injected by the

trypsinized cooked meat medium. The mice were monitored every 2 h for 36 h.

Amplification and sequencing of purified toxin gene

To confirm positive samples, amplification of the purified toxin B gene was performed using the oligonucleotide primers 5′ AGT ACA GGA TCC TTC AGT CCA CCC TTC ATC TTT 3′ and 5′ GGG CCC CCA TAT GAT AGG ATT AGC TTT AAA TG 3′, as previously described (Accession No. JQ964805.1) (Kirma *et al*., 2004). Only one primer pair was chosen to amplify the toxin B gene, but to ensure better results, we cultured the bacteria in a stationary phase to excrete the highest level of neurotoxin.

Amplified genes were finally extracted from gels using a QIA quick gel extraction kit (Bioneer, USA) as described by the manufacturer. Buffer 1 (gel binding buffer) was added to the cut gel, incubated at 60°C for 10 min to solve the gel completely. The solution was transferred to the DNA binding column tube, centrifuged for 1 min at 13000 rpm and poured in the flow-through, re-assembling the DNA binding filter column with 2.0 ml from collection tube. 500 µl of buffer 2 (cell lysis buffer) was added to the DNA binding column tube, centrifuged for 1 min at 13000 rpm., poured in the-through, re-assembling the DNA binding filter column with the 2.0 ml collection tube and dried by additional centrifugation at 13000 rpm for 1 min. This was followed by adding 30 µl buffer 3 (EB) to the center of the DNA binding filter column and letting for at least 1 min at room temperature to elute. The eluted DNA fragment was separated by centrifugation at 13000 rpm for 1 min. The purred products were finally subjected to sequencing (Macrogen, South Korea).

Results

Two specimens (2%) were confirmed as type B *C. botulinum* (Fig. 1). Furthermore, a 1280 bp nucleotide band was amplified on both positive samples, which corresponded to the toxin B gene (Fig. 2).

Typical symptoms were observed in the injected mice including loses of appetite, ruffling of fur, incoordination, weakness of the limbs and impaired movements, labored abdominal breathing, total paralysis, and wasp waist of the abdomen. All mice injected with the positive specimens died during 17 to 33 h post injection. No clinical signs were recorded in the control group.

To analyse the sequencing data, BLAST1 comparison was performed with the NCBI/GeneBank database. The sequence result was 97% identical with the *Clostridium botulinum* strain CDC3281 *BoNT/B* gene sequence.

Discussion

Infant botulism is produced by germination and toxinogenesis of *C. botulinum* in the GI tract. The spores are widely distributed in nature, and are ingested with contaminated honey on the ground (Rebagliati *et al*., 2009; Olivieri *et al*., 2012). The highest prevalence of spores in bee wax suggests the important role of the wax that contaminates honey. The contamination of honey with *C. botulinum* type B spores more likely occurs via dust carried by bees, as a consequence of the frequent existence of spores of serotype B in soil (Nevas *et al*., 2006). As previously described by De Medici *et al*. (2009), despite the presence of PCR inhibitory substances in food raw materials, the multiplex PCR assay can be used in various clinical, food and environmental samples, in routine surveillance monitoring programs. As such, the ability of this technique to detect and differentiate *BoNT* genes encoding the neurotoxins is highly advantageous (Lindström *et al*., 2001).

Of 294 honey samples examined in Denmark, Norway and Sweden, using the mPCR assay for the detection of types A, B, E and F of *C. botulinum*, 26, 10 and 2% were respectively found positive, of which, type B was predominant in Denmark and Norway, while type E was detected in Sweden (Nevas *et al*., 2005). In the current study, the prevalence of 2% with *BoNT/B* was found in the contaminated honey. Contrary to reports with higher contamination rates (Nevas *et al*., 2002; Özlem *et al*., 2006; Ahmed *et al*., 2011), there are studies supporting our work, reporting lower contamination levels in honey (Kautter *et al*., 1982; Rall *et al*., 2003).

Detection procedures of the intoxication are based on culture, mouse bioassay and more recently a multiplex PCR (Ahmed *et al*., 2011). It seemed that The PCR assay is more sensitive for type B, with a smaller size multiplication fragment. Compared to the previously described techniques such as culture and other immunoassay procedures, a marked improvement has been shown both in the identification of the microorganism and its toxin (Lindström *et al*., 2001).

Even though molecular tests cannot detect toxin, they can detect *BoNT* genes if the organism can be cultured. *BoNT* genes can also be directly amplified from food and clinical samples if organisms are still present (Chaudhry, 2011).

Our results showed that the molecular assay, in combination with enrichment, could reliably detect the three important types simultaneously. Furthermore, it quite sensitively identified low numbers of spores in a complex material present in honey (Nevas *et al*., 2002).

The complexity of honey is basically due to low pH, high sugar content, high viscosity, low water activity and existence of hydrogen peroxide which inhibit the normal growth of microorganisms.

In conclusion, the results presented here revealed the hazards of contaminated honey as a cause of infant botulism. Therefore, strict hygienic measures should be taken to reduce the risk of this disease.

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