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Animal botulism in Poland – laboratory and epidemiological investigations

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Abstract

Introduction: The aim of the study was to present cases of botulism in animals found in Poland in 2019–2021. The analytical laboratory diagnosis and difficulties that occurred in the interpretation of the results are described. **Material and Methods:** From 2019 to 2021, samples of serum, intestinal content, liver, spleen, kidney, faeces, wet feed, dry feed, ensilage, water and mixed samples of internal organs associated with 10 suspected animal botulism cases were sent to the National Veterinary Research Institute. Samples were analysed using a mouse bioassay and culture methods in combination with *ntnh* and *bont* gene detection. **Results:** Among the ten putative botulism cases, only four (40%) were confirmed in the laboratory on the basis of the detection of botulinum toxin (BoNT) or the *ntnh* or *bont* genes. The remaining six (60%) were determined as probable despite observable characteristic clinical signs. **Conclusion:** The diagnosis of botulism in animals is a very difficult task, made so by the heterogeneity of *Clostridium botulinum* strains and possible loss of toxinogenicity during laboratory processing or the potential degradation of toxins. Laboratory diagnosis is a complex and problematic process which should utilise different prescribed methods for specific types of sample.

Keywords: botulism in animals, Poland, *Clostridium botulinum*, BoNT, *ntnh*, *bont*.

Introduction

Clostridium botulinum is a species of bacteria of which the strains comprise a heterogenic group of microorganisms known to produce the most potent toxins in the environment (botulinum neurotoxins – BoNTs). These toxins are the aetiological agents of botulism – a highly fatal paralytic disease of humans and animals (1, 12, 18). There are eight recognised BoNT types, designated by the letters A–G (3, 4, 5) and X (34). Approximately 40 subtypes of these toxins are also known (34). The production of BoNTs is not only attributed to the *C. botulinum* species; however, some other strains of *Clostridia* may also show this toxigenicity, *e.g. C. sporogenes*, *C. baratii* and *C. butyricum* (7, 15, 20).

Different metabolic and physiological properties and 16S rRNA gene sequences within *C. botulinum* species are the basis of strain classification into one of the four metabolic groups. To *C. botulinum* group I type A and proteolytic *C. botulinum* type B and F strains are classified. Group II comprises non-proteolytic *C. botulinum* E, B and F strains. The next group consists of *C. botulinum* C, D, CD and DC strains, and to group IV belong strains of *C. botulinum* type G. In addition to *C. botulinum*, unusual strains of related species have been described that were able to produce BoNTs, and the toxins produced by them were the causative agents of botulism cases. These include strains of *C. baratii*, *C. butyricum* and *C. sporogenes* (2, 7, 12, 26). Botulism in animals is mainly caused by toxin types C and D and their mosaic variants (BoNT/CD and DC); however, toxin types A, B and E are also observed as causative agents.

Botulism occurs in wild and domestic animals (7, 12, 19). Among those reared on farms, cattle, poultry and fur animals are the most frequent botulism cases. Botulism symptoms in animals usually develop with symmetrical and progressive flaccid paralysis, observed at the beginning in the hindquarters as muscle tremors, stumbling and weakness spreading to the head and neck. At the end stage of symptom progression,

affected animals assume a lying position. Recumbent animals are usually unable to rise or turn. Death is caused by paralysis of the diaphragm and respiratory muscles. The incubation time of the disease depends on the amount of ingested toxin and may be a few hours or extend to several days (7, 16, 18, 24). Botulism cases are often outbreaks which are difficult to manage. Sick animals are able to spread *C. botulinum* spores in excrement and transmit disease indirectly to healthy animals by contamination of feed and water. Most often, symptoms of the disease appear after intoxication caused by ingestion of BoNTs. Very rarely do symptoms occur as a result of intestinal toxicoinfection caused by the development of *C. botulinum* spores in the lumen of the large intestine and the production of the toxin *in situ*. The symptoms of botulism may also occur as a result of toxicoinfection caused by *C. botulinum* spores in wounds (1, 12).

The aim of the study was to present the laboratory diagnosis of suspected cases of botulism presenting with clinical signs found in animals in Poland in 2019–2021. These cases were investigated and verified using analytical laboratory tools based on culturing, PCR and mouse bioassay (MBA). Difficulties in interpretation of the results are also described.

Material and Methods

Clinical interpretations. Samples were accepted for laboratory diagnosis after collecting data in the form of a questionnaire containing a description of symptoms indicative of botulinum intoxication and the number of affected animals. The following symptoms had been observed by clinicians: difficulties in swallowing, reduced muscle tone, descending, flaccid, symmetrical quadriparesis, breathing difficulties, salivation, constipation, ptosis, dryness of the mucous membranes of the mouth and throat, urination disorder, dilation of the pupil (mydriasis) and diarrhoea. The botulism symptoms were noticed in five cattle, three mink, a chicken and a horse. Depending on the number of affected animals, some cases were incidental and some were part of significant outbreaks (Table 1). The majority of cases were observed in provinces located in eastern Poland, where 6 cases were noted (Podlaskie, Masovia, Warmia-Masuria and Lublin), three others having occurred in the central-northern Pomerania province and the tenth case having originated from Greater Poland, a province of western Poland (Table 1, Fig. 1).

Material. From 2019 to 2021, samples of serum, intestinal content, liver, spleen, kidney, faeces, wet feed, dry feed, ensilage, water and mixed samples of internal organs associated with 10 animal botulism cases were sent to the National Veterinary Research Institute in Poland. In total, 37 samples were analysed. All samples are described in Table 2.

Mouse bioassay. A MBA was performed for serum, liver, spleen, water and kidney samples. The single experiment was carried out following the US Food and Drug Administration (FDA) protocol (27) and Polish Standard PN-R-64791:1994 (21). Mice were injected intraperitoneally with 0.2 mL of serum or a suspension of the other types of sample in phosphate buffer (1:1 ratio). Positive results were confirmed by a seroneutralisation test with antitoxins.

Table 1. Suspected animal botulism cases in Poland of which samples were sent to the National Veterinary Research Institute laboratory from 2019 to 2021

Case	Province	Animal	Approximate number of affected animals	Type of feed	Year	Month	Average temp. (°C)
1	Pomerania	Cattle	6	Silage, TMR	2019	February	2.8°C
2	Masovia	Mink	-	Wet feed	2019	June	21.8°C
3	Podlaskie	Cattle	45	Pasture, silage, TMR	2019	June	21.8°C
4	Lublin	Mink	2,000	Wet feed	2019	August	20.3°C
5	Pomerania	Cattle	-	Silage	2019	November	5.7°C
6	Greater Poland	Mink	2,500	Wet feed	2020	July	18.7°C
7	Podlaskie	Cattle	2	Silage, TMR	2020	September	15.3°C
8	Pomerania	Horse	1	Silage, granulated feed	2020	October	10.4°C
9	Warmia-Masuria	Cattle	8	silage, TMR	2021	November	5.1 °C
10	Masovia	Chicken	18,000	Dry feed	2021	November	5.1 °C

TMR – total mixed ration
Temperatures according to the Institute of Meteorology and Water Management (IMGW) (9)

Table 2. Samples associated with suspected animal botulism cases in Poland sent to the National Veterinary Research Institute laboratory from 2019 to 2021

Case	Samples										
	Serum	Intestinal content	Liver	Spleen	Kidney	Faeces	Wet feed	Dry feed	Ensilage	Water	Mixed samples of internal organs
1	+	−	−	−	−	−	−	−	−	−	−
2	−	+	+	−	−	−	+	−	−	−	+
3	+	+	+	−	−	+	−	−	+	−	−
4	+	+	+	−	−	−	+	−	−	−	−
5	+	−	−	−	−	−	−	−	+	−	−
6	+	−	+	−	−	−	+	−	−	−	+
7	−	+	+	+	+	−	−	+	−	−	−
8	+	+	+	−	−	−	−	−	−	+	−
9	+	−	−	−	−	+	−	−	+	−	−
10	+	+	+	−	−	+	−	−	−	+	−
Total	37										

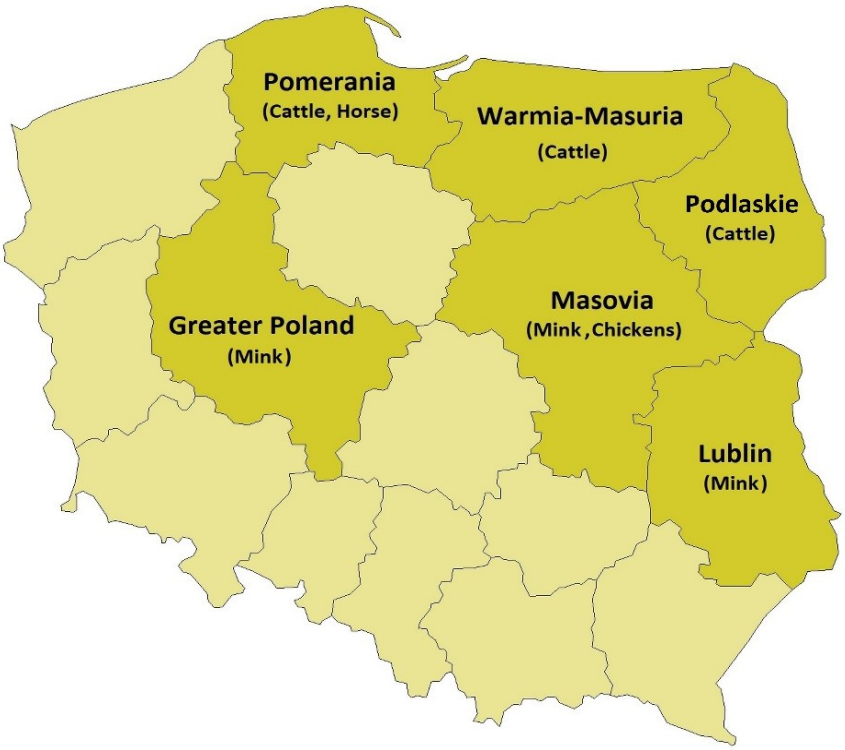

Fig. 1. Polish provinces with suspected botulism cases of which samples were sent to the National Veterinary Research Institute laboratory from 2019 to 2021. Darker regions denote provinces with cases

Culture methods. The samples of intestinal content, liver, spleen, faeces, wet feed, dry feed, ensilage, litter, water and mixed samples of intestinal content were subjected to culturing. Each sample was inoculated into a test tube or bottle with from 10 to 90 mL (depending on the inoculated weight of the sample, which was maximally 10g) of Tryptone Peptone Glucose Yeast Extract Broth (TPGY) constituted by 50 g/L casein enzymic hydrolysate, 5 g/L peptic digest of animal tissue, 20 g/L yeast extract, 4 g/L dextrose, and 1 g/L sodium thioglycolate, with a final pH of 7.0 ± 0.2 at 25°C. Subsequently, the inoculum was pasteurised at 70°C for 15 min in a water bath. The pasteurised inoculum was incubated at 37°C

for 48 h. After incubation, the turbidity of the TPGY was assessed, and 1 mL of liquid culture was inoculated into the tubes with 10 mL of fresh TPGY broth and incubated for an additional 24 h.

After incubation in liquid media, 10 µL of culture was spread on Willis–Hobbs agar containing 10 g/L peptic digest of animal tissue, 10 g/L meat extract, 5 g/L sodium chloride, 12 g/L lactose, 0.032 g/L neutral red, 10 g/L skim milk powder, 2 g egg yolk powder and 10 g/L agar, with a final pH of 7.0 ± 0.2 at 25°C. The same aliquot of culture was also spread on fastidious anaerobe agar specified as 23 g/L peptone, 5 g/L sodium chloride, 1 g/L soluble starch, 0.4 g/L sodium bicarbonate, 1 g/L glucose, 1 g/L sodium pyruvate, 0.5 g/L L-cysteine HCl H_2O, 0.25 g/L sodium pyrophosphate, 1 g/L L-arginine, 0.5 g/L sodium succinate, 0.01 g/L haemin, 0.001 g/L vitamin K, 2 g/L egg yolk powder and 12 g/L agar, with a final pH of 7.2 ± 0.2 at 25°C. The plates with agar media were incubated anaerobically at 37°C for 48 h. The colonies were evaluated for their morphology and biochemistry: colour, surface, shape, size, and lipolytic, proteolytic or lecitinolytic features. The isolated strains suspected of belonging to *Clostridium* spp. were additionally Gram stained.

DNA isolation. DNA was isolated from 1 mL of liquid culture and from a small number of colonies grown on the surfaces of agar plates. Extraction of DNA was performed with a Genomic Mini AX Bacteria kit (A&A Biotechnology, Gdynia, Poland) according to the manufacturer's procedure. The DNA amount used for the PCR varied between 1 and 25 ng. The amount of DNA was estimated with a Nicolet Evolution 300 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The extracted DNA was frozen at −20°C or directly subjected to PCR analysis.

Molecular detection of BoNT-producing *Clostridia*. Analyses were performed using methods for *ntnh* and *bont/A–F* gene detection. The *ntnh* gene is common in all BoNT-producing *Clostridia* toxin types and is an element of the botulinum cluster determining the production of the non-toxic non-haemagglutinin (NTNH) component in the botulinum protoxin complex. The *bont* genes are specific to particular toxin types and responsible for production of BoNTs; therefore, they are active components of the botulinum neurotoxin.

The *ntnh* gene was detected using a set of seven primers and the TaqMan probe according to Raphael and Anreadis (22) and with previously described concentrations of reagents (7). In a few cases, the DNA positive for the *ntnh* gene was investigated for the *bont*/*A–F* genes with the method described by Kirchner *et al.* (11) and the reagent concentrations described by Grenda *et al.* (7). All reactions were carried out using a LightCycler 2.0 instrument (Roche, Basel, Switzerland).

Results

The botulism cases in the 2019–2021 period of this investigation were diagnosed as such on veterinarians' suspicions upon observing clinical signs and on the results of testing the samples sent to the National Veterinary Research Institute. The most frequent botulism symptoms were observed in cattle and totalled five cases. Symptoms also presented in three cases in mink and also in one incidental case in a horse and one outbreak in broiler chickens. Most frequently, a reduced muscle tone was observed, this having been in seven cases. Other frequent symptoms included breathing difficulties, observed in six cases; descending flaccid, symmetrical quadriparesis and salivation and constipation, noted in four cases; difficulties in swallowing, dryness of the mucous membranes of the mouth and throat and ptosis, affecting animals in two cases; and urination disorder, mydriasis, and diarrhoea, recognised in one case (Table 3).

Half of these cases were observed in the summer period (between June and September) when the mean temperature was above 15°C. Only one case was noted in October when the mean temperature was above 10°C, three cases were observed in November and coincided with a mean temperature of above 5°C, and one case came to light in the winter period, in February, in mean temperature conditions of around 3°C (Fig. 2).

Only 4/10 botulism cases (40%) were confirmed in the laboratory on the basis of the detection of botulinum toxin, *ntnh* or *bont* gene in the samples. The remaining cases – 6/10 (60%) – were determined to be probable, even despite the characteristic symptoms observed clinically.

One of the methods of arriving at a laboratory diagnosis was by examination of serum samples collected from sick animals with characteristic botulism symptoms, which was feasible for 4/10 (40%) examined cases. Another fruitful method in 3/10 (30%) cases was the detection of BoNT, *ntnh* and *bont* genes in the samples of liver. Feed was found positive once and in this case *ntnh* and *bont* were detected. Determination of the toxin type using the MBA and *bont* real-time PCR indicated that the considered cases were caused by toxin types C (three cases) and D (one case). Also, in the case of chicken botulism, *C. botulinum* spores of type C were detected in water retained in the drinkers. The feed and water referred to were considered a potential source of the *C. botulinum* spores and toxin triggering the botulism symptoms (Tables 4 and 5).

The isolation of *C. botulinum* strains was possible only in three cases and these isolates were classified to toxin type C using the real-time PCR for *ntnh* and *bont* gene detection.

Table 3. Symptoms manifested during particular botulism cases

Symptom	Case									
	1	2	3	4	5	6	7	8	9	10
	Animal									
	Cattle	Mink	Cattle	Mink	Cattle	Mink	Cattle	Horse	Cattle	Chicken
Difficulties in swallowing	+	–	–	+	–	–	–	–	–	–
Reduced muscle tone	+	+	+	+	+	–	–	–	+	+
Descending, flaccid,	+	–	–	+	–	+	–	+	–	–
Breathing difficulties	–	+	–	+	–	–	+	+	+	+
Salivation	–	–	–	+	–	+	+	–	+	–
Constipation	–	–	+	+	–	–	+	+	–	–
Ptosis	–	–	–	+	–	–	–	–	–	+
Dryness of the mucous	–	–	–	+	–	–	–	+	–	–
Urination disorder	–	–	–	+	–	–	–	–	–	–
Dilation of the pupils	–	–	–	–	–	–	–	–	+	–
Other symptoms	–	–	–	–	–	+ diarrhoea	–	–	–	–

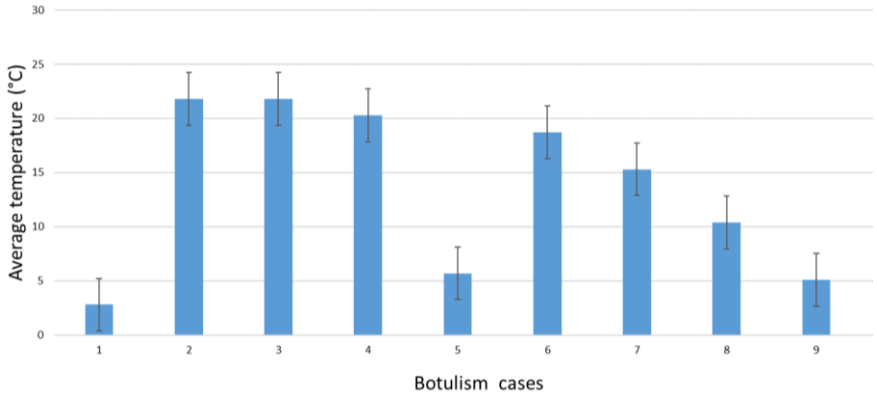

Fig. 2. The average temperatures in the periods and areas affected by botulism outbreaks according to the Institute of Meteorology and Water Management (9)

Table 4. Results of the tests used in laboratory examination and classification of the cases

Case	Test result of samples examination			Classification of case
	MBA	PCR (*ntnh* gene detection)	PCR (*bont* gene detection)	
1	–	–	–	Probable
2	–	–	–	Probable
3	+	+	+	Confirmed in laboratory
4	+	+	+	Confirmed in laboratory
5	+	–	–	Confirmed in laboratory
6	–	–	–	Probable
7	–	–	–	Probable
8	–	–	–	Probable
9	–	–	–	Probable
10	+	+	+	Confirmed in laboratory

Table 5. Results obtained for samples associated with suspected animal botulism cases

Case	Animal	Laboratory confirmation results											
		Serum	Intestinal content	Liver	Spleen	Kidney	Faeces	Wet feed	Dry feed	Ensilage	Water	Mixed samples of internal organs	Toxin type
1	Cattle	−	−	−	−	−	−	−	−	−	−	−	−
2	Mink	−	−	−	−	−	−	−	−	−	−	−	−
3	Cattle	+	−	+	−	−	−	−	−	−	−	−	D
4	Mink	+	−	+	−	−	−	+	−	−	−	−	C
5	Cattle	+	−	−	−	−	−	−	−	−	−	−	C
6	Mink	−	−	−	−	−	−	−	−	−	−	−	−
7	Cattle	−	−	−	−	−	−	−	−	−	−	−	−
8	Horse	−	−	−	−	−	−	−	−	−	−	−	−
9	Cattle	−	−	−	−	−	−	−	−	−	−	−	−
10	Chicken	+	−	+	−	−	−	−	−	−	+	−	C

Discussion

All the samples collected during the experiments were associated with suspected animal botulism cases. Not all cases were confirmed by laboratory results, and in fact only 40% of them were. According to Aniballi *et al.* (1), confirmation could be provided by detection of BoNTs or BoNT-producing clostridia in the serum, liver, gastrointestinal content or a contaminated wound, also in the feed and environment of affected animals. We were able to find *C. botulinum* or BoNT in serum, liver, feed and water samples. The MBA is still considered the gold standard in the laboratory diagnosis of botulism; however, a negative MBA result does not exclude botulism as the cause of the observed symptoms, because the toxin might be present at a level below the limit of detection. The low sensitivity but high specificity of the mouse bioassay for the diagnosis of botulism in horses exemplifies this. According to Johnson *et al.* (10), positive results are highly suggestive of botulism but negative results do not exclude the diagnosis. It is very important to prepare samples such as serum, faecal material and feed as soon as possible for toxin detection, because of the probability of BoNT degradation before analyses. Toxins could be present in a sample at a level below the limit of detection or may have been biodegraded by microbes in the intestinal tract of the animal (1) or by exposure to high temperatures for long enough to degrade them (8). *Clostridium botulinum* spores are not usually present in the alimentary tract of animals; however, their detection in faecal samples collected simultaneously with the observation of botulism symptoms could indicate botulism as a probable cause of disease. The high heterogeneity of BoNT-producing clostridia and the occurrence of strains phenotypically similar to the neurotoxigenic strains of *C. botulinum* but without the ability to produce botulinum toxins is the one of the problems in laboratory diagnosis of botulism (1, 7, 16). Most frequently, animal botulism is caused by group III *C. botulinum* C and D toxin types and their mosaic variants CD and DC. The toxinogenicity of this group is determined by lysogenic bacteriophages harbouring the *bont/C*, *bont/D*, *bont/CD* and *bont/DC* genes. This could be a reason for botulinum toxin production being disabled in laboratory conditions (7, 25, 33). In our study, we used molecular biology methods for specific detection of the *ntnh* and *bont* genes at the liquid culture stage which facilitate detection of botulinum cluster of genes detection before isolation (7). We were able to obtain isolates with proven occurrence of the *ntnh* and *bont/C* genes in three cases. However, we were not able to isolate *C. botulinum* type D.

The cause of the negative results of laboratory examination of the samples of 60% of the suspected cases could be associated with the circumstances described above (erroneous initial clinical diagnosis, a dose of toxin under the limit of detection, degradation of toxin, extended time from symptom onset to sample collection, or loss of specific lysogenic bacteriophages) (1, 13).

Most of the botulism cases were noted in farmed mink (30%) and cattle (50%). In fur animals, the progression of botulism symptoms can be very fast. In some cases, animals which have appeared healthy have died 2 or 3 hours later. Usually, botulism outbreaks affect hundreds or even thousands of farmed mink and they are the cause of considerable economic losses (14, 18, 32). The cases of botulism in mink considered in this study originated in significant outbreaks with 2,000 and 2,500 animals affected. Outbreaks are generally associated with toxin formation in improperly chilled slaughterhouse by-products used in fur animal feed. Mink, along with other fur animals, are susceptible to botulinum toxins, particularly type C (14). The oral lethal dose of BoNT/C for mink was determined by Tjaberg and Skulberg (30) as approximately 5×10^3 mouse lethal dose (MLD)/g. The oral toxicity of BoNT/C toxin to foxes has been reported to be higher and ranged from 10^3 to more than 10^8 MLD per animal (14).

We also confirmed in the laboratory the type C botulism outbreak in chickens. This affected

approximately 18,000 birds and was probably associated with water contaminated with spores of *C. botulinum* type C. Outbreaks in commercial poultry are rare and generally tend to recur on the same farm, often affecting the same houses or pens. Very often, it is almost impossible to find any source of similar outbreaks. There is also a hypothesis that botulism in poultry is a toxicoinfection, also called "gut toxigenesis", which is caused by *C. botulinum* spore germination in the intestine and subsequent production and absorption of the toxin. The present researchers considered this possibly to be associated with botulism in broiler chickens in acknowledgement of the previous demonstration of this mechanism by Trampel *et al.* (31). It is unknown what circumstances influence the germination of spores and subsequent proliferation in the intestine. The literature shows that healthy broilers do not harbour *C. botulinum* type C. This observation suggests that *C. botulinum* is not widespread in broiler populations. Generally, outbreaks are caused by a sporadic colonisation of flocks with *C. botulinum* introduced into a poultry house (23, 28, 31).

Cattle botulism is a rare disease with high mortality (16). Generally, the cause of botulism in cattle is ingestion of preformed toxin-contaminated feed. We appraised five suspected cases of cattle botulism, but only two cases were confirmed by us in the laboratory. These cases were proven to have been caused by *C. botulinum* type C and D. Clostridial spores are natural inhabitants of soils and could contaminate most plants used as feed material. We posit that type C and D spores may have been ingested with the feed of the cattle which gave the samples confirmed for botulism.

It is believed that wet, cool spring weather prevents rapid fermentation of silage, resulting in elevated pH, which could favour the vegetation of *C. botulinum* spores and BoNT formation. Ingestion of tissue from dead animals in hay and silage or ingestion of poultry litter or dead bird parts is also considered a source of BoNTs. Botulism in the form of intoxication is also commonly observed in phosphorus-deficient areas where toxin-contaminated bones are ingested (6, 16). Goldsztejn *et al.* (6) described the statistical significance of the influence of organic fertilisers on clostridia prevalence in silage.

Most frequently, the disease onset is after intoxication (by ingestion of feed or water contaminated by BoNTs). Less often, botulism can be caused by spores which cause the development and the production of toxin to take place in the lumen of the large intestine. Diagnosis is very difficult due to the non-specific clinical symptoms (6, 16). In most cases, a diagnosis of botulinum toxin intoxication is made after excluding other causes of muscle paralysis. According to Moeller (17), the lethal dose of BoNT/C for cattle is equivalent to 3.88 MLD/kg body weight. The course of cattle botulism depends on the amount of toxin absorbed and on its source, *i.e.* whether it came into the body as a result of intoxication or toxicoinfection. Three forms of cattle botulism are differentiated: peracute, acute and visceral (still debated and unexplored) depending on the time from exposition through subsequent symptoms occurrence to death. Generally, clinical signs often develop between 48 and 96 h after ingestion of the toxin, but may appear as early as 24 h or as late as 10 to 18 days after exposure (6, 16).

Most of the cases affected animals in eastern Poland, where animal production is the most intensive (29). Our study's botulism outbreaks took place in the summer and at average monthly temperatures between 15°C and 20°C. According to the FDA report, the minimum temperature for the growth of non-proteolytic strains and toxin formation by *C. botulinum* is 3.3°C. For proteolytic strains, this temperature is estimated at 10°C. It is difficult to evaluate the exact conditions of feed storage before animals received their feed in the cases studied; however, there is a slight possibility of toxin formation at monthly temperatures fluctuating around 0°C. However, the reduction of toxins' potency is also slower at low temperatures. According to Hubalek and Halouzka (8), the time required for a 99% (hundred-fold) reduction of toxicity for BoNT/C is more than 5 years at −70°C or −20°C, 6 months at +5°C, 3 weeks at +20°C, 2 weeks at +28°C, 2 days at +37°C, 9 h at +42°C, less than 30 min at +56°C, less than 20 min at +60°C, and less than 5 min at +80°C. These data suggest that if produced in an ecosystem in a mild climatic zone, BoNT/C toxin could persist there over the winter season and cause intoxication (8).

The diagnosis of botulism in animals is a task which is encumbered by the heterogeneity of *C. botulinum* strains and possible loss of toxinogenicity during laboratory processing. It is very important to collect the samples (serum, internal organs and suspected feed) as soon as possible after the appearance of the first symptoms or after autopsy. Laboratory diagnosis is a very complex and problematic process which should exploit different types of methods in a prescribed way for different types of samples. Reports of animal botulism cases in Poland are not officially collated; therefore, it is difficult to collect data on possible sources or the exact number of animals affected by this disease. This is the first study reporting laboratory diagnosis of animal botulism in Poland, a disease which still remains an underestimated problem in the country. A complex monitoring system and strategy are needed to ensure the microbiological safety of animal production and prevent potential economic losses.

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Animal Rights Statement: The experiments on animals were conducted after approval from the Local Ethical Committee in Lublin.

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