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Evidence for a natural humoral response in dairy cattle affected by persistent botulism sustained by non-chimeric type C strains

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ABSTRACT

Bovine botulism is a sporadic acute disease that usually causes catastrophic losses in the herds. The unusual clinical evolution of a persistent mild outbreak in a dairy herd, prompted us to characterize the neurotoxin gene profile of the strain involved and to evaluate whether seroconversion had occurred.

Diagnosis was based on mild classical symptoms and was supported by PCR and bacteriological findings, which revealed the involvement of a non-mosaic type C strain.

An in-house ELISA was developed to detect antibodies to botulinum neurotoxin type C and its performance was evaluated in a vaccination study.

Fifty days after the index case, fecal and serum samples were collected from the 14 animals of the herd and screened for *Clostridium botulinum* and anti-botulinum neurotoxin antibodies type C, respectively. The in-house developed ELISA was also used to test 100 sera samples randomly collected from 20 herds.

Strong ELISA reactions were observed in 3 convalescent and 5 asymptomatic animals involved in the studied outbreak. The ELISA-positive cows all tested positive for non-mosaic *C. botulinum* type C in the feces and the same strain was also detected in the alfalfa hay, suspected to be the carrier source. Ten out of the 100 randomly collected sera tested positive for anti-botulinum neurotoxin type C antibodies: 7 had borderline values and 3 from the same herd showed titers three times higher than the cut-off.

We concluded that type C botulism in cattle may occur with variable severity and that prolonged exposure to sublethal doses of botulinum neurotoxin C may occur, resulting in detectable antibodies.

1. Introduction

Botulism is a neuroparalytic disease caused by the action of the botulinum neurotoxins (BoNTs) produced by the Gram-positive spore-forming anaerobic bacterium *Clostridium botulinum*. BoNTs are classified into 8 serotypes (A to H) on the basis of their different antigenic properties. In addition to *C. botulinum*, some strains of *Clostridium butyricum*, *Clostridium baratii* and *Clostridium argentinense* are able to produce BoNTs types E, F and G, respectively [1–4].

Serotypes A, B and E are known to cause the majority of human botulism outbreaks whereas serotypes C and D are responsible for most outbreaks in animals. Characterization of isolates of neurotoxin gene type D from cattle in Japan revealed that all tested strains were “chimeric” because they harbored the so-called “mosaic” neurotoxin gene composed of two-thirds type D and one-third type C sequences [5]. Furthermore, the toxins produced by those strains were neutralized in vivo by both antitoxin type D and C [5]. The same authors demonstrated that these so-called chimeric strains type D/C produce BoNTs endowed with higher toxicity than non-chimeric strains. Takeda and co-workers also observed that the C/D mosaic was more lethal to chicken than the non-chimeric type C [6].

Animal botulism can result from the ingestion of BoNTs produced either outside the host (intoxication) or in the gut of the host that has ingested the spores (toxico-infection). One of the most common forms of feed contamination is the accidental inclusion of

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an animal carcass in the forage [7].

Diagnosis of botulism is mainly based on clinical signs characterized by progressive flaccid paralysis ultimately involving the respiratory muscles and causing the death of the animal due to respiratory failure.

Detection of the neurotoxin in biological samples by the mouse lethality bioassay (or mouse test) is the gold standard for confirming the disease but many authors have reported difficulties in confirming the presence of toxin in cattle, despite having a clear symptomatic picture [8–12]. This finding could be due to the lower susceptibility of mice compared to bovines, or to the absence of the neurotoxin at sampling due to sequestration from the bloodstream or gastrointestinal lumen and binding to the cholinergic neuronal terminals [13]. Differences in susceptibility to types C and D have already been demonstrated in different animal species, and ruminants have proven to be more susceptible than small laboratory animals and primates to type D, when the toxin is orally administered [14].

A large number of PCR protocols have emerged as a useful tool for the detection of *C. botulinum* in biological samples and for the identification of the serotype involved. In addition, ethical reasons support the development and employment of diagnostic methods other than the use of laboratory animals [15].

Immune-enzymatic assays (ELISA) for the detection of antibodies to BoNTs have been developed in some studies. These tests have proven useful in discriminating vaccinated from unvaccinated animals and in establishing a protective titer [16,17], but inadequate in retrospective investigations in affected herds [18]. In other studies, serological tests revealed seroconversion to type C in clinically normal animals, but the actual presence of *C. botulinum* or BoNTs in biological samples of seropositive animals was not investigated [19].

In this study, we optimized an in-house indirect ELISA for the detection of antibodies to BoNT type C and applied this test in a persistent outbreak of bovine botulism. Serological results were studied in association with investigations on the actual presence of *C. botulinum* in the feces. In addition, the test was applied to determine whether sub-clinical seroconversion to BoNT type C may have occurred in the population presumed negative.

2. Materials and methods

2.1. Case history

At the end of June 2012, clinical signs referable to botulism were observed in a herd formed by 11 lactating Simmental cows, 4 pregnant heifers and 7 calves of the same breed. Only the cattle fed with alfalfa hay (5 lactating cows and 2 heifers) showed the characteristic symptoms of ataxia, weakness, constipation alternating with diarrhea, dysphagia, emaciation, enhanced salivation, mydriasis, apathy and stiff gait due to non-infectious laminitis. Milk production decreased from a daily average of 25 to 10 kg/cow. Mortality was very low (one heifer) while the majority of diseased animals recovered within five to eight weeks of symptoms onset. The clinical signs appeared five to seven days after consumption of the hay suspected of contamination and the disease showed a chronic course lasting ten weeks. At the start of the disease, blood and fecal samples were collected for diagnostic purposes from two symptomatic animals. Lactating cows and heifers were fed with hay (produced on the farm), alfalfa hay (purchased) and concentrates. One sample of each feed was sent to the laboratory and tested for the presence of *C. botulinum* (see below).

Furthermore, about 50 days after disease onset, fecal and blood samples were collected from 14 animals (9 lactating cows, 1 heifer and 4 calves).

2.2. PCR protocols for detecting and characterizing BoNT encoding genes

One and half grams of each fecal sample were mixed with saline solution (vol/vol) and added to 12 ml of fortified cooked meat medium (FCMM) [20] contained in Pyrex tubes sealed with a screw cap.

Two hundred and fifty grams of each feed sample were placed in a sterile plastic stomacher bag and washed with 250 ml of saline solution. The washing solution was then filtered through surgical gauze, harvested in 50 ml Falcon tubes and centrifuged for 30 min at 5500 × *g*, at 6 °C (Megafuge 1.0 R, Haraeus, Germany). Pellets were pooled and 1.5 g was added to 13.5 ml of FCMM.

Tubes inoculated with feces and the washing solutions of the feed samples were immersed in a hot bath (71 °C) for 10 min, cooled in water and incubated at 37 °C in a Bactron IV anaerobic chamber (Shel Lab, Cornelius, OR, USA). After 48 h of incubation, 175 μl of each broth culture was collected from the bottom of the tubes and DNA was automatically extracted (Microlab Starlet, Hamilton, Bonaduz, Switzerland) using a MagMax Total Nucleic Acid Isolation kit (Ambion/Life Technologies, Carsland, CA, USA). PCR protocols for *C. botulinum* neurotoxin genes types A to F were applied in accordance with previously published conditions [21,22]. Type of mosaic neurotoxin gene was assessed by biomolecular techniques, as described elsewhere [22].

2.3. Clostridium botulinum isolation and BoNTs investigation

PCR-positive FCMM broth cultures were plated on egg yolk medium (EYA) produced with Blood Agar Base No.2 (BAB2) (Oxoid, Hampshire, United Kingdom), as a base, and 50 ml/L of a yolk solution composed of 25 ml of fresh yolk and 25 ml of saline solution. A further 50 μl of FCMM was collected from the bottom of the tubes and plated on BAB2 with 5% defibrinated sheep blood and with agarose content enhanced to 2.5% to contain the spread of swarming clostridia (e.g. *Clostridium sporogenes*) on the agar surface. The EYA and BAB2 plates were incubated for 48 h in an anaerobic cabinet (Shel Lab) with an atmosphere composed of 5% hydrogen, 5% carbon dioxide and 90% nitrogen. Suspected colonies observed in the EYA (lecithinase and small lipase positive colonies) and BAB2 (weakly hemolytic small colonies) were collected and transferred to a further 10 ml of FCMM in tubes. The broth cultures were incubated in anaerobic conditions at 37 °C for 48 h and tested by PCR protocols for toxin types A, B, C, D, E, F as described above.

Supernatants of PCR-positive broth cultures were filtered with a 0.45 μm pore-size filter (Millipore, Tullagreen, Ireland) and tested for BoNTs by the mouse test [23]. The same test was also applied to the sera of the two symptomatic cows.

2.4. Antigen production

An in-house indirect ELISA was developed to detect BoNT-C antibodies according to previously described methods, with some modifications [16,19]. Antigens were produced in a dialysis apparatus to reduce the presence of medium components and to contain any non-specific ELISA reactions. The broth culture for the dialysis was composed of 4% proteose peptone (Bacto Proteose Peptone No. 2, Difco/Becton Dickinson), 2% pancreatic digest of casein (Bacto Tryptone, Difco/Becton Dickinson), 2% extract of autolyzed yeast cells (Bacto Yeast Extract, Difco/Becton Dickinson), and 1% glucose (Sigma-Aldrich, St. Louis, MO, USA) [24]. A dialysis tubing (50-kDa cut-off) (Spectra/Por 6, Spectrum Medical Industries, Rancho Dominguez, CA, USA) was intussuscepted, suspended in the carboy, and fixed to the cap. The dialysis apparatus was sterilized by autoclaving at 121 °C for 30 min and allowed to cool to room