A severe outbreak of botulism in cattle in Central Italy

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Summary
Botulism in cattle is rarely reported in Italy. This study describes an outbreak of botulism in a dairy herd in Central Italy in September 2012, and the notably high mortality rate it caused. Differential diagnoses involving toxicology and bacteriology, and electrolyte imbalances, all proved negative. A multiplex polymerase chain reaction (PCR) for detecting the BoNT gene led to the identification of the causative agent as *Clostridium botulinum* type DC. The presence of the toxin was confirmed subsequently via mouse bioassay. Initially, the peracute deaths and ambiguous clinical signs delayed the diagnosis and, as a result, impeded identification of the source of the infection on the farm. The severity of the outbreak demonstrates that screening for animal botulism should always form part of the diagnostic protocols used to investigate sudden peracute deaths without apparent cause in livestock.

Grave epidemia di botulismo in un allevamento di bovine in Toscana

Parole chiave
Botulismo animale,
Clostridium botulinum,
Bestiame,
Tossina mosaico DC,
Italia.

Riassunto
Questo articolo descrive l'epidemia di botulismo che ha colpito un allevamento di vacche da latte in Toscana nel settembre 2012 . Il botulismo animale si presenta raramente in Italia ma, come in questo caso, può essere causa di focolai di malattia associati ad alta mortalità. Tutte le prove diagnostiche atte ad evidenziare eventuale presenza di batteri, sostanze tossiche o squilibri elettrolitici. sono risultate negative. La *multiplex polymerase chain reaction* (PCR) per rilevare il gene BoNT ha individuato nel ceppo di *Clostridium botulinum* tipo DC l'agente patogeno responsabile della morte degli animali. Le prove biologiche hanno successivamente confermato i risultati del test PCR. L'elevata mortalità e la presenza di un quadro clinico non caratteristico non hanno facilitato il percorso diagnostico determinando un ritardo che ha invalidato la possibile identificazione della fonte dell'infezione. Una diagnosi più veloce avrebbe potuto ottimizzare l'indagine epidemiologica del focolaio, favorendo l'attuazione di misure che prevenissero eventuali casi futuri. La gravità dell'epidemia dimostra che lo screening per il botulismo animale dovrebbe sempre far parte dei protocolli diagnostici utilizzati per indagare i casi di mortalità iperacuta del bestiame senza apparente causa.

Introduction

Clostridium botulinum is a Gram-positive, spore-forming, obligate anaerobic bacterium, commonly found in soils and aquatic sediments (Whitlock 2004, Anniballi *et al.* 2013b). In anaerobic, warm and humid conditions, the microorganism can multiply rapidly, producing the different types of neurotoxins responsible for botulism in humans and animals (Senturk and Cihan 2007). Seven immunologically distinct botulinum neurotoxins (BoNTs) (serotypes A-G) are recognised. Recently, an 8th serotype (BoNT/H) was identified (Barash and Arnon 2014, Rossetto *et al.* 2014). This serotype appears to be a genetic arrangement (mosaic) of types A and F toxins, fully neutralised by the type A antitoxin (Maslanka *et al.* 2015). Botulinum neurotoxin types A, B, E, and F are responsible for botulism in humans. Types C, D, mosaics CD and DC toxins have been related to animal disease, though type A, B, and E toxins have also been recovered from animals (Bianchi 1950, Haagsma and Ter Laak 1979, Neill *et al.* 1989, Jean *et al.* 1995, Ortolani *et al.* 1997, Heider *et al.* 2001, Senturk and Cihan 2007, Lindström *et al.* 2010, Krüger *et al.* 2012, Payne *et al.* 2011, Anniballi *et al.* 2013b). Botulin neurotoxin/DC comprises the L chain (catalytic domain) and HN domain (translocation domain) of serotype D and the HC domain (binding domain) of serotype C, whereas BoNT/CD consists of the L chain and HN domain of serotype C and the HC domain of serotype D (Rossetto *et al.* 2014). The neurotoxins are produced during the growth and autolytic phases of the bacterium (Bohnel *et al.* 2001, Senturk and Cihan 2007).

Literature describes different forms of botulism for both humans and animals. In animals, the most common forms are due to the consumption of contaminated feeds (feed-borne) or as a consequence of toxico-infections. However, wound botulism is also recognised (Whitlock 2004, Anniballi *et al.* 2013b). Dead rodents, birds, or reptiles accidentally contained in feeds as well as decaying vegetable material are all substrates in which *C. botulinum* can proliferate and produce toxins (Neill *et al.* 1989, Jean *et al.* 1995, Wobeser *et al.* 1997, Galey *et al.* 2000, Senturk and Cihan 2007). Furthermore, hay stored in plastic bags, along with high pH forages and silages, seem to encourage the germination of spores (Whitlock 2004).

Different symptoms are described in the literature, and these relate to varying levels of susceptibility to BoNTs depending on species, breed, and individual (Bohnel *et al.* 2001, Anniballi *et al.* 2013b). The most frequent symptoms reported in cattle are muscular weakness, ataxia, progressive paralysis, dysphagia, loss of tongue tone, decrease in salivation, bradycardia, decreased rumen movement, and recumbency (Ortolani *et al.* 1997, Bohnel *et al.* 2001, Heider *et al.* 2001, Braun *et al.* 2005, Zarenghi *et al.* 2006, Senturk and Cihan 2007). Small doses of BoNTs may not lead to clinical signs for 5-10 days, while high doses, administered experimentally to cattle, lead to recumbency and death in 18-24 hours. Thus, the course of illness in an outbreak may vary from 2-30 days, depending on the size of the dose ingested by the animals (Whitlock 2004).

Although rare, cattle botulism outbreaks have been reported from various parts of the world (Galey *et al.* 2000, Heider *et al.* 2001, Martin 2003, Advisory Committee on the Microbiological Safety of Food 2006, Aish *et al.* 2006, Senturk and Cihan 2007, Payne *et al.* 2011), including Europe. Between 2003 and 2009, suspected botulism emerged in ruminants in England and Wales; 168 cattle and 19 sheep died, with in-herd mortality rates ranging between 5% and 80% (Payne *et al.* 2011). Outbreaks in France increased significantly in the mid-1990s, peaking at 42 outbreaks in 1995 (AFSSA 2002); while Finland reported its first case of bovine botulism in 2008 (Myllykoski *et al.* 2009). Only 3 outbreaks of cattle botulism have been reported in Italy so far. In 1950, Bianchi studied an outbreak of botulism amongst cattle in the province of Modena (Bianchi 1950). The source of the toxin was identified in the carcass of a cat that had contaminated the cattle feed. A strain of *C. botulinum* type D was subsequently isolated from the viscera of 2 of the dead animals (Bianchi 1950). Zarenghi and colleagues described a high mortality rate due to botulism in dairy heifers (Zarenghi *et al.* 2006). During this outbreak, 99 out of 230 (43%) animals died in 1 month, which amalgamated to an 80% morbidity rate (Zarenghi *et al.* 2006). Rosignoli and colleagues described an outbreak of botulism in a herd of cattle in which 8 Friesian heifers and a weaned calf were involved (Rosignoli *et al.* 2000). The clinical signs were suggestive of botulism, and the clinical diagnosis was confirmed by the detection of the BoNT in 4 blood samples, but not in the feed (Rosignoli *et al.* 2000).

In the present study we report on an outbreak of botulism in Italian cattle. The outbreak yielded a high mortality rate, due to the *C. botulinum* type DC mosaic toxin.

Case history, clinical symptoms, pathological findings, and diagnosis

In September 2012, an outbreak of botulism affected cattle on a farm in the province of Grosseto in the region of Tuscany (Figure 1). At the time of the outbreak, the dairy herd of 480 Friesians included 210 lactating cows. The herd was divided into 2 groups (group A and group B), separated by the foraging line and allocated to separate feeding

Figure 1. *Location of the outbreak.*

alleys. Group A consisted of 365 cows, while group B consisted of 115 animals. The 2 groups shared the same external paddock and a common milking compound. All the animals received the same feed ration. The disease was severe, and caused 48 deaths amongst the animals belonging to group B, while the animals in group A showed no signs of illness. The disease manifested on 9 of September 2012 with the sudden death of the first 3 animals (day 1). New cases appeared over the following 12 days (Figure 2). The disease evolved over a maximum of 8 days, even though the precise duration could not be assessed in the 24 animals that were euthanised for ethical reasons. The last 4 animals were euthanised on the 15th day of the outbreak (Figure 3). After the first 3 asymptomatic deaths, the whole group B exhibited decreased feed intake, while those showing symptoms reported progressive muscle weakness (characterised by a slow gait), ataxia and lateral recumbency, followed by death. The animals were also hypothermic (min 37.1 °C - max 38.3 °C), aware of their surroundings, and able to move their tails and heads (often turned into the flank). Muscle fasciculation in the forelimb was also noted. The animals were treated with saline solution (0.9% NaCl), antibiotic, cortisone, vitamin B, and methionine, with no signs of improvement. On the second day, a commercial clostridial vaccination against *Clostridia chauvoei, Clostridia novyi, Clostridia haemolyticum, Clostridia septicum, Clostridia perfringens*, and *Clostridia tetani* was administered to all animals, the stable was cleaned, and litter and water were changed. During cleaning, 1 of the 5 water troughs was seen to have run dry and smelled of sulphite.

By day 6, 7 animals had been necropsied and their organs sent (refrigerated) to different laboratories for testing, along with 7 blood samples (5 from affected animals, and 2 from animals of the healthy group A). Animal feed and drinking water were also analysed (1 kg of corn silage, 1 kg of banded alfalfa, 1 tanked milk sample, 8 x 500 mL water samples from 4 drinking troughs used by the affected group (B), and sediments from the water trough found empty).

The main post mortem findings in the 7 animals included petechial haemorrhages on the epicardium, haemopericardium, and enteritis of the small intestine. The main histological findings on the liver were steatosis associated with centrolobular congestion, edema, and microhaemorrhages. Perivascular edema, and the presence of micro intracerebral haemorrhages were recorded in the brains. The heart showed myocardial congestion and perivascular edema with subepicardial microhaemorrhages; multifocal exudative glomerulonephritis, associated with tubular nephrosis, and numerous haemorrhages

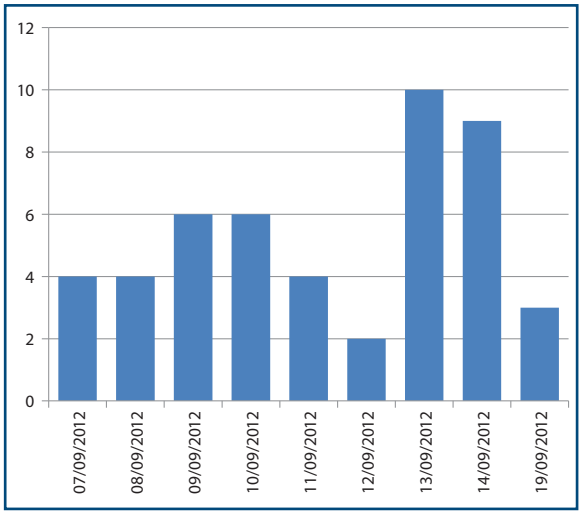

Figure 2. *Daily incidence of new clinical cases occurred in dairy cattle in province of Grosseto, Italy (September 2012).*

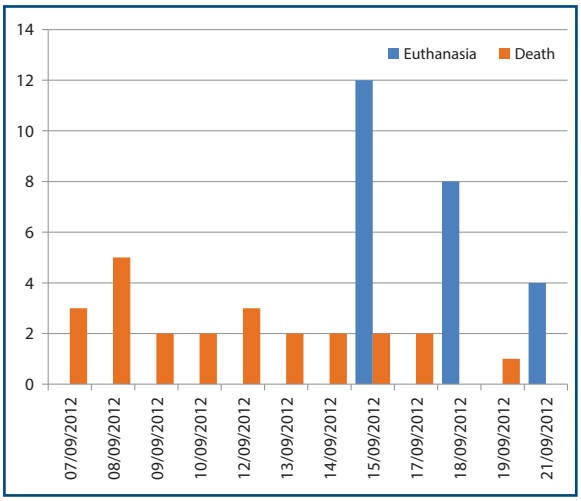

Figure 3. *Daily mortality over 15 days registered during the botulism outbreak occurred in dairy cattle in province of Grosseto, Italy (September 2012).*

on the medullary were seen in the kidney; the intestine showed a severe enteritis with areas of de-epithelialisation of the mucosa and microhaemmorrhages, characterised by the presence of extensive lymphocytic and eosinophilic infiltrates in the lamina propria. Routine bacteriological analyses were conducted on the organs and biologic liquids (livers, kidneys, intestines, spleens, intracardiac coagulums, pericardiac liquids, and milk) of the deceased animals. Unfortunately, they did not reveal the presence of any relevant pathogenic bacterium as a possible causative agent. All the intestine samples were positive for *C. perfringens*, but an ELISA test (BioXDiagnostic®) revealed no toxins (alpha, beta, gamma, delta).

The microbial counts of clostridia were below 10^8 CFU/g. Two spleen and kidney samples were positive for *Clostridia sordelli*, 1 animal resulted positive for *Klebsiella pneumoniae* (brain, lung, and intra-cardiac coagulum), while a further 3 animals were positive for haemolytic *Escherichia coli*. The complete blood cell counts revealed no additional abnormalities other than a slight neutrophilia. Serum biochemistry indicated an increase in creatine phosphokinase (CPK) in 3 out of 5 animals tested. Two animals showed a slightly low ureic (BUN) value, and a further 2 had low iron (Fe) levels. The cholinesterase values of the affected group B were similar to those of group A. All liver and kidney samples were negative for organochlorates, carbamates, organophosphates, as well as the associated gastric contents. All water samples chemically matched the parameters of potable water and were negative for pathogenic bacteria, organochlorates, carbamates, organophosphates. The sediments of the empty water trough were negative as well. Three liver samples were found positive for *C. botulinum* producing mosaic DC toxin using a multiplex PCR method (Anniballi *et al.* 2013a). The PCR method was performed following the guidelines provided by the Italian National Reference Centre for Botulism (ISS, Rome, Italy). The ISS confirmed these PCR results, and conducted the mouse bioassay that demonstrated the presence of toxin in 1 of the cow liver samples (ISS 2013).

Discussion

In Italy, botulism in large animals is a rare occurrence. However, the number of outbreaks in Italy and Europe has increased in the last decade. The rarity of the disease in combination with the lack of pathognomonic symptoms in animals can lead to a misdiagnosis, or late diagnosis, which results in economic losses for farmers.

In the outbreak reported here, the first clinical suspicion consisted of *C. perfringens* enterotoxaemia. Accordingly, a commercial vaccine against *Clostridia* was administrated, along with vitamin E and selenium, because of the myopathy seen during necropsy. The initial results from blood tests showed an increase in creatine phosphokinase. This was interpreted as muscular damage caused by recumbency, and the neutrophilia as stress-related. The low BUN was interpreted as a sign of hepatosis, linked to high milk production in Friesian cattle. A metabolic disturbance was also considered as a possible diagnosis, but later excluded because the blood test results did not show hypomagnesemia, hypokalemia, or hypocalcemia. Organophosphate intoxication was excluded because there were no differences in the cholinesterase blood levels between the animals of both groups A and B. This conclusion was subsequently confirmed by the negative toxicological results arising from the assays for organochlorates, carbamates, and organophosphates that were conducted on the liver, kidney, and water samples. The absence of *C. perfringens* toxins, and the relatively low bacterial counts from the intestine, make it improbable that the bacterium underlays the high mortality rate. Once botulism was suspected, 3 of the previously obtained liver samples were analysed using a multiplex PCR for *C. botulinum*; these tested positive for the mosaic DC gene. For 1 of the samples, the presence of the BoNT was confirmed via mouse assay. In the 2 remaining samples, the apparent absence of BoNTs may be due to the integrity of the toxins being compromised following the repeated thawing and freezing of the samples between one laboratory and the next.

A belated diagnosis of the cause of this outbreak impeded identification of its origin. Various factors may have played a role. For example, it may be worth noting that the animal feeds included newly imported banded alfalfa; it is known that cut-grass that is moist from rain is able to provide an optimal anaerobic environment suited to the production of BoNTs (Braun *et al.* 2005). In addition, after being processed in the nearby bio-waste compound, the litter from the neighbouring broiler farm, could have been recycled by being spread over the farm's adjacent fields. It has been reported that broilers may subclinically carry *C. botulinum* type D in their gut flora, so this type of contamination would pass unnoticed in poultry (Krüger *et al.* 2012, Payne *et al.* 2011). Finally, it is feasible that the original source of the intoxication was the empty waterhole that reeked of sulfite (which is often produced by clostridia). This was suspected during a previous outbreak of botulism in Italy (Zarenghi *et al.* 2006).

Unfortunately, because all feed and water were removed, and the stable was cleaned for biosecurity reasons at the start of the outbreak, nothing could be test for the presence of *C. botulinum* and its

associated toxins; thus no laboratory data exist on the possible source of contamination. To better understand the epidemiology of these outbreaks, and to circumvent future outbreaks, it is fundamental that an in-depth investigation of the environment is preceded by the rapid and accurate diagnosis of the causative agent.

Identifying the cause of this outbreak in cattle was hindered by the absence of pathognomic symptoms and the paucity of cases reported in cattle in Italy. Botulism was suspected based on the high mortality rate, but only after organophosphate, organochlorate, and carbamate poisoning, toxico-infections, and metabolic disturbances had been excluded. The final diagnosis was obtained only after *C. botulinum* type DC and related toxins had been detected in the liver samples taken from deceased animals. While the public health risk linked to cattle botulism caused by BoNT type C and D is considered to be low (AFSSA 2002), it remains particularly relevant to animal health and welfare. The high mortality rates characterising this outbreak mirror those reported previously. Based on our experience, botulism should form part of the diagnostic protocols that deal with cases of sudden death without apparent cause, especially when encountering laterally recumbent cattle amongst non-periparturient, afebrile, and alert animals. Finally, every effort should be made to identify the source of the contamination within the immediate environment in order to circumvent reoccurrence of the disease. In this instance, this was not achievable.

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