

Occurrence of *Clostridium botulinum* in Four Species of Fish in the Persian Gulf

ORIGINAL

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Abstract

Background: Food poisoning (FP) caused by *Clostridium botulinum* is the most serious feature of FP in man consuming the contaminated food stuffs specifically seafood. The occurrence of the contamination to different species of the bacteria in four species of fish in Persian Gulf was investigated using selective culture and multiplex PCR (mPCR) assays.

Methods: The samples were initially enriched in cooked meat broth for 7 days at 30 °C in an anaerobic condition followed by subculture onto blood agar. DNA of the samples were then extracted for further mPCR assay using three species-specific pair of primers to amplify the 782, 205 and 389 bp fragments corresponding to the A, B and E types of the microorganism (MO).

Results: Out of 120 specimens, *C. botulinum* was detected in 5 samples (4.2%). Altogether, five and two samples were respectively positive in the mPCR and culture assays which were significantly different ($p < 0.05$).

Conclusions: No evidence of type E was shown; which, was possibly resulted from the limitation of our study. Our results have suggested a reliable molecular assay to employ in the food Laboratories for rapid identification and differentiation of various kinds of *C. botulinum*.

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Introduction

Botulism is a paralytic disease mainly caused by *C. botulinum* and rarely *C. boturicum* and *C. barrati* [1]. The disease is more evident in children under one year of age because there is more chance to germinate spore in the gastro-intestinal tract (GIT) and so productions of more exotoxins [2]. species of *C. botulinum* are classified in I to IV subgroups, from which, the I and II groups (A, B, E, F) and B, C and D were respectively identified in human and cattle [2, 3, 4, 5]. The groups of bacteria are phenotypically dissimilar, for instances, the biochemical characteristics and production of metabolites in different types of the bacteria are not appropriate enough to differentiate them [6]. Considering the different growing conditions of the groups of microorganism, members of group I are growing in the temperature higher than 10 °C, pH ~ 4.5 and the concentration of 10% NaCl, while, the psychrotrophic members of group II are able to grow at 3 °C, pH ~ 5 and lower temperature than 10 °C and 5% concentration of NaCl [7, 8]. Mortality resulted by the consumption of foods contained clostridial neurotoxins, was previously reported about 3-5% [7, 9]. The greatest epidemic clostridial food poisoning via the consumption of homemade canned food was occurred in 2006, in which, 209 people showed the typical symptoms of the FP [7]. There are many reports on the prevalence of the strains of group II in seafood, elsewhere, for example the prevalence of 40-70% was reported from the sea products [10, 11].

In the traditional foods, the highest rate of botulism in Iran was observed following consumption of vegetables and fish [12]. Since, the most prevalent clostridial food poisoning was earlier reported from the consumption of fish and other homemade foods, four main species of fish was selected in the current study [13]. Contamination of fish to *C. botulinum* depends on various factors including types of fish, nutritional habitants, feeding methods and

environments. The contamination in farmed fish is originated from sucking sludge, and thus, gills are well thought-out as the main sources on infection. As such, evisceration is considerably reduced the number of microorganism [12, 14]. Although, most studies were focused on various epidemiological forms of the poisoning, the prognosis of disease is favorable and mainly depends on to promptly detect the spores and/or toxin, using serological and molecular approaches [1]. In order to overcome the problems arising from a quick and reliable detection of different types of the bacteria, present study was conducted to compare the selective culture and mPCR assay. Previous studies were mainly performed in the Iranian Northern Provinces [12, 14]; therefore we have decided to work on the possibility of contamination in the fishes of Persian Gulf, South of Iran.

Methods

A total of 120 samples including 30 of each species of *Tylosurus crocodiles*, *Scombromorus guttatus*, *Otolithes ruber* and *Scombromorus commerson*, were chosen. Fresh procured slaughtered and washed fishes were prepared from Bushehr, in order to meet the standard methods recommended by APHA and FDA. 10 g of intestine and gill was mixed in a same amount of phosphate buffer saline (pH= 6-6.2), as was recommended by others [1, 14, 15]. The samples were then incubated in water bath at 65 °C for 30 min to inactivate non-spore forming bacteria. The samples were then centrifuged at 9000 × g for 30 min followed by heat shock (80 °C for 10 min). For enrichment of the samples, each precipitate resulted from the centrifugation was transferred to a 10 ml test tube containing Cooked Meat (Merck, Germany) which was anaerobically incubated at 30 °C for 7 days. The samples were subsequently cultured onto blood agar and incubated at 35 °C for five days [16]. In order to perform fur-

ther DNA extraction and PCR assay, one ml of each enriched sample was simultaneously transferred to a 1.5 ml microtube.

DNA extraction and PCR assay

DNA extraction was performed using a Cinnapur DNA kit (Cinna Gen, Iran). Briefly, 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 5 °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 pair of primers was used as previously described: species specific 205 bp, fragments 782 and 89 bp, which was subjected to serotypes B, A and E, respectively [6] (Table 1).

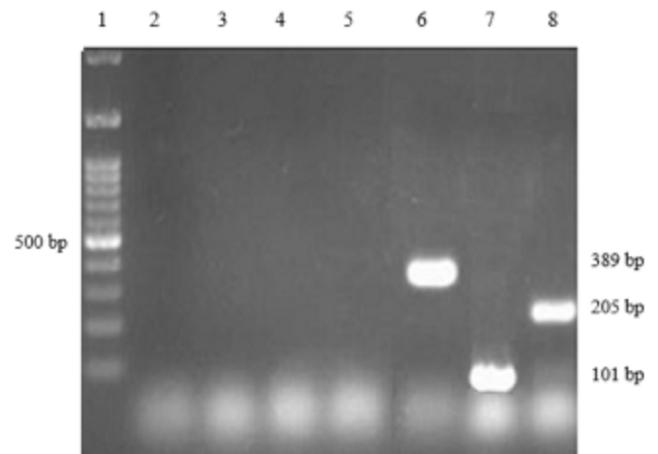
Table 1. Primers used for specification of BoNT/A, BoNT/B and BoNT/E amplicons corresponding to types A, B and E of *C. botulinum* Primers oligonucleotid sequence (5' 3') Product size.

Primers	Primer sequences	Product size (bp)
Af	GGGCCTAGAGGTAGCGTACTG	101
Ar	TCTTGATTTCAGAAAGCATATTTT	
Bf	CAGGAGAAGTGGAGCGAAAA	205
Br	CTTGCGCCTTTGTTTTCTTG	
Ef	CCAAGATTTTCATCCGCCTA	389
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 MgCl₂

[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, toxin B was further amplified [6] (Figure 1).

Figure 1. Multiplex PCR analysis of the samples. Size markers (DNA ladder 100bp), Lanes 2 to 5: the negative examples, Lane 6: product of type B, Lane 7: product of type A, Lane 8: mPCR product of type E.



Results

Out of 120 specimens, *C. botulinum* was detected in five samples (4.2%), in which, types A, B were identified in two samples of *Tylosurus crocodiles* 3/30 (10%), Types A and B were respectively identified in the samples taken from one case of each *Otullitus rubber* 1/30 (3.3%), and *Scombrormorus*

Table 2. Comparison between a conventional culture and mPCR assays to detect different types of *C. botulinum* in fish samples

Species of fish	Number of Samples	Number (%) of positive samples by culture assay	Number (%) of positive samples by PCR assay	Types and number of the bacteria
<i>Scombromorus guttatus</i>	30	0 (0)	0 (0)	-
<i>Scombromorus commerson</i>	30	0 (0)	1(3.3)	B
<i>Tylosurus crocodiles</i>	30	2 (6.7)	3(10)	A (1), B (2)
<i>Otolithes ruber</i>	30	0 (0)	1(3.3)	A

commerson 1/30 (3.3%) (**Table 2**). The contamination of *Tylosurus crocodiles* was higher than that of other species, even though, this was not statistically significant. However, a significant difference was found between culture and PCR assays ($p < 0.05$).

Discussion

In the present study, three species out of four main fish species of the Persian Gulf fishes were found positive. *C. botulinum* type A and B were respectively identified in two and three cases. Out of 428 captured fishes from Baltic Sea, the contamination to the A and E was 24.8% [9]. According to a study by Huss et al. on 1407 captured fishes from Scandinavia North Sea and North Atlantic, the contaminations were respectively 0%, 12.4% and 4.27% [17]. 5.4% of 214 vacuumed packed fishes were contaminated to type E. Results of the current and previous work elsewhere, revealed the association between consumption of seafood, especially the traditionally processed ones (salted, smoked and fermented) and botulinal food outbreaks poisoning.

During 1368-1382, total of 341 suspected cases of botulinal food poisoning were recorded, in Iran, from which, 31.08% caused by salted and spawn, therefore, the seafood considered as most contaminated food stuffs [14, 18]. Cold smoked preparations of fishes (at 30-35 °C) and consumption of its undercooked foods was the major cause of

botulinum in the Northern, Iran [19]. Isolation and identification of *C. botulinum* is not usually straight forward and depends on the mouse bioassay, a time consuming and expensive method, which may replace by a reliable and quick molecular approach [6, 20]. In a study completed by Ahmed et al. (2011) in Egypt, various types of the bacteria were confirmed with the prevalence of 26%, 19% and 21% in honey, seafood and meat products, respectively [7, 21]. Even though, the prevalence of the food poisoning, bacteria and its toxin was investigated in the Northern provinces of Iran, little is known about the disease in the Southern areas of Iran.

Surprisingly, no evidence of type E was identified in our samples which was likely due to the limitation of our work both in the number and species of fish employed, which could be resolved in the future studies. Prohibiting consumption of raw or undercooked products is the major concern to prevent the poisoning. Finally, we recommend a closer investigation on the fresh and processed seafood to employ accurate and rapid diagnosis of botulism.

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Conflict of interest statement

The authors declare no conflict of interest.

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