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A survey of traditional Iranian food products for contamination with toxigenic *Clostridium botulinum*

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Summary This study aimed to determine the rate of *Clostridium botulinum* contamination in some traditional Iranian food products (cheese, *kashk* and salted fish) and evaluate the efficacy of the mouse bioassay method in detection of *C. botulinum* toxins in these foods. A total of 131 samples (57 cheese, 11 *kashk* and 63 salted fish) were collected and examined to determine the rate of contamination by *C. botulinum*. Standard monovalent anti-toxins were used to determine the types of toxin. *C. botulinum* bacteria were detected in 4.58% of the examined samples (1.52% of cheese and 3.06% of salted fish samples). While no contamination was detected in the *kashk* samples, *C. botulinum* types A and E were found to be dominant in cheese and salted fish samples, respectively. These results indicate—some traditional Iranian foods may be contaminated with different types of *C. botulinum*, and the consumption of these products, either raw or cooked, may contribute to food-borne intoxications.

Introduction

Clostridium botulinum has long been recognised as an aetiological agent of food-borne botulism and is a worldwide contaminant of fish [1]. This bacterium

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can produce the most poisonous poison with a lethal dose as low as 10^{-9} g/kg. Botulinum is a neurotoxin, which, on absorption, blocks the secretion of acetylcholine from cholinergic nerve endings, which leads to paralysis, especially of the respiratory system. Although approximately 40—60% of patients develop antibodies to botulinum neurotoxin, only 2—5% ultimately form antibodies that activate the neurotoxin [2,3]. *C. botulinum* produces seven distinct toxins (types A—G) of which types A, B, E and rarely F are toxigenic to humans. These toxins have been reported as significant food safety hazards [4,5]. Types A and B are found in soil and animal fertilisers and, therefore, might be found in food—of vegetable origin, including tomatoes, spinach and beans [6]. Type E, in contrast, is found in aquatic environments, sea food and marine sediments. This toxin is a known source of botulism—and has been reported internationally [7—11].

Botulism is a rare disease with four naturally occurring syndromes: food-borne botulism is caused by ingestion of foods contaminated with botulinum toxin, wound botulism is caused by *C. botulinum* colonization of a wound and in situ toxin production, infant botulism is caused by intestinal colonization and toxin production, and adult intestinal toxemia botulism is an even rarer form of intestinal colonization and toxin production in adults. There are many reports in Iran on food-borne botulism due to consumption of traditional salted fish, dairy products (e.g., cheese and *kashk*) and canned foods [12—15].

Kashk is a dairy product prepared commercially and traditionally in Iran. Some Iranians consume the traditional *kashk* along with some of the local foods, which possibly lead to growth and toxigenity of *C. botulinum* in these foods (increasing of pH).

A number of assays determining *C. botulinum* contamination exist (polymerase chain reaction (PCR), enzyme-linked immunosorbent assay (ELISA), FA and bioassay) [16]. However, the sensitivity of the commercialised ELISA and PCR techniques is much lower than the mouse bioassay [17—19]. At the present time, the mouse bioassay is still a gold standard for detection of botulinum toxin [20—23]. This study has been approved by the Ethics Committee of Research Center of the University.

Materials and methods

This study was conducted in 2007. A total of 131 samples were evaluated in the study period (57 cheese, 11 *kashk* and 63 salted fish). The fish samples were collected from the Caspian Sea in the Gillan province, and traditional cheese and *kashk* samples were obtained from the markets of this region. These traditional food products, typical of northern Iran, are freshly prepared by the local vendors without conforming to any health standards. We chose to study these foods because of their high consumption among Iranians and the associated frequent reports of food-borne botulism related to their consumption.

The cheese and *kashk* samples were collected randomly from the place of processing or from the markets and the fish samples were collected from a pond in sterile 30 cm × 50 cm polythene pouches and aseptically transported to the laboratory. All of the fish samples were eviscerated and traditionally salted (2—3%), by mixing salt at room temperature (about 22 °C), and kept in a watertight container for 4 days.

Sample preparation

5—10 g of each sample was mixed with an equal volume of gelatine phosphate buffer (pH 6—6.2) and shaken vigorously for 10 min. For each sample, two test tubes containing 5 ml of cooked meat medium (CMM, Merck, Darmstadt, Germany) were taken and 3—5 g of homogeneous sample was added to the CMM and incubated in a water bath at 60 °C for 10 min to activate the *C. botulinum* spores. Then the samples were anaerobically incubated at 30 °C for 7 days. Following incubation, enriched cultures were centrifuged at 10,000 × *g* and 4 °C for 20 min and each supernatant was adjusted according to standards designated in the FDA Bacteriological Analytical Manual [16].

Contemporaneous cultures were examined for turbidity, gas production and digestion of meat particles (as per the APHA standard method) since proteolytic types of *C. botulinum* are amenable to meat analysis. Cultured specimens were Gram-stained. The centrifuged samples were refrigerated and the supernatant fluid was used for toxin assay.

Mouse bioassay methodology

The supernatant fluids were divided into three portions: one remained untreated (for non-proteolytic types); the second was heated at 100 °C for 10 min for toxin neutralisation (to act as a control), and the third was trypsinised to demonstrate the presence of inactive pro-toxin (for proteolytic types). For the trypsinised sample, we prepared trypsinising solution (1 g trypsin 1/250 Difco + 10 ml sterile

Table 1 Frequency of Botulinum toxin in examined traditional food samples (according of toxin type).

Sample	Number	(*n*) % Positive cases	Toxin type
Cheese	57	(2), 1.52%	A (2)
Kashk	11	(0)	—
Salted-smoked fish	63	(4), 3.06%	E (2), B (1), A (1)
Total	131	(6), 4.58%	E (2), B (1), A (3)

water with pH 6.2), mixing 0.1 ml of this solution with 0.9 ml of supernatant.

Characterisation of botulinum toxins from culture supernatants

About 0.5 ml each of sample solution was injected intraperitoneally into a pair of mice (weighing 20—25 g each) and both mice were observed periodically for 96 h for symptoms of botulism. The typical signs of botulism in mice usually begin in the first 24 h. Polyvalent ABE and standard monovalent anti-toxins (supplied by Iranian Red Crescents and Ministry of Health and Medical Education from Russia (Soto-Verjak Research Center, Moscow, Number 121002041)) were used to ascertain the toxin types.

Results

The results are shown in Table 1. From a total 131 samples examined, *C. botulinum* toxins were detected in six (4.58%), including two (1.52%) of cheese samples and four (3.06%) of salted fish samples; however, no contamination was seen in the *kashk* samples. Therefore, the contamination rate in salted fish samples was 1.8% more than that in cheese samples. Further we found toxin types A and E were dominant in cheese and salted fish samples, respectively. In the entire study sample 50%, 33.3% and 16.6% of the positive samples were related to type A, type E and type B, respectively (Table 1).

After incubation of the culture media, we observed turbidity, gas production and digestion of the meat particles in the test tubes. Gram staining revealed Gram-positive bacteria with sub-terminal spores. We confirm that the cultures were presumptively positive because broth Gram stains were consistent with *C. botulinum*, and the broth subcultures with solid media produced typical colonies after anaerobic incubation.

Final confirmation of presence of *C. botulinum* was with the mouse bioassay where the mice had all the typical signs of botulism, including ruffling of fur, followed in sequence by laboured breathing, weakness of limbs and, finally death due to respiratory failure.

Discussion

The present study shows that 4.58% of the studied samples of traditional Iranian foods were positive for *C. botulinum*. The higher rate of contamination in salted fish samples which we measured may be related to the kinds of fish and the process of salting and smoking, which may result in anaerobic conditions and could lead to contamination. Changes in oxidation—reduction environment (Rh), pH, heat and level of salt are factors which could provide a favourable environment for toxin production [2,3,5]. Proteolytic types of *C. botulinum* are able to produce toxins even in 5% salt, while only 2—3% salt concentration is used in the preparation of salted fish in Iran [28,29].

The consumption of different kinds of traditional fish products has been increasing in Iran in the past decade. From 2002 until 2007, a total of 260 cases of botulism were detected in Iran; of which 38 cases were related to consumption of traditional dairy products (non-pasteurised local cheese and *kashk*) and 88 were due to salted sea foods (salted fish, smoked fish, canned beans) [12,30—32].

This study demonstrates that *C. botulinum* toxin type E was dominant in salted fish samples we studied. Similar results were found in the Vahdani and Tavakoli studies (Table 1). In these studies, different types of *C. botulinum* were isolated from the sediments and sea foods, meat and vegetable cans [14,33]. This has been suggested by the results of other researchers as well [18,20,34].

In contrast, within dairy products, *C. botulinum* toxin type A is predominant. In a report on Iran published by the Pasteur Institute, 27 people were poisoned on consumption of traditional cheese in the Qazvin province Northern Iran; of these, botulinum toxin (type A) was detected in 10 patients [30].

Angulo et al. showed that 30 persons were intoxicated with *C. botulinum* type A after consuming potatoes [35], and Porshafi et al. revealed type A as predominant [30]. Our study is consistent with these reports since it also determines type A as the dominant type in cheese samples and both the positive cases (100%) related to this type (Table 1). Based on data gathered by Townes et al., during

the botulism epidemic in Atlanta (USA), eight people were contaminated by consumption of cheese sauce, of which four were hospitalised and one died [25].

In 1988, Hydarnia and Memarbashi documented botulinum toxin in *kashk*. As a result the sale of pasteurised *kashk* in Iran was obligated by the Iranian health ministry. In the Hydarnia and Memarbashi study, it is believed that *C. botulinum* can grow in anaerobic environment and produce toxin in *kashk* [36]. In recent years, despite mandated pasteurization of *kashk*, nine cases of botulism intoxications due to consumption of these foods have been reported. In our study, no contamination was seen in the *kashk* samples because botulinum toxin is not produced at a pH below 4.5, while increasing pH to 5.5—6.5 could result in the growth of *C. botulinum* and toxin production.

In addition, infant dairy products may be contaminated by *C. botulinum* spores, which is very dangerous. Brett detected botulinum toxin in baby milk powder using the AFLP technique [34].

Globally, there are many reports about botulism intoxication by consumption of traditional foods. For example, in 1991, an epidemic of botulism was reported from Egypt, in which 99 individuals were intoxicated and 18 of them died because of traditional, smoked, salted fish consumption. Studies in China, France, Canada, Alaska and Japan showed botulism poisoning following ingestion of raw, salted, smoked and vacuumed fish [7,9,10,21,24—27].

Although there are various methods (PCR, ELISA and FA) for detection of botulinum toxin, the mouse bioassay is currently considered gold-standard despite a number of shortcomings [9,18,21,30,37,38].

Conclusion

In conclusion, the results of this study and similar ones in other countries demonstrate that different types of *C. botulinum* may be present in traditional Iranian food products (cheese, *kashk* and salted fish). Since these products are usually consumed raw or undercooked, their consumption may pose a risk of food-borne botulism intoxication. Therefore, public health education improvement of all aspects of food processing, avoidance of raw food consumption and, finally, regular quality control education of food managers can be effective in preventing food poisoning or other infectious complications following consumption of these products when contaminated by *C. botulinum*.

Conflict of interest statement

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