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BOTULINUM NEUROTOXIN SEROTYPES INVOLVED IN FOOD-BORNE BOTULISM OUTBREAKS IN ROMANIA IN THE LAST FIVE YEARS

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Abstract

This paper summarizes five food-borne botulism outbreaks occurred in Romania from January 2010 till the beginning of 2015. In this period, from 54 food samples received from human botulism suspicion cases and 140 self-control samples, only five samples were positive to the botulinum neurotoxin detection by mouse bioassay. Traditional prepared food seems to be the most common way to get the causative bacteria from specific poisoning areas. The food matrices positive for BoNT were pork and fish meat, all of them made at home in a traditional way. The most frequent BoNT serotype incriminated was B, found in three outbreaks associated with homemade salted and smoked dried pork and one outbreak with a homemade salted and smoked-dried fish meat. Only in one case, two BoNT serotypes A and B were detected in the same sample (salted and smoked- dried pork meat). For certain regions, seems to be incriminated a certain type of BoNT. Amongst the five outbreaks, three were reported in the North-Western, one in the Western and one in the Southern area of the country, thereby these places could be assigned like botulism poisoning zones, but studies need to be continued.

Key words*: botulism, outbreaks, BoNT serotypes, Romania.*

INTRODUCTION

Botulinum neurotoxins (BoNTs) are produced by six anaerobic spore-forming Gram positive phylogenetically and physiologically distinct clostridia (*Clostridium botulinum* Groups I-IV and some strains of *Clostridium baratii* and *Clostridium butyricum*) (Peck, 2009). BoNTs are the most acutely lethal and powerful naturally occurring toxins known to science, leading to neuroparalytic illness by inhibition of acetylcholine release in synapses (Sharma and Whiting, 2005; Vossen et al., 2012).

Food-borne botulism is rare but must be considered a *life-threatening* emergency, requiring rapid recognition of the disease and identification of the source and type of the toxin (CDC, 1998). Knowing the toxin type is important in selecting an antitoxin for treatment (antitoxin produced against one type is not protective against others). The causative bacteria and spores are ubiquitous in nature but the distribution of strains can vary with the geographic area. The bacteria/spores alone do not cause the disease, only their production of botulinum toxin in anaerobic conditions renders them pathogenic.

In the last two decades, various BoNT detection methods appeared like ELISA (Abbasova et al., 2011), Endopep-MS (Hedeland et al., 2011), ELISA-PCR (Fach, 2002) but the "gold standard" is still the mouse bioassay (Quinn et al., 1994). There are seven types of BoNT recognized (A to G) and 32 subtypes (Barash and Arnon, 2014), but the prevalent in human botulism are A, B, E and F types (Barr et al., 2005). Most strains produce only one type of toxin, but strains producing multiple toxin types are not unprecedented (**Gimenez and Gimenez**, 1993; Santos-Buelga et al., 1998; Barash and Arnon, 2004). In the last few years, Romania has experienced several food-borne botulism outbreaks, as follows: two outbreaks with 27 human cases in

2003 (two deaths), 10 outbreaks with 18 cases in 2004, three outbreaks with 21 cases in 2005, two outbreaks with 23 cases in 2006 (one death), five outbreaks with 110 cases in 2007 (three deaths) and one outbreak with 11 cases in 2008. From all cases, 98.75% were B type and only 1.25% were E type. (Ivana et al., 2009).

MATERIALS AND METHODS

This study is concerns to all samples of food products analysed for BoNT detection and typing between 2010 and 2015 in the Institute for Hygiene and Veterinary Public Health (IHVPH) from Bucharest, Romania. The method used for detection and identification of botulinum neurotoxin in food was the mouse bioassay followed by sero-neutralisation in according to the Romanian standard procedure SR 13419/1998. The antitoxin antibody serums to A, B, E and F used for the mouse toxin neutralisation test were supplied by Microgen-Russian Federation (equine serum used at 1 UI/ reaction) and Metabiologics Inc.-USA (goat serum used at 20 µg/reaction). The mice (16-24 g, NMRI breed) were acquired from the National Institute of Research and Development Microbiology and Immunology "I. Cantacuzino", Romania. For preparation of test samples an extraction of 20 g sample was made in 40 ml buffer, a clear supernatant from this homogenous suspension being obtained after centrifugation. Positive controls included a suspension of food samples without antitoxins, and negative controls were prepared by boiling samples (heated to 100^0C for 10 min.), because BoNT is thermolabile. The toxin activation by 5% trypsin solution was used for all sample extracts, because in food can also multiply nonproteolytic *C. botulinum* strains that lack the activation botulinum toxin proteases. Mice were injected intraperitoneal (Figure 1) with 0.5 ml of sample extract alone for toxin detection and titration, mixed with polyvalent antibodies for confirmation and mixed with monovalent serum antitoxin A, B, E and F for serotype identification. We used five mice per dillution for BoNT titration and two mice for each A, B, E and F BoNT identification by sero-neutralisation assay. To estimate BoNT concentration in 20 g of sample we used Spearman-Karber method (*the formula WHO, 1996*). Every time we did a titration of the neurotoxin from food extracts determining the lowest lethal dilution of the toxin (MLD_{50} – mouse lethal dose which kills 50% of the mice), because we wanted to use small quantities of antitoxin serums and to be sure that the neutralising process will be completed. The specific signs of botulism in mice (ruffled fur, labored breathing, weakness of limbs, followed by total paralysis and death due to respiratory failure) were recorded daily for 96 hours (Figures 2 and 3).

All identifying information of the outbreaks, collection time of samples and characteristics of the people involved were recorded from official papers of the Public Health and Sanitary Veterinary Directorates (SVD) of Romania.

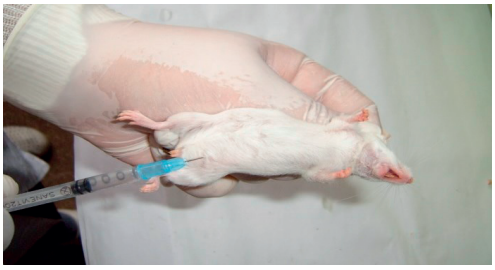

Figure 1. Intraperitoneal inoculation of mouse

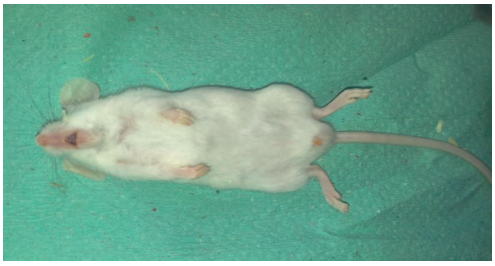

Figure 2. View of a dead mouse due to respiratory failure caused by descending paralysis

Figure 3. View of a dying mouse with *wasp*-like narrowed waist *due to* labored abdominal breathing

RESULTS AND DISCUSSIONS

Five food-borne botulism outbreaks occurred in Romania from January 2010 till January 2015 after 54 samples were analyzed from suspected clinical cases of botulism in humans who ate preserved meat. Additionally, 140 self control samples from different retailers were tested, but all of them were negative to the BoNT mouse bioassay (Figure 4).

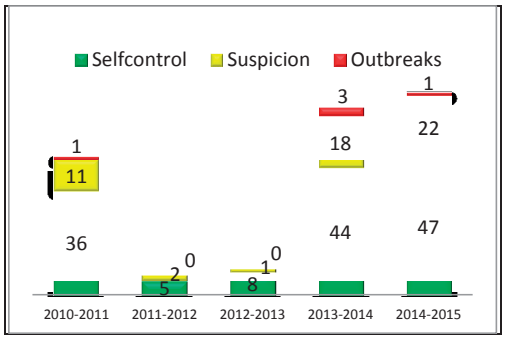

Figure 4. Samples tested for BoNT detection between 2010 – 2015

The mouse bioassay is still the only definitive and accepted test that certifies both the presence and the biological functionality of BoNTs extracted from contaminated samples (Sachdeva et al., 2010; FDA, 2012), beside the in vitro tests (ELISA, PCR, etc) which can only detect the toxin presence but cannot guarantee that it's active or not.

In October 2010, an outbreak was announced by Public Health Directorate in the University campus of Timisoara. Here, a 27 years old student has participated to a party and consumed sushi and smoked dried pork. He arrived to the Victor Babes Hospital of Infectious Diseases from Timisoara with diplopia, dysphagia, drooping eyelids, vomiting, mydriasis, abdominal pain and slurred speech. The pork meat was received from a person from Remetea village, Timis county. When was contacted, this person also was found with the same botulism symptomatology.

The outbreak was investigated by a team of inspectors from SVD Timis who initiated epidemiologic investigations to determine the source of infection. They subsequent collected the two remaining meat pieces and sent these samples to the IHVPH laboratory. BoNT detection method successfully identified both two samples as the vehicle of intoxication. The toxin concentration was estimated to 13967 MLD_{50}. After confirmation by a A/B/E/F polyvalent antitoxin mixture, we detected two BoNT serotypes A and B by mouse sero-neutralisation, as follows: initially all mice died when they were injected intraperitoneal with separate A, B, E or F monovalent antitoxin and, only when they received a mixture of A and B antitoxins, all the mice survived (Table 1). Our results clearly indicate that the sample was presumably contaminated by either a mixed culture of bacteria harboring different BoNT serotypes (A and B) or possibly there was a single strain with the ability to produce multiple toxins AB or BA.

In November 2013, a three person family from Targoviste town developed botulism symptoms (drooping eyelids, slurred speech, difficulty swallowing, dry mouth and muscle weakness) because they consumed smoked dried carp fish given by a neighbour who caught the fish by himself in a pond in Gogosari village, Giurgiu county. The fish was prepared in house by the fisherman in Targoviste town by one day salting, three days drying and one day smoking. Unlike the neighboring family, the fisherman's family did not developed any signs of illness. The remaining fish was sent to our laboratory for BoNT detection. The result was a BoNT B type with 4690 MLD_{50}/20 g of sample. Taken into account that the illness occurred in humans in the Targoviste, the outbreak has been established by the Public Health Directorate in this city even if the source of the disease could it be the spores from the fish from the pond of Gogosari village, Giurgiu county.

Another outbreak occurred in April 2014 when a family (three members) from Bogdand village, Satu Mare county, consumed smoked salted pork and homemade pork sausages. The animal provenience was Craidolt village from the same county. Only two family members who ate the pork meat fell ill with diplopia, dysphagia, blurred vision, abdominal pain. A family member had symptoms earlier than the other (at 24 hours) but he reached medical assistance at seven days after the onset of symptoms, together with his brother who developed abdominal pain, diarrhea and

swallowing disorders. Vet inspectors contacted by Health Public Directorate immediately sent samples from pork to the IHVPH laboratory for investigations. The sample was positive to the BoNT mouse bioassay detection. The concentration of the BoNT type B in 20 g sample was 910 MLD_{50}.

In the Arad county, Magulicea village, Health Public Directorate declared an outbreak of botulism in June 2014 when at 24 hours later, after eating smoked pork meat traditionally made in house, a woman started to have vision disorders, photophobia and deglutition problems. Her son who only tasted the meat (he hasn't liked the taste of meat) developed milder symptoms. Vet inspectors sent pork meat and sausages samples to the IHVPH. Only pork meat was positive to BoNT detection method. The BoNT present in meat was a B type and the concentration was 1400 MLD_{50}/20 g of sample.

In the Bihor county in January 2015 a botulism outbreak occurred within a family formed from two persons who have been hospitalized with signs of diplopia, deglutition disorders and abdominal pains. Following Health Public Directorate notification about this outbreak, a SVD team went to the family address in Tarian village, where they seized all smoked salted pork and sent samples to the IHVPH laboratory. After BoNT testing, the type resulted was B and the concentration was estimated at 22135 MLD_{50}/20 g of sample.

Because detection limit on mouse bioassay (1 MLD_{50}/ml) is about 0.03 ng BoNT (Lindstrom M. and Korkeala H., 2006) and taking into account that the human oral toxic potentially fatal dose begins with ~70 ng (Peck M.W., 2009; CIDRAP, 2012), we can see in the table 1 how dangerous can be the BoNT contaminated food for consumers.

Unlike other countries where cheese or vegetables are most incriminated (CDC, 1998), the basis of all outbreaks in Romania is mostly pork meat, traditionally prepared in house. Therefore the real source of infection seems to be placed in rural areas where backyard pigs are usually slaughtered at home. BoNT is produced usually during home preparation. In Romania, the traditional preservation is mostly done by immersion several weeks in saline solution at low temperature (9-12^{0}C) followed by 24 hours of warm smoking process. To kill the spores is needed a heating for 30 minutes at 121°C. BoNT can be destroyed by boiling or heating to 80°C for 10 minutes (Shahcheraghi et al., 2013).

It seems that the concentration of toxin in 20 g of food is not so high, but the disease gravity could rise if the consumer eats too much, leading to fatal ending. In just one case a person said that the taste of the meat is changed, so, because of the preparation mode with saline solutions and a lot of smoke, the action of the bacteria on the organoleptic properties of the food could be concealed.

The botulism is a telluric disease due to certain areas where this traditional preservation of meat is ancient. The spores are well maintained in anaerobic conditions in soil and sediments for decades just like a kind of "biological cycle" that could be firmly related to intestinal flora of warm blood animals, particularly pigs (FDA, 2012). Regarding the regional distribution of the food – borne botulism outbreaks between January 2010 and January 2015, the majority of them were situated in the West, North – West region and just one in South of Romania (Figure 5).

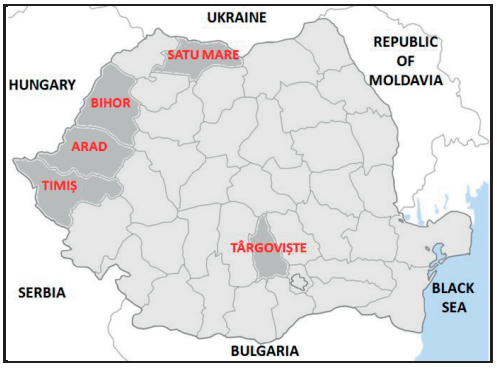

Figure 5. Romanian food-borne outbreaks between 2010-2015 - geographical distribution

Table 1. BoNT types and concentration in samples from each botulism outbreak

	Timis outbreak		Targoviste outbreak		Satu Mare outbreak		Arad outbreak		Bihor outbreak	
	BoNT A and B		BoNT B		BoNT B		BoNT B		BoNT B	
Dilution (dilution factor 10)	positive	negative	positive	negative	positive	negative	positive	negative	positive	negative
10^{0}	5	0	5	0	5	0	5	0	5	0
10^{-1}	5	0	4	1	3	2	4	1	5	0
10^{-2}	4	1	0	5	1	4	0	5	4	1
10^{-3}	0	5	0	5	0	5	0	5	1	4
10^{-4}	0	5	0	5	0	5	0	5	0	5
Calculation step:										
- $\log_{10}$ of the reciprocal of the lowest dilution at which all the animals were positive	1		0		0		0		1	
- the total number of positive animals at all dilutions	14		9		9		9		15	
- $\log_{10}$ of the reciprocal of the end-point dilution	2.3		1.83		1.1		1.3		2.5	
- end point dilution	$10^{-2.3}$		$10^{-1.83}$		$10^{-1.1}$		$10^{-1.3}$		$10^{-2.5}$	
- MLD_{50}/0.5ml	199.5		67.6		12.6		19.9		316.2	
- MLD_{50}/20g sample[1]	13967		4733		881		1397		22136	
- Oral human toxic dose/20g sample[2]	~ 6		~ 2		~ 0.4		~ 0.6		~ 9.5	

[1]The mouse lethal dose is initial calculated in 0.5 ml which is the quantity inoculated intraperitoneal in mouse. One mouse lethal dose is multiplied by 70 because ~35ml supernatant results from the extraction of the 20g of sample). [2]Oral human toxic dose is the total amount of MLD_{50}/20g sample divided by 2333 which is the number resulted from the difference between one oral human toxic dose – 70 ng and a MLD_{50} – 0.03 ng.

CONCLUSIONS

Detection and typing of *BoNT* using the mouse bioassay is still the reference method for food samples analysis (Skarin et al., 2013) that confirms in the same time the presence and the biological functionality of BoNTs in contaminated food samples. An ISO describing a molecular method for *Clostridium botulinum* detection from food has appeared in September 2013 but the toxin can't be detected in this way.

Different studies (Negut and Rafila, 2009; Neghina et al., 2010; Ivana et al., 2009) performed on BoNT detection have showed that mainly BoNT serotype in Romanian's traditional food were B and E, but to the best of our knowledge, for the first time, we detected a mix of BoNT A and B in the same sample. Because the bacteria isolation from this sample couldn't be achieved, we do not know if there was a mix of two bacteria type A and B or a single bacterial cell AB that produced both neurotoxin types.

Being linked to soil and water, spores persistence is a regional problem. In Romania, the poison excreted by this bacteria in food is occurring when a traditional preparation is made in the backyards in rural areas during smoking process or preservation of meat. Until now the most poisonous areas in Romania remains the West, North-West and South regions, close related to the traditional conservation of the pork and fish meat.

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