Artykuł przeglądowy *Review*

Botulism: Current problem in veterinary medicine

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Summary

The study presents up-to-date information on the occurrence of botulism in animals. Clostridium botulinum and toxins produced by this microorganism have been characterized. Detailed data on the structure of botulinum toxins (BoNTs) and on their mechanism of action in causing botulism symptoms have been described. The study also describes botulism symptoms in selected animal species with particular emphasis on cattle and birds. In addition, it provides general information on the laboratory diagnosis of botulism and on difficulties in detecting C. botulinum and botulinum toxins in samples related to botulism in animals.

Keywords: botulism, Clostridium botulinum, botulinum toxin, BoNT

Clostridium botulinum is considered as a species of pathogens capable of producing botulinum toxins marked by the letters from A to H. These toxins (BoNT) are believed to be the most toxic substances occurring in nature.

Generally, strains classified in the *C. botulinum* species are those that can produce one of the serologically distinct botulinum toxins (BoNTs). This definition of *C. botulinum* was first suggested by Prévot (28). However, neurotoxin production is not a stable phenotype in many *C. botulinum* strains. Other bacteria (e.g. *C. baratii* and *C. butyricum*) are also known to produce BoNT. It was found that that some *C. botulinum* strains can produce simultaneusly two types of BoNTs and that some nontoxigenic strains contain silent BoNT genes (4). Therefore, nomenclature based on BoNT production is inadequate.

Strains capable of producing botulinum toxins are characterized by high biochemical and genetic diversity. Early biochemical and physiological studies of *C. botulinum* indicated that the strains were physiologically distinct and could be divided into four groups, which were designated as I, II, III and IV (10). Group I includes non-toxigenic *C. sporogenes* and the proteolytic *C. botulinum* strains capable of producing A, B, and F toxins. Group II consists of *C. botulinum* serotypes A, B, E which are proteolytic. Group III includes proteolytic and non-proteolytic *C. botulinum* C, D as well as *C. novyi* type A. Group IV consists of *C. botulinum* type G, *C. subterminale* and *C. hastiforme* (10, 12, 34). These physiological groups were based on proteolytic and saccharolytic features, acid and alcohol fermentation end products, ability to grow at low temperatures, heat resistance of spores, and ability to grow in the presence of acids, alcohols, and salts (30).

Molecular and taxonomic studies have confirmed the diversity of BoNT-producing clostridia (4). Bacteria from the genus *Clostridium* were divided into four major groups, I-IV, based on the G + C content of their DNA and rRNA genes. *Clostridium botulinum* strains, because of their low GC content, were classified as group I and separated into groups I-A, I-F, and I-H, based on rRNA homologies (15). The classification of *C. botulinum* into four groupings, which correspond to the four physiological groups I-IV was supported by the 16S rRNA analyses of the genus *Clostridium* (4, 15). An analysis of 174 *C. botulinum* strains by amplified fragment length polymorphism (AFLP) and by the sequencing of 16S rRNA and BoNT genes confirmed the existence of at least four distinct genomic backgrounds, each of which has independently acquired BoNT genes through horizontal gene transfer (14). The proteolytic group I strains of *C. botulinum* expressing toxin types A, B, and F are closely related to *C. sporogenes*. The saccharolytic group II strains expressing toxin types B, E, and F are related to *C. butyricum*, *C. beijerinckii*, and *C. acetobutylicum.* The group III strains expressing toxin types C and D cluster with *C. novyi* and *C. haemolyticum*, and the group IV strains expressing toxin type G cluster with *C. subterminale*, *C. proteolyticus*, *C. argentinense*,

and *C. schirmacherense* (30, 32, 35). The lipids of *C. butyricum*, *C. beijerinckii*, and *C. acetobutylicum* have been studied intensively (27, 30).

Botulism in animals

Botulism is a severe flaccid paralytic disease caused by eight different neuroparalytic toxin subtypes (A to H) (27, 30). All BoNT subtypes block the release of acetylcholine, which leads to a flaccid paralysis (24). BoNTs types A, B, E, more rarely F, and the recently discovered serotype H are responsible mainly for human botulism, whereas toxin types C and D are involved in animal botulism worldwide (2, 27). The animal botulism is most commonly caused by contamination with poultry litter, soil, or carcasses (21, 23, 34). BoNTs are proteases remarkably specific to only three targets. They are called SNARE proteins of the presinaptic membrane. BoNT/B,/D,/F, and/G cleave VAMP, each at a single site (24, 30). BoNT/A and/E cleave SNAP-25, each at a single site, whereas BoNT/C cleaves both syntaxin and SNAP-25. BoNT/B cleaves VAMP (11, 14, 24, 26).

The signs of botulism are caused by flaccid muscle paralysis and include progressive motor paralysis, disturbed vision, difficulty in swallowing and chewing, disturbed vision, and progressive paresis. Death is usually due to respiratory or cardiac paralysis. The toxin prevents the release of acetylcholine at motor endplates (neuromuscular junction). The passage of impulses down the motor nerves and muscle contractility are not hindered. During the action of BoNTs, no characteristic gross or histological lesions develop, and pathologic changes may be ascribed to the general paralytic action of the toxin, particularly in the muscles of the respiratory system, rather than to the specific effect of the toxin on any particular organ (5, 30).

Botulism cases are common in wild and domestic animals. They occur sporadically as well as massively all over the world (27, 30). The most affected species of animals are cattle and birds, although botulism cases are also frequently found among horses, sheep, and goats (17, 22, 23, 30).

Botulism in cattle. In most cases, botulism in cattle is caused by the consumption of feed contaminated with botulinum toxins or, much more seldom, with *C. botulinum* spores. An environment favorable to *C. botulinum* growth and toxin production are anaerobic conditions inside silos or silage bales. Essential in the silage preparation process is a proper pH value, which should be between 4 and 6. BoNTs are produced when the pH value is higher than 4.6. Silages with a high content of dry matter (*e.g.* when feed material is collected late in the vegetation season) can be favorable for *C. botulinum* growth and BoNT production. The sources of *C. botulinum* in silages can be soil (*e.g.* enriched in organic matter from floodplains), carcasses, or poultry manure (30). Recently, the fertilization of crops with biogas plant residues has also been suspected as a potential cause of botulism in cattle. Frequent occurrence of chronic cattle botulism cases has been observed in northern Germany, which has the largest number of biogas plants in the country (16, 29).

The signs of botulism that can first be observed in cattle include muscular weakness, depression, and incoordination. Paralysis and muscle weakness usually begin with the hind legs and progress to the forequarters, neck, and head. The animal may become aggressive. On falling down, the cow may appear very weak and have great difficulty in rising. It may not be able to lift its head (limber neck). Initially, the cow lies in a normal resting position with its head on the ground or turned towards the flank (sternal recumbency). During the further progression of symptoms, the animal may become semiconscious, and when it goes onto its side (lateral recumbency), it is unlikely to get up again. Resulting from an uncontrolled paddling movement of the legs, semicircular marks on the ground may be the only evident sign when a carcass is found. Death is usually caused by respiratory failure or exposure between one to four days after the onset of clinical symptoms – sometimes, the progress of the disease can take up to 14 days. Epidemics have occurred in dairy herds, in which up to 65% of adult cows developed clinical botulism and died 6-72 hours after the onset of recumbency. Initially, clinical signs are similar to parturient paresis, but cows do not respond to parenteral calcium therapy. In intensively farmed herds, botulism cases are responsible for high mortality (3, 17, 30, 33). From 2003 to 2009, 168 cattle botulism cases (25) were reported in England, caused mainly by *C. botulinum* type C. One bovine botulism case, caused by type C (unpublished data), has been recorded in Poland. The first case of a bovine type C botulism outbreak was also reported in Finland in 2008 (35).

Botulism in birds. Avian botulism is a paralytic, often fatal, disease of birds that results from the ingestion of BoNT. Waterfowl die-offs due to botulism are usually caused by type C toxin. Deaths among fish-eating birds, *e.g.* gulls and common loons, are sporadically caused by type E (21). Disease in birds is also caused by type A botulinum toxin, most frequently in domestic chickens. The symptoms of botulism in poultry and wild birds are usually seen as a flaccid paralysis of the legs, wings, neck (twisting), and eyelids. Botulism in broiler chickens with the toxicoinfectious form may also manifest itself by diarrhea with excess urates and respiratory problems. Healthy birds, affected birds, and dead birds in various stages of decay are commonly found in the same area. Generally, the symptoms occur during 24 h. Birds are unable to use their wings and legs normally or to control the third eyelid, neck muscles, and other muscles. The animals with paralyzed neck muscles cannot hold their heads up and often drown. Death can also result from water deprivation, electrolyte imbalance, respiratory failure, or predation (9, 21, 30).

Many species of mammals and birds are affected by type C botulism. In the wild, the greatest losses are among waterbirds, but most birds are susceptible to type C botulism. An exception are vultures, which are highly resistant to type C toxin. The most significant host determinant for botulism is foraging behavior. Filter-feeding and dabbling waterfowl and probing shore birds appear to be among the most affected species. Mortality from botulism among wild raptors has been associated with an improper disposal of poultry carcasses. Among captive and domestic birds, poultry, pheasants and waterfowl are the most frequently affected. Losses vary significantly from year to year at site-specific locations and from species to species. More than a million deaths from avian botulism have been reported in relatively localized outbreaks in a single year, and outbreaks with losses of 50,000 birds or more are relatively common (21, 30, 34). Avian botulism is considered as the most important disease of migratory birds. Five outbreaks of botulism in water birds were reported over a 5-year period from 2004 to 2008 in Korea. In October 2008, an outbreak of avian type C botulism affected approximately 2,000 wild water birds in the Namdong flood control basin, Incheon, South Korea (31). In Europe, several cases of animal botulism have been reported in recent years. In Sweden, from 2000 to 2004, more than 10,000 seabirds died from type C botulism in the Blekinge archipelago in southeastern Sweden (21). Recently, two botulism outbreaks in wild birds have been noted in Poland (9, 34).

Reported clinical signs in horses are very similar to those in cattle, with progressive muscle paresis, recumbency, dysphagia, and decreased muscle tone (tail, tongue, jaw), respiratory distress, and death. Horses are very susceptible to botulinum toxins. It is estimated that a dose of BoNT sublethal for laboratory mice can cause death in adult horses (30).

Foals are particularly sensitive to botulinum toxins, and botulism symptoms in their case are described as the shaker foal syndrome. Foals under 4 week of age are usually the most susceptible to this syndrome. They may be found dead without premonitory signs, but in most cases they exhibit signs of progressive symmetric motor paralysis. Stilted gait, muscular tremors, and the inability to stand for 4-5 min are salient features. Other clinical signs include dysphagia, constipation, mydriasis, and frequent urination. As the disease progresses, dyspnea with extension of the neck and head, tachycardia, and respiratory arrest occur. Death, due to respiratory failure, ensues most often 24-72 hours after the onset of clinical signs. The most consistent necropsy findings are pulmonary edema and congestion, as well as excessive pericardial fluid containing free-floating strands of fibrin (25, 30).

Botulism in other species. Goats and ranch mink are also susceptible to type C botulism. Although dogs and cats are usually regarded as resistant to type C toxin, a few cases have been reported in dogs, which is a factor to consider when dogs are used to retrieve carcasses during outbreaks. Type C botulism also occurred in captive African lions that were fed toxin-laden chickens (30).

Structure of botulinum toxins

All toxin serotypes are synthesized as a single-chain polypeptide with a molecular mass of approximately 150 kDa. When produced by the bacterium, the toxin molecule associates with additional non-toxic proteins from a range of macromolecular complexes between 300 and 900 kDa. To obtain maximum biological activity, the 150 kDa toxin polypeptide must be cleaved into a 100 kDa heavy chain and a 50 kDa light chain, which remain connected by a disulphide bond. All toxin serotypes act by preventing acetylcholine release at peripheral nerve endings, thus inducing a temporary denervation and relaxation of muscles. Botulinum toxins are collected in the cytosol of bacterial cells. BoNTs are released at the stage of the logarithmic growth of bacteria and intensively increase during autolysis. Botulinum toxins consist of several components, which form protoxins. The protoxins are formed of active (BoNT), haemaglutinin (HA) and non-toxic non-heamaglutinin components (NTNH). Three different forms of botulinum protoxins with various molecular weights are distinguished: M – consists of BoNT and NTNH with a sedimentation coefficient of 12S and molecular weight of 300 kDa, L – consists of BoNT, NTNH, and two HA components with a sedimentation coefficient of 16S and a molecular weight of 500 kDa, and LL – consists of two L forms with a sedimentation coefficient of 19S and a molecular weight of 900 kDa. *Clostridium botulinum* type A can produce each of these forms, *C. botulinum* types B, C, D are able to produce M and L, whereas E and F strains produce only M. BoNTs are released from the protoxin complex by the action of proteases which are produced by the host organism or by proteolytic strains of *C. botulinum*. BoNTs possess three functionally different domains:

1) zinc-dependent metalloprotease – light chain (Lc);
2) transporting domain (H_N);
3) toxin-binding domain on the presynaptic membrane surface (it consists of two subdomains marked NN and HC) (5, 14, 24).

Significant heterogeneity exists among serotypes C and D, which are of great importance to veterinary medicine. Their diversity has probably arisen from the recombination or mutation of phage genomes that is thought to occur during the cycles of curing and reinfection of type C and D strains in the environment. The toxins of group III organisms (*C. botulinum* C and D, and *C. novyi*) are encoded on separate pseudolysogenic bacteriophages (6, 7). Cultures of toxigenic strains can be cured of their prophages and stop producing toxins, but they can be converted to the toxigenic state through reinfection by phages (15,

22). The type of toxin produced is determined by the infection with a specific phage. Type C strains can be reinfected by bacteriophages C or D, but the strains of type D are infected only by the homologous phage. *C. novyi* can be converted to either *C. botulinum* type C or *C. botulinum* type D depending on the phage type. A cured type C organism may continue to produce C2 toxin. This suggests that Cβ strains are derived from Cα strains upon the loss of their prophage. A cycle of phage loss and reinfection is thought to occur *in vivo*. Type C strains consist of two distinct subtypes, Cα and Cβ. Type Cα produces C1 and lesser amounts of C2 and D toxins; Cβ produces C2 toxin, and type D produces predominantly type D toxin along with smaller amounts of C1 and C2. The exoenzyme C3 is produced by both C and D. Oguma et al. (22) found that 23 biochemical characteristics of *C. botulinum* types C and D differentiate them into four groups and not three (Cα, Cβ, and D), as it had been thought. It was also found that the classification of Cα, Cβ, and D is incorrect according to toxin reactions to antibodies raised against Cα, Cβ, and D (30). Strains producing interserotype recombinant toxins, primarily the C-D and D-C mosaic subtypes, have also been reported. Recently, Wourdsta et al. (35) have shown that D-C types are highly represented among bovine isolates, whereas C-D types are highly represented among isolates from several avian species. Interestingly, all positive samples were recorded as mosaic types C-D or D-C, irrespective of the nature of samples and the regions where they were collected (13, 20, 35).

Diagnosis of botulism

In most cases, botulism takes a form of intoxication, and diagnosis should be based on the detection of BoNT in samples related to the symptoms of this disease. In some cases, e.g. disturbances in the natural gut microflora caused by antibiotic treatment, the toxicoinfection form may occur (30).

Apart from clinical manifestations, diagnosis is based on positive laboratory findings. The detection of the toxin in the serum and feces of animals remains the standard method. The detection of *C. botulinum* in feces, feed, gastric and intestinal contents supports diagnosis, but should not be considered specifically indicative of the disease. Sometimes, the presence of several toxin-producing strains, not only BoNT, may complicate diagnosis. There are tests for botulinum toxin that can be used to identify it in the gut contents and blood of affected animals, as well as in samples of suspect feed and water. The success rate of testing for the toxin varies, and, in many cases, botulism diagnosis is made on the basis of clinical signs and by excluding all other possible causes. Another test looks for antibodies to botulinum toxin in animals that survive. This test may support diagnosis if the vaccination status of the animals is known prior to the outbreak, and the animals have not subsequently been vaccinated. The diversity among *C. botulinum* strains and the occurrence of *C. botulinum*-like strains cause problems in the isolation process, and consequently may cause difficulties in diagnosing botulism. The growth of this species requires obligatory anaerobiosis. Different nutritional requirements for particular metabolic groups determine the usage of a proper culture method. The main factors that influence the isolation of *C. botulinum* from a sample are incubation temperature, pH value, content of preservatives, and presence of competitive microflora (8, 18).

The extreme potency of botulinum neurotoxin requires rigid safety standards to ensure the safety of laboratory workers. Despite the potency of its neurotoxin, the non-invasive and non-contagious *C. botulinum* has been graded as a class II pathogen. The appropriate biosafety level 2 containment facilities and trained personnel are therefore a minimum requirement when dealing with *C. botulinum*. Additional contingencies should be considered whenever aerosol or droplet formation from toxic material is expected. The definition of additional contingency measures should be based on a risk assessment of activities in each laboratory dealing with *C. botulinum* and its toxin. The efficacy of the pentavalent toxoid formerly used to immunize laboratory staff worldwide has been shown to be lower than expected. Therefore, special attention must be paid to the safety of laboratory staff (18). Generally, detection of *C. botulinum* is based on proving the ability of suspicious strains to produce botulinum toxins (BoNTs). Most methods are based on biological and immunological techniques, such as the mouse bioassay or ELISA for botulinum toxin detection after the culturing of suspicious strains. In recent years, chromatographic and molecular biology methods have become much more popular (1, 8, 9, 18, 34, 35).

Despite the fact that botulism is highly fatal, the effective laboratory diagnosis of this disease remains limited. Generally, diagnosis is based on the mouse bioassay, which is considered as a "gold standard" in laboratory practice. The usage of serological assays could be time-consuming and moderately specific. To ensure feed and animal safety, proper prevention should be considered, which could be achieved by introducing new methods and standards in microbiological laboratories connected with the official laboratory control of animal and human samples. Prevention should be ensured in every link of the food chain, "from field to table", especially by introducing the HACCP system and Good Manufacturing Practice (GMP).

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