LABORATORY RESULTS ASSOCIATED WITH AUTOCHTHONOUS CASES OF BOTULISM (2003 - 2017)

Daniela Lemeni[1a], Ioana Macovei[1a]*, Mircea-Ioan Popa[1,2]

[1]Cantacuzino National Medico-Military Institute for Research and Development, Bucharest, Romania

[a]The Laboratory of Anaerobic and Zoonotic Infections

[2]Carol Davila University of Medicine and Pharmacy, Bucharest, România

ABSTRACT

Botulism occurs by ingestion of canned food contaminated with *Clostridium botulinum*. In most cases, the incriminated canned foods are homemade; commercial products only occasionally produce the disease, due to improper handling and storage after purchase. In Romania, the most common sources of food botulism are homemade pork products (ham, bacon, salami, meat and sausages preserved in lard). The disease occurs in small outbreaks. Out of several serotypes of *C. botulinum*, the predominant serotype in our country is type B. The Laboratory of Anaerobic and Zoonotic Infections in Cantacuzino Institute performs *in vivo* reference laboratory diagnosis of botulinum food poisoning, using the mouse lethality assay for detection of the toxin in the sample and for toxin typing. Between 2003 and 2017, our laboratory tested 576 biological samples for the diagnosis of botulism: patient blood serum samples (80%), food samples (19.02%) and autopsy fragments from deceased patients (0.8%). Type B botulinum toxin was detected in 50% of the total number of blood serum samples received for testing and a single serum was positive for type E toxin. 5% of the tested food samples were positive for type B toxin. Until 2007, the geographic origin of positive samples that we received for testing showed high incidence of food botulism in the center and west of the country. Starting 2007, we received more samples from several eastern counties, many of them being confirmed as positive. Until mid 2009, we frequently received insufficient amounts of patient serum, and we could not determine the toxin serotype.

Keywords: botulism, serotype, botulinum toxin, lethality test on mice, Romania

REZUMAT

Botulismul survine prin ingestia conservelor contaminate cu spori de *Clostridium botulinum* rămași viabili din cauza sterilizării insuficiente. Frecvent, conservele incriminate sunt preparate în casă; produsele comerciale produc ocazional boala, datorită manipulării și depozitării necorespunzătoare după cumpărare. În România, cele mai frecvente surse de botulism alimentar sunt produsele din carne de porc (șuncă, salam, carne și cârnați conservate în untură). Boala apare în focare mici: 1-3 pacienți, reprezentând numărul obișnuit de persoane care consumă aceleași conserve. Predominant în România este serotipul *C. botulinum* B. Laboratorul de Infecții Anaerobe și Zoonotice din Institutul Cantacuzino efectuează diagnosticul de laborator de referință *in vivo* al toxiinfecției alimentare botulinice, determinând prezența toxinei botulinice în produsul patologic și tipul de toxină prin utilizarea testului letalității la șoareci. Între 2003 și 2017, laboratorul nostru a testat 576 de probe biologice pentru diagnosticul botulismului: sânge (80%), alimente (19,02%) și fragmente necroptice de la pacienți decedați (0,8%). Toxina botulinică de tip B a fost detectată la aproximativ 50% din numărul total de probe de ser primite pentru testare; un singur ser a fost pozitiv pentru toxina de tip E. Dintre probele alimentare testate, 5% au fost pozitive pentru tipul B. Până în 2007, originea geografică a probelor pozitive primite pentru testare a arătat o incidență ridicată a botulismului alimentar în centrul și vestul țării. Din 2007, am primit probe și din județele din estul țării, multe fiind confirmate ca pozitive. Până la jumătatea anului 2009, am primit frecvent cantități insuficiente de ser, fără a putea determina serotipul toxinei.

Cuvinte-cheie: botulism, serotip, toxina botulinică, testul letalității la șoareci, România.

**Corresponding author:* Ioana Macovei, Cantacuzino National Medico-Military Institute for Research and Development, Bucharest, Romania, email: ioanas.macovei@gmail.com

INTRODUCTION

Botulism is a serious, non-contagious disease caused by a proteic neurotoxin, the most active of the biological toxins produced by *Clostridium botulinum* [1].

The disease occurs after the ingestion of preserved food in which there have been anaerobic conditions favorable for the germination of *C. botulinum* spores remaining viable as a result of insufficient sterilization. In most cases, household canned foods are incriminated; industrially prepared products occasionally produce outbreaks, generally due to inappropriate handling after purchase. The nature of the cans varies by country: in the US, the maximum incidence occurs in canned fruits and vegetables; in Russia, it is mostly attributed to fish cans; in Poland – in those of pork; in Romania – pork meat preparations (ham, bacon, salami, meat and sausages in pork lard) [2-4]. The outbreaks comprise one to three people, representing the usual number of those who consume the same can. In this form of botulism, mortality is 5-10% [5, 6].

There are seven types of *C. botulinum*, designated from A to G, differentiated by the antigenic structure of neurotoxins, although all cause the same clinical illness, botulism [5]. Toxins are strictly type specific, being neutralized only by the corresponding antitoxins. Types A, B and E cause human botulism and the other 4 types produce animal botulism. Based on our records, we can speculate that in our country, only type B was isolated, with two exceptions: one type A in the 1980s and one type E in 2007 (unpublished personal data: registers of samples received in the Anaerobic Laboratory for the period 2003-2017).

Besides the food poisoning form, wound botulism was first reported in 1943 [7]. The disease occurs by cutaneous infection, when traumatic injuries are contaminated with soil (produced by firearms, traffic accidents, fall from height), but also to intravenous drug users. Located in the wounded tissue, spores germinate, producing bacilli that multiply and express the toxin, which is then released into the systemic circulation [8]. Mortality is 5% [6]. Between 1976 and 1978, a third variant was described - infantile botulism - due to spore germination and toxin production in the infant's intestine (under 24 months), fed with honey, vegetables or uncooked meat. Mortality, in the case of infantile botulism, is less than 1% [6].

C. botulinum, a Gram positive, long, straight or curved, mobile bacillus containing a subterminal spore, strictly anaerobic, was isolated in 1896 by van Ermengem, during a food intoxication with sausage (*botulus* - sausage in Latin) [9].

Botulinum toxin is the most potent biological toxin [10]. It was the first bacterial toxin isolated in absolute purity in crystalline form by Lamanna *et al.* in 1946 [11]. The lethal dose of type A toxin for humans is ≤ 0.001 μg/kg [12]. Botulinum toxin is thermo labile: it can be destroyed in 30 minutes at 80°C and in 10 minutes at 100°C [13]. The spores are destroyed by autoclaving for 30 minutes at 120°C [14]. It is the only bacterial exotoxin that resists the action of gastric acid [15]. When introduced in the intestine along with the food bowl, it is resorbed and distributed through the circulatory system, where it is found for a long time even after a certain amount is attached to the nerve endings. Poorly sterilized cans, held at ambient temperature, provide good growth conditions for *C. botulinum*. As a result of gas accumulation, these cans may bulge or swell due to butyric acid accumulation, a phenomenon that can also be induced by the growth of other bacterial species (e.g. *E. coli*) [16]. The disease incubation period ranges from a few hours to eight days, averaging 18-36 hours [17]. The shorter the incubation, the more critical is the poisoning. In cases with less than 24 hours of incubation, chances of survival are very low [18]. Short incubation is the consequence of either introducing a higher amount of toxin into the body (possibly by ingestion of a larger amount of food), or by the presence of a more toxic *C. botulinum* strain [17].

There is no natural immunity against botulism, so receptivity is general. Active immunization is necessary for workers who manipulate botulinum cultures and toxins [19]. Passive immunization is mandatory in the treatment of declared botulism. The botulinum antitoxin only neutralizes free molecules

of toxin (unattached to the central nervous system), which is why it is necessary to be administered as early as possible [20].

Laboratory diagnosis

The clinical samples collected from patients with suspicion of botulism are: serum, incriminated food, faeces, gastric and intestinal contents, wound swabs and necropsy fragments [7]. Three milliliters of serum are required for diagnosis in order to detect the presence of toxin and to perform the type determination. Botulinum toxin production is evaluated by the mouse lethality assay [21]. This assay consists of intraperitoneal inoculation of mice with the clinical sample, followed by 4 days of daily observation. Death occurring during this time indicates the presence of botulinum toxin [22]. Determination of the toxin type is done by an *in vivo* neutralization bioassay. The clinical sample investigated is incubated with A, B, or E antitoxin and then inoculated intraperitoneally in mice; the animals are observed for 4 days and only those injected with the specific antitoxin survive [8]. Epidemiological diagnosis of a limited outbreak involving people who have ingested the same contaminated food is made by detecting the toxin in both patient's serum and the food consumed [23].

In case of an outbreak, intervention measures comprise taking samples from both the patient and the possibly contaminated food that was consumed so as to confirm the presence of the toxin and to prevent further consumption [24].

In Romania, patients with suspected clinical botulism are hospitalized in an infectious disease unit and the antitoxin is administered for neutralization of the circulating toxin [24]. To determine the presence of botulinic toxin and to identify the toxin type, blood is collected from the patient prior to antitoxin administration. Samples are sent to Cantacuzino Institute, at the Laboratory of Anaerobic Infections.

MATERIALS AND METHODS

SAMPLES: Patients' serum/incriminated food/necropsy fragments

Serum samples: Blood is collected at the onset of the disease to detect the free botulinum toxin; 2.5 ml serum/patient are sent to the Anaerobic Laboratory in Cantacuzino Institute as soon as possible, in hermetically sealed and appropriately labeled vials.

Blood samples collected long after the appearance of clinical signs have a risk of providing false negative results as the botulinum toxin will attach to the nerve endings.

Incriminated food samples: A few grams of the incriminated food must be collected in a sterile, tightly closed container. As the botulinum toxin does not diffuse, portions with modified aspect are of particular interest.

During the study period, the food received for testing was represented by home-canned meat, smoked pork, fried pork, lard, marinated fish stored in the house, home-made sausages, sausage lard, home-marinated mackerel and home-canned vegetable stew.

Method: The determination of botulinum toxin presence in serum/food or necroptic samples was performed by using the **mouse lethality assay.** Animals were inoculated in the peritoneum with serum or food extracts and followed for 4 days. Animal death occurring within this period confirms the presence of botulinum toxin. The toxin type was determined by ***in vivo*** **neutralization bioassay.** As described before, the clinical sample investigated was incubated with the A, B, or E antitoxin and inoculated intraperitoneally in mice afterwards. After 4 days from inoculation, only the mice injected with the specific antitoxin survived.

RESULTS

Between 2003 and 2017, 682 samples, including 529 sera, 149 food samples and 4 necroptic fragments were received by our laboratory for confirmation of food botulism. Sera represented 77.5% of all tested products, food samples represented 21.8%, and necroptic fragments 0.7% (Table 1).

The number of samples ranged between 17 (2004) and 108 (2007) (Fig. 1).

Over half of the samples (50% being sera) tested positive (Table 2). Type B botulinum toxin was detected in all positive cases, with the exception of a type E sample isolated from a

patient who consumed a fish can from Thailand in 2007. The circulating toxin could be detected if the sample was collected in maximum 14-20 days from the onset.

Tested food samples were home-made canned foods such as larded sausages, smoked pork, fried meat, marinated fish, home-canned vegetable stew, as well as commercial canned food: most commonly liver-paté, but also canned fish (mackerel, tuna, sprat salad, marinated fish), honey, melted cheese or parmesan. Type B botulinum toxin accounted for 5% of the tested food samples. The presence of botulinum toxin in food was confirmed when the food container that the patients consumed was tested. Necroptic fragments were received only once, in 2009, and were negative. The tested samples and the confirmed cases with their distribution per year between 2003 and 2017 are shown in Figs. 2 and 3.

Table 1. Samples received for testing

Type of samples	Total requests	Positive (%)
Serum	**529**	**279 (77.5)**
Food	**149**	**6 (21.8)**
Necroptic fragments	**4**	**0 (0.7)**
Total (n)	**682**	**285**

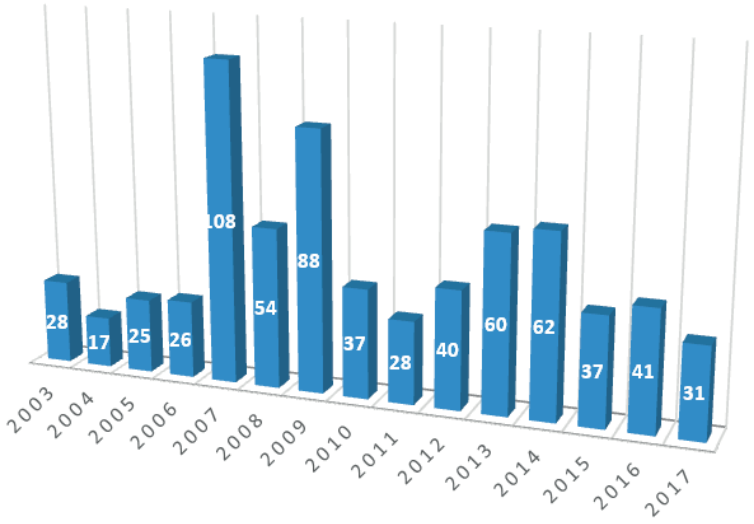

Fig. 1. The number of tests performed between 2003 and 2017

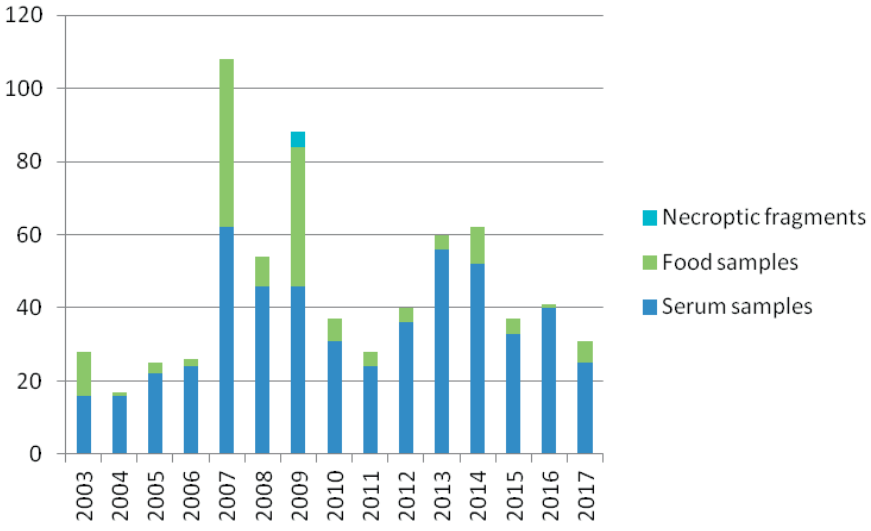

Fig. 2. The distribution of samples tested between 2003 and 2017

Table 2. A summary of the number of samples received by the Laboratory of Anaerobic Infections between 2003 and 2017

Year	Total samples received (n)	Serum samples				Food samples			Necroptic fragments
		Submitted	Positive	Samples with typed toxin	Insufficient quantity for typing	Submitted	Positive	Toxin type	
2003	28	16	7	1	6	12	0	-	0
2004	17	16	8	2	6	1	0	-	0
2005	25	22	12	5	7	3	0	-	0
2006	26	24	9	7	2	2	0	-	0
2007	108	62	34	23 (22 type B and 1 type E)	11	46	1	B	0
2008	54	46	26	18	8	8	1	B	0
2009	88	46	31	30	1	38	1	B	4 (negative)
2010	37	31	20	17	3	6	0	-	0
2011	28	24	16	15	0	4	1	B	0
2012	40	36	16	16	0	4	2	B	0
2013	60	56	23	23	0	4	0	-	0
2014	62	52	31	31	0	10	0	-	0
2015	37	33	16	16	0	4	0	-	0
2016	41	40	16	16	0	1	0	-	0
2017	31	25	13	13	0	6	0	-	0

Over a year period, the frequency of requests and the geographical distribution of cases were relatively homogeneous. Most cases of botulism confirmed by our laboratory came from the following counties: Bucharest (NIID Matei Balș, 91), Bihor (62), Iași (62), Harghita (32), Arad (29), Timiș (29), Satu-Mare (21), Mureș (19), Galați (17) and Suceava (10) (Fig. 5). Before 2007, the counties with the highest incidence were those in the central and western parts of the country, while in the years after, the incidence started to rise in many eastern counties as well.

A very large age spectrum was noticed among the patients, ranging from 3 to 79 years old as outbreaks were confirmed within families, comprising 2-3 cases of people who consumed the same food (usually parent-child, husband-wife, brothers or friends). As the toxin does not diffuse in food, not all people consuming the same product became ill.

DISCUSSION

This article presents a review of suspected cases of botulism in Romania based on the samples received by the Laboratory of Anaerobic Infections in Cantacuzino Institute during a 15-year period (2003-2017). As the incidence of botulism has dropped dramatically in the last decades, fewer reports concern the epidemiology of this disease worldwide. A study which compared botulism incidence in Romania before and after the Romanian Revolution in December 1989 showed an increase in reported

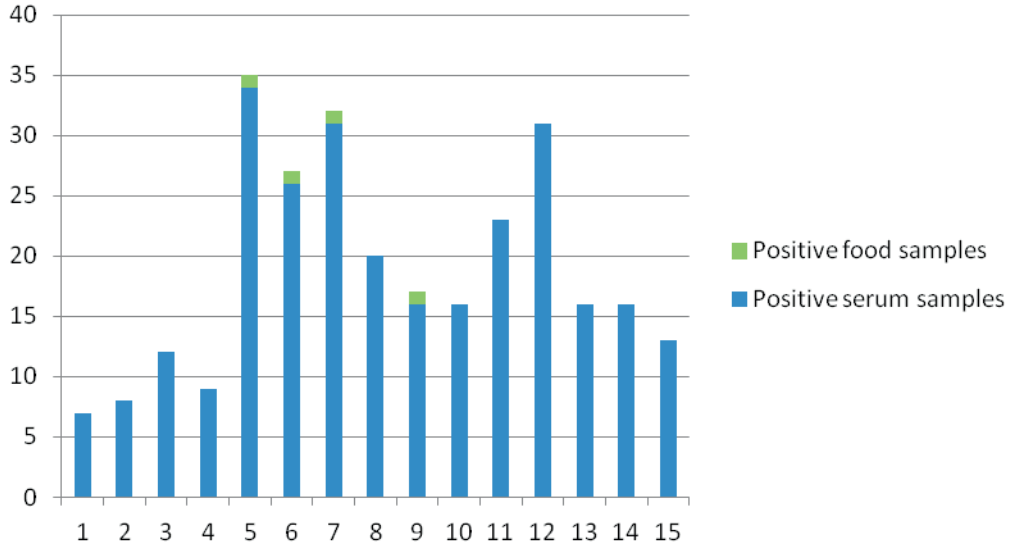

Fig. 3. The distribution of confirmed cases between 2003 and 2017

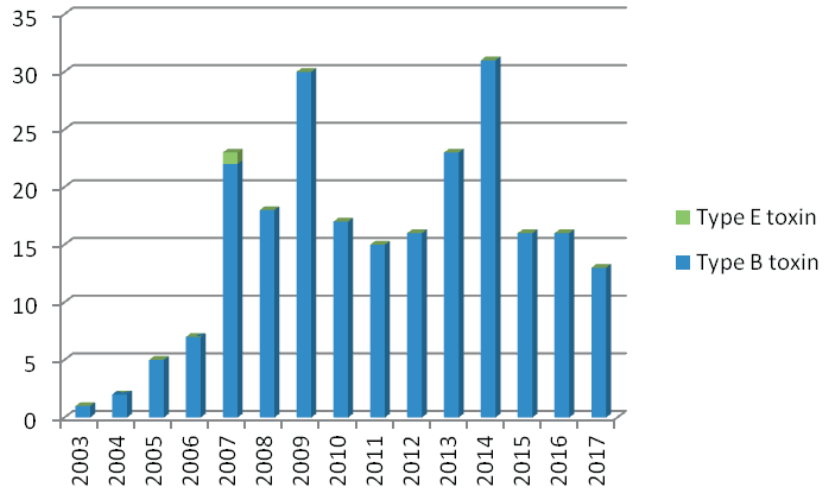

Fig. 4. Botulinum toxin types identified by Cantacuzino Institute from confirmed cases (2003-2017)

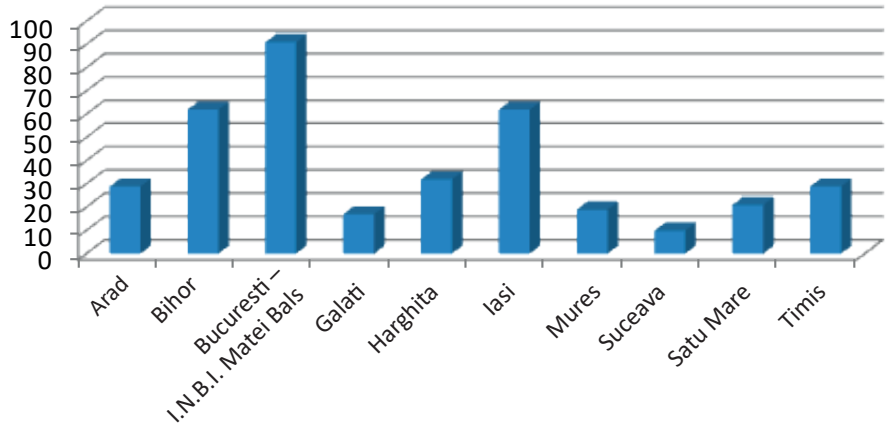

Fig. 5. The geographical distribution of botulism cases

cases after 1990. The main reasons stated for this situation were poverty and food shortage, lack of reliable energy sources and lack of strict control practices within canning factories [4].

The rise in cases that we observed in the eastern part of the country after 2007 might be explained by the growing consumption of home-canned food or perhaps by a change in older and perhaps safer conservation techniques. This might also be caused by a deficit of reports before 2007, especially given the fact that patients do not seek medical attention at the first symptoms and might die before they are diagnosed and treated.

The study has several limitations. The most important limit is the fact that there is no national overview regarding the samples that are sent to Cantacuzino N.M.M.I.R.D. Thus, because we cannot be certain that all samples have been submitted to the Reference Center, the real number might be higher. There is also a high heterogeneity regarding the number of samples sent from different municipalities (highest number in Bucharest, lowest in Suceava). Another limit is the lack of clinical data for each case and the fact that the source was not always identified.

The lack of access to this type of data in most cases limits us in obtaining a solid correlation in our study or in performing a wider analysis including cases of wound or infant botulism occurring in our country. However, additional information that we sometimes received confirms previous observations that the symptomatology is directly proportional to the amount of ingested toxin [25].

The low number of similar recent published studies in our country also limits the possibility to compare our results, thus making the analysis difficult.

A centralized national database with multidisciplinary access (including clinicians, epidemiologists, microbiologists and food surveillance authorities) would be helpful in control and prevention as well as in maintaining a realistic image regarding its epidemiology.

The impact of the socioeconomic status on food consumption practices is highly important. As Romania continues to have high rates of poverty, especially in rural areas where many people are retired or unemployed, it is doubtful that the classical preservation methods will be replaced. Besides the lack of means, some of the techniques are part of tradition such as pork slaughtering for Christmas and will, therefore, be hard to change.

CONCLUSIONS

Food botulism remains a serious public health problem considering its outbreak potential although the epidemiology in the last fifteen years has not changed dramatically.

For a continuous surveillance of this infection throughout the country, all suspected patients should have samples collected and sent to the Laboratory of Anaerobic Infections in Cantacuzino Institute. High incidence regions must be closely followed. As the majority of cases are associated with pork-based foods, education regarding safe conservation and consumption practices is very important.

The good collaboration between clinicians, public health authorities and the National Reference Center located in Cantacuzino Institute is of critical importance.

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