Monitoring genes encoding botulinum neurotoxins type A B, E and F in selected samples of food and feed

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Abstract

Clostridium botulinum, as an ubiquitous bacterium, may occur in a great many foods of both plant and animal origin. Spores of *C. botulinum* are present in soil, lakes and coastal waters, and occur widely in the digestive tract of fish and other animals. Their occurrence in foods represents a great risk to man. The phenomenon of food poisoning is associated with the germination of spores, the growth of microorganisms and the production of neurotoxins in food. Many food products support the growth of *C. botulinum*, even in the case of aerobic storage, since the environment inside foods is easily anaerobic enough for the growth of bacteria and the subsequent formation of toxin. The aim of this work was to investigate foods and feeds in various categories by means of a molecular method and to determine the presence in them of genes encoding botulinum neurotoxins. Although 8 spreads, 4 sausages, 3 jams, 3 ready-to-eat foods and 5 feeds were tested, genes responsible for the production of botulinum neurotoxins type A, B, E and F were not confirmed in any of them.

Botulism, Clostridium botulinum, foods, neurotoxins

Introduction

Clostridium botulinum bacteria are gram-positive, spore-forming strict anaerobes. They are classed in groups I – IV according to the types of toxins they produce and a number of other biochemical properties (proteolysis, lipolysis, fermentation of sugars, etc.). Strains classed in groups I (proteolytic) and II (non-proteolytic) represent the greatest risk to man because it is only in these groups that the production of type A, B, E and F toxins associated with food poisoning has been recorded. While proteolytic strains cause problems following the ingestion of vegetables or meat conserved or preserved at home (Lindström 2003), the occurrence of non-proteolytic strains is associated with packed foods with an extended period of consumption stored at low temperatures (Peck 1997). The association between the distribution of strains and the character of the foodstuff is directly correlated with the optimum and minimum growth temperature of strains and with the degree of resistance of spores to the action of heat (Table 1).

Table 1. Growth conditions and resistance of spores of *Clostridium botulinum* bacteria to the action of heat (Rossetto et al. 2014)

Group	Optimum heat	Minimum growth heat	Inactivation of spores by the action of heat
Proteolytic strains	35 – 40 ºC	10 – 12 ºC	121 °C ~ 3 minutes
Non-proteolytic strains	28 – 30 ºC	3.3 ºC	90 °C ~ 10 minutes

The mechanism of action of botulinum neurotoxins consists of blocking the release of acetylcholine in the synapses of the peripheral nervous system which results in paralysis and, in extreme cases, in respiratory failure. There are various forms of botulism. Toxin-producing *C. botulinum* bacteria reproducing in food which is subsequently ingested without heat treatment result in intoxication by the toxin that is already present in the food.

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Cases have, however, been recorded of toxin-producing *C. botulinum* bacteria reproducing in the digestive tract with subsequent toxicoinfection. When wound botulism occurs, toxin-producing bacteria reproduce in damaged tissues from where the neurotoxin is absorbed into the organism. The final form is infant botulism in which the reproduction of bacteria and production of toxin take place in the infant's gastrointestinal tract. Infant botulism is associated with the ingestion of honey by children less than one year old (Rossetto et al. 2014).

The growth of proteolytic stains of *C. botulinum* in group I is retarded at 15 °C and halted by a temperature of 10 – 12 °C. In addition to this, their growth is accompanied by an unpleasant aroma which immediately makes food unacceptable. Non-proteolytic strains of *C. botulinum* in group II are, in contrast, capable of reproducing and forming toxin at temperatures beneath 7 °C (a minimum of 3.3 °C) which are commonplace in food distribution chains, while temperatures in household refrigerators may be 10 – 12 °C. The growth of *C. botulinum* bacteria in group II is not accompanied by an aroma and food looks normal even when it may contain a lethal dose of botulinum neurotoxin. A dose of 30 ng of neurotoxin is required to cause illness or, in isolated cases, even death in man. Consumption of just 0.1 g of food in which *C. botulinum* bacteria are growing may cause botulism (Peck et al. 2006).

The most effective way of inactivating spores of *C. botulinum* in food is heat, although the thermal resistance of spores is affected by the properties of the food itself (pH, water activity, fat content).

Materials and Methods

A total of 23 samples were selected for monitoring – 18 (78.3%) samples of various kinds of food (Table 2) and 5 (21.7%) samples of feed. The group of tested feeds included feed for cattle, feed for fish, a feed mix for pigs, a complete feed for early-weaned pigs and a mixed sample of wheat, barley and maize. The samples were taken during official controls or were sent to the Veterinary and Food Institute in Dolný Kubín during the performance of checks on other target parameters in parallel with which the potential occurrence of genes encoding botulinum neurotoxins types A, B, E and F was also tested.

Table 2. Overview of types of food tested

Type of food	Number of samples	Percentage
Spreads	8	44.4
Sausages	4	22.2
Jams	3	16.6
Steamed ham	1	5.6
Herring fillets in oil	1	5.6
Filled baguette	1	5.6
Total	18	100

The samples of food and feed were processed and tested in accordance with the valid ISO 17919:2013. After 24-hour anaerobic cultivation of 25 g of food in the non-selective reproduction medium TPGY (tryptone-peptone-glucose-yeast extract broth) at 30 °C, two representative samples of a volume of 1.0 ml were taken in parallel, from which DNA was extracted by an isolation method based on the use of cetyltrimethylammonium-bromide (CTAB method). If the first PCR reaction produced a negative result, a further PCR was performed from the reproduction medium cultivated for another 48 ± 2 hours, diluted 1:10 in fresh TPGY medium and cultivated for another 18 ± 2 hours. Anaerobic cultivation took place in a Bug Box anaerobic workstation (Ruskin Technology Ltd, UK). A qualitative multiplex real-time PCR method was used to detect genes encoding botulinum neurotoxins types A, B, E and F (Messelhäusser et al. 2007). This involved triplex PCR detecting genes encoding toxins A and B and an internal amplification control (IAC) of DNA plasmid pUC19 (Plate III, Fig. 1) and duplex PCR detecting genes encoding toxins E and F (Plate III, Fig. 2). This method is based on the principle of enzymatic amplification of gene fragments using classical PCR technology with primers and TaqMan® fluorescence reporter probes. Real-time PCR took place on iQ5 real-time PCR cyclers (Bio-Rad, USA).

Genomic DNA isolated from reference strains of *C. botulinum* producing type A, B, E or F neurotoxins was used as a positive control. Reference strains were provided by the National Reference Centre for Botulism (Istituto Superiore di Sanità, Rome, Italy).

Results

Spreads (44.4%), sausages (22.2%) and jams (16.7%) were the foods most strongly represented among the tested food samples. The test set also included a sample of steamed ham, herring fillets in oil and a filled baguette. All the samples of food tested for the presence of genes responsible for the production of botulinum neurotoxins types A, B, E and F associated with food poisoning were found to be negative. Similarly, the presence of genes encoding the studied botulinum neurotoxins was not found in any of the 5 tested samples of feed. Internal amplification control (IAC) of DNA plasmid pUC19 was performed in all the tested samples which indicates the credibility of the results obtained. The inhibition of amplification was ruled out as a cause of negative results.

Amplification curves with values of $Ct > 30$ represent amplification of the internal amplification control system (IAC) of DNA plasmid pUC19 which is conducted to rule out the process of inhibition during amplification as a possible cause of negative results. Amplification curves with values of $Ct < 20$ belong to positive controls in which genomic DNA of a reference strain of *C. botulinum* producing neurotoxins A or B was used as a template for amplification.

Amplification curves with values of $Ct \sim 20$ belong to positive controls in which genomic DNA of a reference strain of *C. botulinum* producing neurotoxins E or F was used as a template for amplification.

Discussion

A multiplex real-time PCR method in accordance with ISO 17919:2013 based on the work of Messelhäusser et al. (2007) was introduced, validated and accredited at the Veterinary and Food Institute in Dolný Kubín in 2016. The implemented method makes it possible to detect the presence of genes encoding botulinum neurotoxins types A, B, E and F in foods propagated in a liquid cultivation medium with a limit of detection less than or equal to 10 CFU/ml in two separate reactions. A method has appeared recently in scientific publications that enables the concurrent detection of the genes of all four neurotoxins and makes a significant reduction to the costs of testing possible (Kirchner et al. 2010). The given method has not yet, however, gone through the process of international validation and does not, therefore, have the status of a normative method.

Monitoring was performed focusing on the presence of genes responsible for the production of toxins in selected samples of food and feed in connection with the implementation of the method at the Veterinary and Food Institute in Dolný Kubín. Consideration was given to the probability of the potential occurrence of spores of *C. botulinum* in food during the selection of the types of samples. For this reason, sausages and jams were included in the test set of foods in addition to spreads. Steamed ham, herring fillets in oil and a filled baguette were also included among the foods tested. As animals may also be exposed to toxicoinfection, samples of feed were also included in the monitoring. In the past, this disease was considered a problem that occurs at home when meat products (and sausages in particular, hence the term "sausage poison") are not subjected to adequate thermal treatment or when vegetable products (home preserves) are not conserved properly. The Centre for Disease Control and Prevention states that 210 epidemics of food botulism were recorded in the years 1996 – 2014, of which 145 were caused by home foods and 43 (30%) by home preserves of plant origin (CDC 2016).

Under certain conditions, spores of *C. botulinum* may survive the thermal treatment of foodstuffs, though their properties (a_w, low pH, sugar content) mean that they can only germinate and cause disease in isolated cases. When this happens, spores germinate thanks to the anaerobic environment created when oxygen is removed from product packaging during the heat treatment process. As a result of the subsequent storage of these foods at room temperature, vegetative forms of spores produce a dangerous neurotoxin which, following consumption, leads to symptoms typical of botulism (Anonymous 2013). Modern technology in the canning industry was developed in order to destroy spores. In contrast, botulinum toxins are, however, thermolabile and are all inactivated when foods are heated to 85 °C for 5 minutes (Sobel 2005).

Lalitha and Surendran (2002) tested samples of fish in India, where they found a 19% occurrence of *C. botulinum* in fresh fish and as much as 25% in oysters. Its occurrence in salted dried fish was as much as 48%. Although Fujisawa et al. (2000) also tested samples of fish and fish products and recorded positive findings for the presence of clostridia (22%), they did not record any bacteria of the species *C. botulinum* or *C. perfringens*.

Conclusions

In view of the widespread occurrence of *C. botulinum* in the environment, this bacterium and the toxins it produces represent a risk to the health of consumers, particularly in the case of the failure of the production process for food/feed or incorrect storage conditions. The method implemented reliably detects the presence of the genes responsible for the production of dangerous botulinum neurotoxins. The presence of these genes does not, however, confirm the presence of the toxin itself, but merely the bacteria's potential to produce it. Since botulism represents a serious risk to human health, positive findings made using molecular-biological methods must be followed up by biological proof of the toxin ("mouse bioassay") or direct genetic evidence of the isolated suspect strain. All samples of food and feed tested at the Veterinary and Food Institute in Dolný Kubín were shown to be negative.

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ISO 17919:2013 International Organization for Standardization: Mikrobiológia v potravinárskom reťazci. Polymerázová reťazová reakcia na dôkaz patogénov z potravín. Dôkaz klostrídií produkujúcich botulínový neurotoxín typu A, B, E a F (In Slovak)

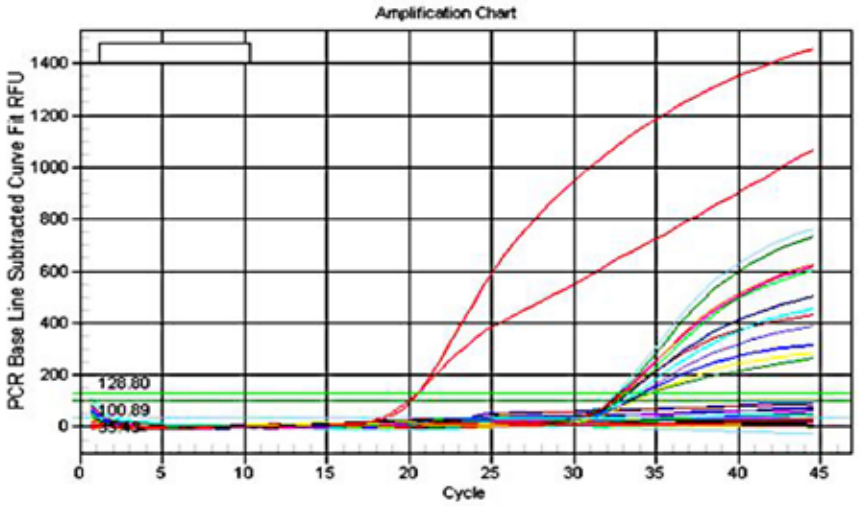

Fig. 1. Graphic representation of the results of triplex real-time PCR with concurrent detection of botulinum neurotoxins type A and B and internal amplification control (IAC)

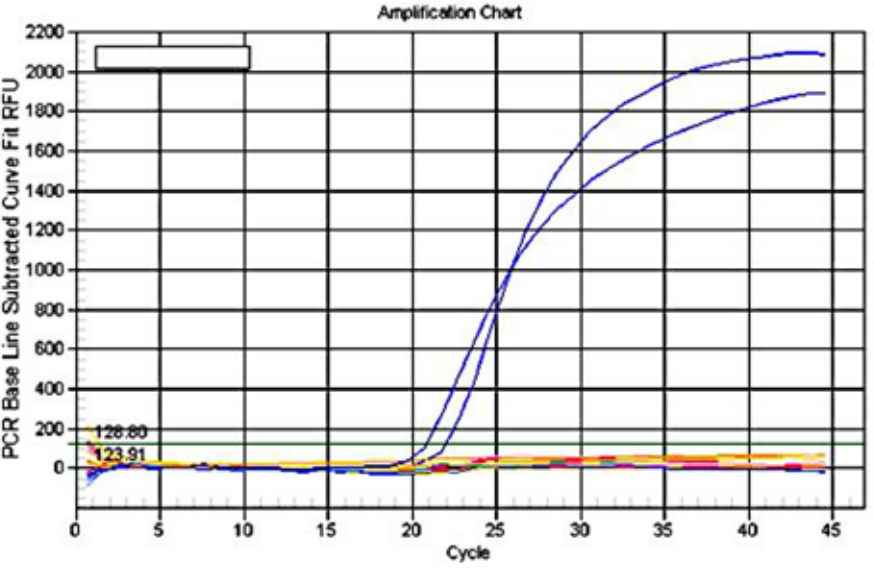

Fig. 2. Graphic representation of the results of duplex real-time PCR with concurrent detection of botulinum neurotoxins type E and F