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Short Communication

Occurrence of *Clostridium botulinum* neurotoxin in chronic disease of dairy cows

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ABSTRACT

Botulism caused by neurotoxins of *Clostridium (C.) botulinum* is a rare, but serious life-threatening disease in humans and animals. Botulism in livestock is usually caused by the oral uptake of *C. botulinum* neurotoxins (BoNT) via contaminated feed and is characterized by flaccid paralysis. In the recent past a new syndrome caused by BoNT in dairy cattle was postulated. It was supposed that *C. botulinum* is able to colonize the lower intestine and may subsequently produce neurotoxin. The continuous resorption of small amounts of these BoNT may then provoke the so called syndrome of "chronic" or "visceral" botulism involving unspecific clinical symptoms, reduced performance of dairy cows and massive animal losses in the affected herd. To test this hypothesis a case-control study was conducted involving 92 affected farms and 47 control farms located in Northern Germany. Fecal samples of 1388 animals were investigated for the presence of BoNT to verify the key requirement of the hypothesis of chronic botulism. BoNT was not detected in any of the fecal samples using the most sensitive standard method for BoNT detection, the mouse bioassay. Therefore, the existence of "chronic" or "visceral" botulism could not be proven.

1. Introduction

Botulism is caused by the bacterium *Clostridium* (*C.*) *botulinum* and is usually an intoxication in humans and also in animals, when the *C. botulinum* neurotoxins (BoNT) are ingested with contaminated food or feedstuff, respectively (Hatheway, 1990). So far, seven serologically differentiable toxin types (A–G) are known, a new serotype H was proposed recently which remains to be verified (Rossetto et al., 2014). Serotypes A, B, E and F are causing human and animal botulism, whereas types C and D are reported to cause predominantly animal botulism (Lindstrom and Korkeala, 2006; Lindstrom et al., 2010). Furthermore, BoNT serotypes are divided into subtypes based on their nucleic acid and amino acid sequence heterogeneity (Hill and Smith, 2013). Remarkable morphological and physiological differences are seen between

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C. botulinum strains isolated from different sources. Four physiologically different BoNT producing groups of *C. botulinum* (I–IV) are described (Hatheway, 1990; Rossetto et al., 2014). Modern comparative sequence analysis also confirms that there are significant genetic differences between these groups (Rossetto et al., 2014). These findings also point to the fact that at present several different *Clostridium* species, the common characteristic of which is the production of orally effective neurotoxins, are subsumed under the name *C. botulinum*. Additionally, some strains of *C. baratii* and *C. butyricum* produce BoNT of the serotypes F and E, respectively (Rossetto et al., 2014).

BoNT are highly potent poisons and are considered to be the most toxic substances produced by living organisms (Gill, 1982). The seven known serotypes cause muscular paralyses by blocking the release of acetylcholine at the neuromuscular synapses. Due to the complexity of their toxic effect, the detection of *C. botulinum* neurotoxins represents a considerable diagnostic challenge (Dorner et al., 2013; Lindstrom and Korkeala, 2006). So far, BoNT are the only known bacterial toxins which are protected against environmental influences by non-toxic accompanying proteins. Based on current knowledge, these accompanying proteins are important for maintaining the toxicity of BoNT in the environment and during gastrointestinal passage. They also play an important role in passing epithelial barriers (Benefield et al., 2013; Gu et al., 2012; Lee et al., 2013, 2014).

Establishing the diagnosis bovine botulism is difficult and mainly based on the clinical picture and the exclusion of differential diagnoses (Stöber, 2002). As small amounts of BoNT are sufficient to cause severe disease, it is often difficult or impossible to detect the toxin in food or feed products, especially since it is not homogenously distributed throughout the matrix. Toxin detection in serum, organs or intestinal contents is also not always successful (CDC, 1998; Hatheway, 1990). As the pathogen occurs ubiquitously (Bell and Kyriakides, 2000; Espelund and Klaveness, 2014), detection of *C. botulinum* in the environment, in organs or in the intestinal contents is no proof of botulism. Therefore, the detection of BoNT is a prerequisite for the laboratory based diagnosis of botulism. In the recent years a number of detection methods for BoNT had been developed, one of these, the Endopep-MS, is demonstrating extraordinary high sensitivity for BoNT of several types in food (Kalb et al., 2015), but none has fully replaced the mouse bioassay (Dorner et al., 2013; Singh et al., 2013). The mouse bioassay still is the most sensitive and reliable detection method for biologically active BoNT in clinical samples. It is considered to be the standard method for botulinum toxin detection worldwide (Dorner et al., 2013; Lindstrom and Korkeala, 2006; Singh et al., 2013).

Human foodborne botulism is well known, but other forms of botulism are also recognized (Rossetto et al., 2014). Botulism can present as intestinal toxemia e.g. in infant botulism, when *C. botulinum* colonizes the intestine and then releases neurotoxin, which is absorbed into the bloodstream. As a result, infants suffer from descending motor weakness and flaccid paralysis (Midura and Arnon, 1976; Pickett et al., 1976). The insufficiently developed intestinal flora of infants up to one year may favor colonization with *C. botulinum*. Spores of *C. botulinum* are possibly ingested with honey (Hatheway, 1990). Infection of wounds with *C. botulinum* and subsequent botulism is known as wound botulism mainly in persons who inject drugs (Lindstrom and Korkeala, 2006; Rossetto et al., 2014).

A relatively common form, BoNT type C botulism, is seen in aquatic birds, particularly wild birds (Songer, 1997), but it is sometimes also seen especially in the summer season in domestic poultry (Songer, 1997). The bacterium *C. botulinum* is widespread in the environment, but botulism in livestock especially in cattle occurs only sporadic (Lindstrom et al., 2010). Botulism in cattle is mainly caused by BoNT types C and D. An association with poultry feces or litter has been observed (Livesey et al., 2004; Payne et al., 2011). BoNT types A and B were only rarely diagnosed in botulism cases of cattle (Lindstrom et al., 2010). In general, cattle intoxication is often caused by feed products which had been contaminated with carcasses, e.g. silages (Songer, 1997).

In addition to foodborne botulism in domestic animals, another form of disease in cattle has been discussed controversially. An untypical form of botulism is supposed to be caused by the colonization of the lower intestine with *C. botulinum* bacteria, subsequent production of BoNT and continuous resorption of small amounts of toxins resulting in chronic wasting of the affected dairy herd involving a complex of unspecific clinical symptoms, reduced performance and massive animal losses (Bohnel et al., 2001). The presumed trigger is a microbial imbalance in the digestive tract favoring the multiplication and toxin production of *C. botulinum*. It is assumed that this syndrome is multifactorial. This hypothesis has been proposed as "visceral" form of botulism (Bohnel et al., 2001). Especially, a significant number of dairy herds affected by chronic wasting condition in Northern Germany were suspected to suffer from "visceral" botulism (Bohnel and Gessler, 2012). Due to the unspecific clinical picture, a reasonable case definition based on clinical findings is still pending.

"Visceral" botulism was also discussed to be a sporadic, but severe zoonosis, when farmers or members of their families developed illness involving neurological syndromes (Dressler and Saberi, 2009).

The overall aim of this collaborative study was to investigate the cause of chronic disease in dairy cattle herds, which is associated with gradual wasting and can affect entire holdings. The crucial point of the hypothesis of "visceral" botulism is the production of BoNT by viable *C. botulinum* bacteria in the intestine of affected animals. The presence of BoNT in feces at detectable amounts is the key requirement of the hypothesis of chronic botulism. In fact, Bohnel et al. based their hypothesis on detection of BoNT in feces of affected animals using the mouse bioassay (Bohnel et al., 2001). Therefore, the confirmation of the presence of BoNT in the feces of affected animals applying the most sensitive analytical method for botulinum toxin detection i.e. the mouse bioassay can add more certainty to the hypothesis of "visceral" botulism in dairy cows.

2. Materials and methods

2.1. Study design

In order to investigate the association between chronic unspecific disease ("visceral" botulism) on cow and herd level and the presence of BoNT in fecal samples, a case-control study was carried out.

Criteria for case farms were: (1) a markedly reduced milk yield (>15% over a period of at least 3 month), (2) increased losses due to fatalities or euthanasia (>5% during the last 12 month), (3) a markedly increased replacement rate (>35% of the herd during the last year or an increase of > 10% compared to the year before), (4) an increased percentage of recumbent animals (>10% during the last 12 month) and (5) the subjective impression of the farmer or veterinarian of chronic herd health problems. Case farms (F) had to match at least three of these five criteria while control farms (K) had none of these problems. Case-1-farms (F1) had no history of clostridial vaccination, while case-2-farms (F2) had been vaccinated against clostridial diseases except *C. botulinum* intoxication (in Germany, clostridial vaccines are available covering diseases caused by *C. perfringens* type A, *C. perfringens* type B, *C. perfringens* type C, *C. perfringens* type D, *C. chauvoei*, *C. septicum*, *C novyi* type B, *C. sordellii*, *C. haemolyticum* and *C. tetani*). Further criteria for all farms were no vaccination against botulism, recording of milk production data, housing in free stalls, and a minimum number of 30 cows in the herd.

The study area was set to Northern Germany including the federal states of Schleswig-Holstein, Lower Saxony und North Rhine-Westphalia north of the federal motorway A2, where most of "visceral" botulism herds were supposed to be located (Bohnel and Gessler, 2012) and a homogeneous target population was apparent (Merle et al., 2012). All farmers participated voluntarily and subscribed either by themselves or by their veterinarians or other advisors. Eligibility criteria were requested in a telephone interview with the farmer and were verified, where possible, based on the milk recording data, prior to the farm visit.

Based on the hypothesis that there is an association between the occurrence of *C. botulinum* or BoNT and the case-control status of the farm, the sample size on farm level was assessed (confidence 95%, power ≥80%, detectable odds ratios 4, prevalence of controls 50%; calculated using NCSS Pass® (Hintze, 2007)) as 46 farms in non-vaccinated (F1), vaccinated (F2) and control farms (K) each. Possible influence of seasonality was accounted for by a balanced investigation of case and control farms over the study period.

During the farm visit, lactating cows and dry cows were scored with respect to lameness (locomotion score (Sprecher et al., 1997)), body condition (Edmonson et al., 1989), cleanliness (Cook and Reinemann, 2007) as well as skin and leg lesions (Reubold, 2003). During the herd scoring cows, which were eligible for being chosen as suspect, and control animals were preselected and marked. At each farm, five chronically sick animals (suspect animals) and five animals without symptoms (control animals) were selected randomly by the study team. Each of these 10 animals was subjected to a thorough clinical examination including neurological examination according to Dirksen et al. (2012), and samples of rumen content and fecal samples were taken. The sampling and clinical investigations at the farms were performed by veterinarians of the Clinic for Cattle of the University of Veterinary Medicine Hannover (TiHo). The study team consisted of veterinarians with advanced clinical experiences, who were trained additionally prior to the investigations. The main criteria for suspect animals were emaciation and habitus of a chronically sick animal (Dirksen et al., 2012) and showing at least one symptom of "visceral" botulism (Bohnel et al., 2001) such as reduced milk yield, locomotion score ≥ 3 (Sprecher et al., 1997), paresis, paralysis, ataxia, indigestion (constipation, diarrhea), behavioral change (apathy), asthenia, sensory disorders (eye lid closure reflex, ear reflex, skin reflex reduced or missing), respiratory disorders, circulatory disturbance (congested jugular vein), bulbar paralysis, chronic laminitis, retracted abdomen, respectively. Control animals were not allowed to show any of the mentioned symptoms and had no signs of acute disease and a locomotion score of 1 according to Sprecher et al. (1997).

2.2. Sampling

Fecal samples were taken from the rectum by a veterinarian; approximately 100 ml were placed in a screw cap sample tube (120 ml, Sarstedt, Nürmbrecht) and were then frozen at −20 °C. Frozen fecal samples were sent to the laboratory and were kept at −20 °C until processing.

2.3. BoNT detection by mouse bioassay

As the laboratory detection of BoNT was controversially discussed in the context of the hypothesis of "visceral" botulism, a protocol for the mouse bioassay was applied which was developed in collaboration with four German laboratories in a laboratory comparative test effort (Dlabola et al., 2013) to achieve maximum specificity and sensitivity. Twenty g of fecal sample were mixed with 20 ml of gelatin buffer (2 g/l gelatin, 4 g/l Na_2HPO_4) in filter bags and homogenized in a paddle blender (Stomacher, Seward, UK). The mixture was stored at 4 °C over night to extract the toxins. The mixture was centrifuged ($10{,}000 \times g$) to remove particles and was then sterile filtered (0.20 μm Minisart High Flow syringe filter, Sartorius, Germany). Two mice each were injected intraperitoneally with 0.5 ml extract which was diluted 1:2 in gelatin buffer. Two further mice were injected with trypsin treated extract (to activate potentially inactive BoNT of some types (Cook et al., 1998)) which was also diluted 1:2 with gelatin buffer. Another two mice received extract (1:2 in gelatin buffer) which was heated to 100 °C for 10 min as control, because BoNT is inactivated by this heat treatment. The mice were then observed for BoNT specific symptoms like wasp waist, labored abdominal breathing, paralysis and death within four days. In case of BoNT specific symptoms and death, mouse bioassay serotyping was supposed to follow in order to prove the presence of BoNT and to determine the serotype according to existing protocols (CDC, 1998; Cook et al., 1998). Mice

receiving a neurotoxic sample and a type specific antitoxin (A, B, C, D, E, and F) would survive.

The mouse bioassay was done according to the German Animal Welfare Act with permission of the respective authorities (Thuringia, State Office for Consumer Protection, reg. no. 22-2684-04-04-104/11).

2.4. Statistical analysis

All data collected in this study was entered in a web-based, relational SQL database individually designed for the project. Data was imported either manually or via import from Excel 2010 (Microsoft Corporation, Redmond, Washington).

Statistical analysis was carried out using SAS 9.3 (Cary, North Carolina). The unit of the statistical analysis was the farm and the animal, respectively. The case-control status of the farm as well as that of the animals was defined as dependent variable. The mouse bioassay test results were qualitative data and defined as explanatory variables.

3. Results and discussion

3.1. Participating farms and animals

During May 2012 and December 2013, 287 farms registered for study participation of which 141 farms were visited. As two farms were not located in the study region, 139 farms (45 F1-farms, 47 F2-farms and 47 K-farms) were included in statistical analyses, whereby the predefined sample size was reached. The distribution of the farms according to the federal states and epidemiological category (F1, F2, K) as displayed in Table 1 reflects the distribution in the target population. The over representation of farms in Lower Saxony and especially in Schleswig-Holstein is due to the higher reported occurrence of chronic wasting conditions in dairy herds at the time of sampling. Mean herd size was higher on control farms (K: 145 cows) compared to case farms (F1: 119 cows, F2: 116 cows). Most farms had mainly Holstein Friesians, but 17 farms (3 F1, 6 F2 and 8 K) had mainly Red Holstein or crossbreeds. Milk yield was higher on control farms (average: K: 26.0 kg per cow and day, F1: 22.8 kg, F2: 21.7 kg) due to the first eligibility criterion.

Identification of five control and five suspect animals was possible at all of the included farms. Besides the main criteria "emaciation" and "habitus of a chronically sick cow", all cows fulfilled at least one further criterion. Most suspect cows were lame with a locomotion score ≥ 3 ($n = 571$, 82.2%). Moreover, circulatory disturbances ($n = 206$, 29.6% of suspect cows), a reduced milk yield compared to pen average ($n = 122$, 17.6%), and retracted abdomen ($n = 115$, 16.5%) were assessed frequently. Also, indigestion (constipation, diarrhea) and respiratory disorders were diagnosed in 66 cows (9.5%) and 44 cows (6.3%), respectively. Neurologic disorders (paresis, paralysis, ataxia), sensory disorders, neurological disorders describing a bulbar paralysis like decreased reflexes at the head, and behavioral changes were assessed in less than 5% of suspect cows.

3.2. Detection of C. botulinum neurotoxin

1388 fecal samples (the fecal sample of one animal was not available and one animal was finally excluded because it was a heifer), were investigated for the presence of *C. botulinum* neurotoxins in the mouse bioassay. BoNT was not detected in any of the fecal samples and none of the mice died with specific symptoms. 33 mice died with unspecific symptoms (0.4% of 8512). It has to be kept in mind that death without clinical signs is not adequate evidence that botulinum toxin was present in the material injected (CDC, 1998). Furthermore, referring to the USDA/FSIS Microbiology Laboratory Guidebook (Cook et al., 1998), death in the absence of neurological symptoms is not an acceptable indication of mouse botulism; death may be non-specifically caused by other microorganisms, chemicals present in test fluids or injection trauma. Three samples were again tested in the mouse bioassay, because two mice each died with unspecific symptoms. One of these three samples revealed repeated unspecific toxicity in the mouse bioassay, this sample was negative for BoNT genes according to Takeshi et al. (1996) (data not shown). In a further analysis, *C. perfringens* epsilon toxin gene was detected in DNA extracted directly from the fecal sample in a PCR according to Baums et al. (2004) (data not shown). Even though *C. perfringens* epsilon toxin was not demonstrated itself, we speculate that it could have been the reason for the toxicity of the sample. Clinical signs in mice evoked by epsilon toxin include depression, ataxia, circling, dyspnea and death (Garcia et al., 2013) and these clinical signs are not in conflict with unspecific mouse deaths observed here.

The mouse bioassay was used to detect BoNT in fecal samples. This assay is still regarded as the most sensitive and standard method to detect BoNT in clinical samples. The investigations of the fecal samples gave no hint for the presence of BoNT. The mouse bioassay was also applied in

Table 1
Distribution of farms [*n*, (%)] by federal states and case-control status of farms (F1 = non-vaccinated case farm, F2 = vaccinated case farm, K = control farms).

	Lower Saxony	Northern Parts of North Rhine-Westphalia	Schleswig-Holstein	Total
Study population	65 (47)	7 (5)	67 (48)	139
K-farms	24 (51)	2 (4)	21 (45)	47
F1-farms	23 (51)	3 (7)	19 (42)	45
F2-farms	18 (38)	2 (4)	27 (58)	47
Target population[1]	13161 (60)	3756 (17)	5050 (23)	21967

1 = Milk producing farms; Source: Statistical offices of the federal states - agricultural census 2010, personal communication, October, 2013.

the study describing "visceral" botulism, in which the detection of BoNT in the feces of affected animals was the main point of the hypothesis (Bohnel et al., 2001). The explanation for this discrepancy can only be speculative; perhaps the misinterpretation of unspecific mouse deaths may play a role. Another possibility is that "visceral" botulism is an extremely rare disease in cattle. The study was thoroughly planned and the criteria for case farms and suspect animals were selected according to the published description of the observed herd and animal symptoms, and so the relevant study population was examined. Therefore, as a main outcome of this study we conclude that the hypothesis of "chronic" or "visceral" botulism could not be proven.

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