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Pathogenesis and Toxins

Chronic botulism in a Saxony dairy farm: Sources, predisposing factors, development of the disease and treatment possibilities

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ABSTRACT

The aim of this study is to investigate *Clostridium botulinum* at a Saxony dairy farm with 159 cows and 18 heifers. The animals exhibited clinical symptoms of chronic botulism. To determine the source of the infection, feces, blood, organs, and gastrointestinal fluids of dead or euthanized cows; as well as soil, water, silage and manure were tested for *C. botulinum* spores and BoNTs using ELISA. BoNT/C and *C. botulinum* type C were detected in 53% and 3% of tested animals, respectively, while BoNT/D and *C. botulinum* type D were detected in 18% of the animals. *C. botulinum* also was detected in organs, gastrointestinal fluids, drinking water and manure. To evaluate possible treatments, animals were given Jerusalem artichoke syrup (JAS), Botulism vaccine (formalinised aluminum hydroxide gel adsorbed toxoid of *C. botulinum* types C and D) or a suspension of *Enterococcus faecalis*. After four weeks treatment with JAS, BoNT/C and *C. botulinum* type C were not detected in feces. In contrast, BoNT/D and *C. botulinum* type D were not significantly influenced by the JAS treatment. Vaccination with botulism vaccine and the *E. faecalis* suspension significantly decreased BoNT/D and *C. botulinum* type D. A significant increase of *Enterococci* was detected in animals treated with *E. faecalis*. Interestingly, there was a negative correlation between the detection of both BoNT and *C. botulinum* with the concentration of *Enterococci* in feces. Although *C. botulinum* C and D antibodies increased significantly ($p < 0.0001$) after vaccination with the botulism vaccine, the reduction of *C. botulinum* and BoNT in feces did not result in recovery of the animals because they were deficient of trace elements [manganese (Mn), cobalt (Co), copper (Cu) and selenium (Se)]. Animals treated with trace elements recovered. It appears that intestinal microbiota dysbiosis and trace element deficiency could explain the extensive emergence of chronic Botulism.

1. Introduction

Chronic botulism is an increasing problem in dairy cattle in Germany and other European countries [1,2]. It is a multifactorial clinical syndrome induced by toxic metabolites (neurotoxins) of different types and subtypes of the anaerobic spore forming *Clostridium botulinum*. The botulinum neurotoxin (BoNT) may be taken up orally if preformed in food or feed (intoxication) or it may be produced inside the gastrointestinal tract (infection). Ingested spores of *C. botulinum* can germinate, multiply and produce BoNTs which are responsible for botulism [1–4]. Notermans and coworkers [5,6] described different forms and courses of the disease from sub-acute and chronic intoxication over a few days to long-lasting infection over weeks to months. Causes of the increased occurrence of chronic disease since 2000 are mostly unexplained, but it could be attributed to dysbiosis of gastrointestinal microbiota [7–9]. Oral metronidazole treatment of mice induced dysbiosis of gastrointestinal tract microbiota which led to colonization by *C. botulinum* types A and B [6]. The common beneficial bacterium *Enterococcus* spp. exerts an antagonistic effect on *Clostridia* [10,11] so that a reduction in *Enterococcus* spp. and other beneficial microbiota could lead to overgrowth by *Clostridia* and resulting pathologies [9]. Currently, the only way to prevent this acute botulism is by vaccination; however, only *C. botulinum* types C and D vaccines are currently available. Vaccination reduces BoNT and *C. botulinum* CD in feces of vaccinated animals [12]. Since 1% fructose prevents BoNT/E production *in vitro* due to reduced

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C. botulinum growth, there is a strong correlation between BoNT expression and growth of the bacteria [13]. Similar results were obtained with *C. botulinum* type B and C using 0.05 and 0.5% Jerusalem artichoke powder or lactulose. Lactulose (0.5%) was able to inhibit BoNT production by *C. botulinum* type B in bacterial suspensions of 10^3 and 10^4 spores/mL incubated for 7 days. The same inhibition was seen with *C. botulinum* type C but only with 10^3 spores/mL [14]. This paper describes the ways we identified the sources, predisposing factors and possible treatments to combat *C. botulinum* types C and D infection in a Saxony dairy farm suffering from chronic botulism.

2. Material and methods

2.1. Description of the cows' health and farm situation

An investigation for *C. botulinum* types CD at a Saxony dairy farm with 159 cows and 18 heifers was initiated in March of 2011. The cows (about 30%) exhibited clinical symptoms typical for "chronic botulism" or secondary complications. The main signs were locomotor disorders, swollen joints, suppuration of skin wounds, viscous saliva (Fig. 1A), regurgitation and spitting out food wads (Fig. 1B), and droopy heads and tails. The behavior of the animals was very listless, without agility and curiosity. Diseased cows died within few weeks after showing these complicated signs. The situation within the stable was very silent. Surprisingly, the cows had reduced water uptake from 15–16 m^3 to 7–9 m^3 per day for the herd. Milk production for the herd dropped from 3000 L to 1500 L per day. Calves suffered from dysfunctional suckling. The beginning of this problem was first observed in 2005 when there was an increase in mortality (about one to two per month); however, by 2011, mortality reached four animals per week. Due to high mortality, additional cows had to be bought from other farms but these replacement animals developed symptoms several months after introducing them into the herd.

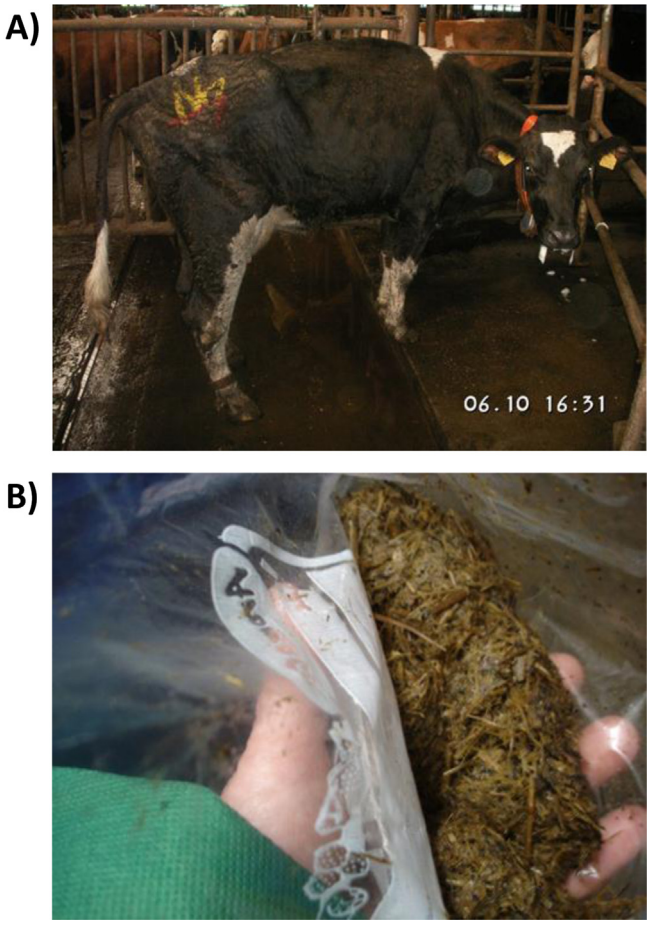

Fig. 1. Viscous saliva of a cow with chronic botulism (A) and feed wrappings spitted out from cows suffered from chronic botulism (B).

2.2. Treatment of cows and collection of specimens

All treatments and collection times of specimens are shown in Fig. 2. Animals were given 250 mL per animal daily of Jerusalem artichoke syrup (JAS, Lieven GmbH, Germany) for four weeks. About one month later, an *Enterococcus faecalis* suspension (10^5/mL, Chevita, Germany) was given orally (500 mL per/animal daily for 9 weeks). All animals were vaccinated with botulism vaccine (formalinised aluminum hydroxide gel adsorbed toxoid of *C. botulinum* types C and D, Onderstepoort Biological Products, South Africa), five weeks apart. Feces and blood samples were collected four times (S1–S4) from the rectum (Ampulla recti) and Vena coccygea mediana, respectively (Fig. 2). The organs of 20 dead or euthanized cows also were investigated. Different organs (liver, kidney, lung, masseter muscles, and gluteal muscles) and gastrointestinal fluids (rumen, colon, jejunum, ileum and cecum) of dead or euthanized cows were collected and tested for *C. botulinum* spores and BoNT.

2.3. Preparation of fecal samples for detection of BoNT/A-E

Fecal samples were diluted 1:3 in PBS (Dulbecco, pH 7.4) with 0.1% Triton X-100, 0.1% Tween 20 and 10 mM EDTA, thoroughly mixed and frozen at −20 °C. After thawing, the diluted fecal samples were centrifuged at 7000 × g for 15 min and the clarified supernatants were analyzed with BoNT-ELISA.

2.4. Indirect detection of C. botulinum spores

Fecal specimens or soil samples were diluted 1:10 (0.5 g in 4.5 ml) in Reinforced Clostridium Medium (RCM, Sifin, Germany), vigorously mixed, and heated at 80 °C for 10 min. Samples were incubated at 37 °C for 7 d under anaerobic conditions and subsequently stored at −20 °C until tested. After thawing, a culture sample was centrifuged at 7000 × g for 15 min and the clear supernatant was analyzed for the type-specific soluble antigens of *C. botulinum* types A-E by ELISA.

2.5. BoNT-ELISA

BoNTs/A-E were determined as described by Krüger and coworkers [12]. Briefly, the standard volumes were 100 µl per well and the standard incubation condition was 1 h at room temperature (1 h at RT) on a microtiter plate shaker (400 rpm). The coating buffer was 0.1 M NaHCO3 and the wash solution (WS) was 0.9% NaCl with 0.05% Tween 20 (Sigma–Aldrich, Taufkirchen, Germany). All washing steps were done in a Nunc-Immuno-Washer 12 (Nunc, Wiesbaden, Germany). After coating the ELISA wells with capture antibodies (3 µg/ml, BoNT-immunoaffinity purified-IgG from rabbits against BoNT/A-E, Institute of Bacteriology and Mycology, University of Leipzig, Germany) overnight at 4–6 °C, they were incubated with 150 µl per well of 1% gelatin from cold water fish skin (Sigma–Aldrich, Taufkirchen, Germany) in 0.9% NaCl-solution for 1 h at RT. The wells were washed twice with WS and loaded with the prepared fecal samples diluted 1:2 in 20 mM Tris, pH 8.0, assay buffer [adjusted with 1 M HCl] containing 0.9% NaCl, 5 mM EDTA, 1% gelatin from cold water fish skin, 0.2% bovine serum