Original article

Is downer cow syndrome related to chronic botulism?

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Abstract

The present work was directed to investigate the relationship between Downer cow syndrome (DCS) and chronic botulism in dairy cattle. For this purpose, a total of 52 fresh calving downer cows and 206 apparently healthy cows at 14 dairy farms were investigated for *Clostridium botulinum* ABE and CD antibody levels, *C. botulinum* and botulinum neurotoxin in rumen fluids as well as in faeces. Results indicated that the downer cows had higher IgG titers for *C. botulinum* ABE and CD than the healthy cows. All tested rumen fluids were negative for BoNT and *C. botulinum*. BoNT/D, however, and *C. botulinum* type D spores were detected in faecal samples of healthy and downer cows in the selected farms. In conclusion, the presence of a significantly higher *C. botulinum* ABE and CD antibody levels in DCS cows than in the healthy animals suggests that chronic *C. botulinum* toxico-infection could be a predisposing factor for DCS.

Key words: Downer cow syndrome, *Clostridium botulinum*, BoNT, ELISA, chronic botulism, antibodies

Introduction

Downer cow syndrome (DCS) cow is an animal that has been recumbent for 24 hours or more, is in sternal recumbency, and is not suffering from hypocalcaemia, mastitis or any obvious injury of the limbs or spine (Eddy 2004). The development of DCS varies; however, splay leg and damage of muscles and nerves are the causes in 70% of DCS cases while 10% of cases are induced by myocarditis, 8% by hepatitis, 5% from persistent hypocalcaemia and 7% are of unknown etiology (Pehrson 2002). At necropsy, there is traumatic injury to limb muscles and nerves, ischemic necrosis of limb muscles, myocarditis, and fatty infiltration and degeneration of the liver. The disease occurs most commonly in the first 2 or 3 days after calving in heavy milk producers and often occurs concurrently with parturient paresis. One casual hypothesis is that the hypocalcaemia state or ischemia due to prolonged recumbency may increase cell membrane permeability of muscle fibers to allow the loss of potassium from the cell which, in turn, causes myotonia and the apparent basis for DCS (Goff 2004). Recumbency is also a diagnostic finding of chronic botulism

(Böhnel et al. 2001, Lund and Peck 2001, Holzhauer et al. 2009). Paralysis; apathy; edema in legs, udder, dew-lap; retracted abdomen; forced respiration and unexpected death are often seen in cases of chronic botulism (Krüger et al. 2012). Until recently a relationship between DCS and botulinum neurotoxin absorption in the large intestine had not been investigated. Botulism is a fatal disease characterized by progressive muscle weakness that leads to paralysis and possible death from respiratory failure (Keller 2006, Steinmann et al. 2007). *Clostridium botulinum* is an ubiquitous Gram-positive, spore forming obligatory anaerobic bacterium that is present in soil, dust and other organic matter such as feces of animals and man, manure, slaughterhouse waste, biogas plant, residues and bio-compost. It produces seven highly toxic neurotoxin isoforms (BoNT A-G) (Williamson et al. 1999, Böhnel and Lube 2001, Long and Tauscher 2006, Bagge et al. 2010). All isoforms, together with the related tetanus neurotoxin (TeNT) secreted by *C. tetani*, are $Zn2++$-endoproteases. The immunologically distinct neurotoxins (A-G) of *C. botulinum* are homologous proteins consisting of a long and short chain linked by an essential disulfide bridge. The short chain blocks the release of acetylcholine at the neuromuscular junction. Human cases of botulism are primarily caused by types A, B, or E, while animal diseases are mostly caused by types C and D (Böhnel et al. 2001, Foran et al. 2003, Popoff 2014). Recently, a new *C. botulinum* neurotoxin (Type H) was identified from a child with infant botulism (Barash and Arnon 2014, Dover et al. 2014). This new toxin, BoNT/H, cannot be neutralized by any of the currently available anti-botulinum antisera, which means that this toxin can't be diagnosed by available antisera (Relman 2014). Thus, animals or humans die but the disease is not diagnosed as botulism. Prevalence of the bacteria in soil is linked to an increased risk of botulism. *C. botulinum* may be acquired from the soil to colonize the intestines of food producing animals (cattle, pigs, chicken). The animals may then be asymptomatic carriers or may develop chronic or acute disease. *C. botulinum* may enter the food chain through these animals to cause food-borne botulism in man (Lund and Peck 2001). In recent years, an increased number of a newly described form of bovine botulism has been described. This form of botulism differs from regular food-born botulism by its slow and chronic development with various unspecific symptoms. This protracted form may develop when small, sub-lethal amounts of BoNT are taken up and/or are absorbed over several days (Böhnel et al. 2001). The main characteristics are increased cow mortality with the highest incidence during the peripartal period (Krüger et al. 2012). Recently, a relationship between gastrointestinal dysbiosis and *Clostridium botulinum* in dairy cows was described (Krüger et al. 2014). The objective of the present paper was to investigate the relationship between DCS and the detection of C. *botulinum* toxin or *C. botulinum* spores in rumen fluids or antibodies in blood serum of DCS cows at 14 German farms.

Materials and Methods

Animals and collection of specimens

A total of 52 fresh calving downer cows and 206 apparently healthy cows at 14 dairy farms were investigated. Downer cow samples were collected 6-48 h after detection of downer cow syndrome by the farmer. At the same time, about 6% (3 until 4 animals of the same lactation group per downer cow) of apparently healthy cows of the same herd were sampled. The age of the cows was between 2 and 11 years. The average body weight of the cows was 550-600 kg. Downer cows with hypocalemic parturient paresis were single treated intravenous with 500 ml Sorbivert® and C-B-Gluconat 24%® (Alvetra GmbH, Neumünster, Germany). The animals were injected again intravenously with 500 ml Sorbivert®, C-B-Gluconat 24%® and 20 ml Metaphosol® (Toldimfosnatrium-Trihydrat). Blood specimens were taken from the *Vena coccygea mediana*. After coagulation and centrifugation at 3,000 x g for 15 min, the serum samples were stored at -20ºC. Rumen fluid samples (1,000 mL per animal) were taken orally using a stomach pump tube. Rumen fluid samples and urine were homogenized and stored at -20ºC until used. Fecal samples were taken from fresh calving cows (farms No. 2, 4, 7, 13 and 14) six months later after taking samples from DCS and healthy cows at each farm. The investigated farms are characterized in Table 1.

Analysis of *C. botulinum* antibodies using ELISA

Solid phase antigen for ELISA

C. botulinum types A (7272), B (7273), C (2300), D (2301), and E (2302) obtained from the National Collection of Type Cultures (NCTC) were used to prepare ELISA antigens. Culture supernatants from *C. sporogenes* and *C. perfringens* (isolated and identified by the Institute of Bacteriology and Mycology, Faculty of Veterinary Medicine, Leipzig University) were used as a control antigen to study cross reactivity. All strains were cultured in reinforced clostridial medium (RCM; Sifin, Berlin, Germany) and incubated

Table 1. Characterization of investigated farms.

Farm No.	Milking cows	Total animals	Stillbirth frequency (heifers %)	Stillbirth frequency (cows %)	Culling rate %	Average downer cows/ month	Downer cows %	Biogas plant	Main disease problem	Animals tested	
										Downer cows	Healthy cows
1	164	326	8	6	30	1	2	no	infertility	1	6
2	329	946	>5	>5	37	4	3	Yes(2005	lameness	8	25
3	570	1254	5	6	35	5	5	No	lameness	6	9
4	164	394	8	10	25	5	10	Yes(2012)	infertility	9	15
5	64	211	16	5	25	3	10	No	infertility	1	4
6	662	1718	8	7	30	2	3	Yes (2013)	infertility	7	24
7	845	1932	9	4	31	4	2.5-4.5	Yes (2003)	no information	7	35
8	661	865	4	3	15	5	15	No	lamemess	2	43
9	94	219	>5	>5	20	1	2	No	mastitis	1	4
10	112	254	6	5	35	1	2	Yes (2008)	lameness	2	4
11	78	170	5	5	25	1	5	No	downer syndrome Lactation4	1	4
12	166	313	>5	>5	30	1	>1	No	lameness	2	10
13	213	414	7	12	25	2	3	No	infertility	1	12
14	149	444	9	9	15	4	10	Yes (2003)	No information	4	11

anaerobically at 37°C for 7 days followed by freezing at -25°C. Supernatants were checked for BoNT type with type specific ELISA (Krüger et al. 2012). After thawing and mixing, the culture suspension was centrifuged at 10,000 g for 15 min and the clear supernatant was decanted. BoNT proteins in the supernatants were detoxified with 20 mM formaldehyde (four additions weekly) and incubated at 37°C. Active formaldehyde groups were blocked by the addition of 100 mM lysine and 100 mM glycine in 100 mM Tris/HCl (pH 8.0) solution and incubated at RT for 24 h. Complete detoxification was verified with the mouse test by Dr. F. Gessler (Miprolab, Göttingen, Germany). The antigen preparation was washed against PBS (pH 7.4) and concentrated with ultrafiltration at a molecular weight cut-off of 50 kDa (Viva-Vivaspin 20, Sartorius Stedim Biotech, Göttingen, Germany). The protein concentration was measured with a spectral photometer (MBA 2,000) and its integrated software (PerkineElmer, Norwalk, Connecticut, USA) and adjusted with PBS to 1 mg/mL.

Detection of IgG-anti *C. botulinum* antibodies by ELISA

ELISA plates were coated with 100 μl/well of detoxified antigen from *C. botulinum* (1 mg/ml in 0.1 M $NaHCO_3$) and incubated overnight at 4-6°C. Coated plates were washed twice with 0.9% NaCl with 0.05% Tween 20 (Sigma-Aldrich, Taufkirchen, Germany) followed by blocking with 135 μl of blocking solution (1% bovine casein and 0.9% NaCl, Sigma Aldrich, Taufkirchen, Germany) for 30 min followed by addition of 15 ml diluted serum samples (1:10 in 50 mM Tris buffer, pH 8, containing 0.9% NaCl, 10 mM EDTA, 1% yeast extract, 1% BSA, 20% RCM and 1% Tween 20) and incubated for 1 h at RT on a microtiter plate shaker. After four washes, a 1:20,000 dilution of IgG from rabbits against bovine IgG (Fc) conjugated with horse radish peroxidase (HRP) (Dianova, Hamburg, Germany) in assay buffer (50 mMTris pH 7.4, 0.9% NaCl, 0.2% yeast extract, 0.1% BSA, 0.1% bovine Casein, 2% RCM and 0.1% Tween 20) was added to each well and incubated 1 h at RT. HRP activity was determined by adding 100 μl/well of 3 mM H_2O_2 and 1 mM 3,3',5,'5- tetramethylbenzidine (TMB) in 0.2 M citrate-buffer (pH 4.0). The substrate reaction was stopped with 1 M H_2SO_4 (50 ml/well) and the optical density (OD) was measured with an ELISA-reader at 450 nm. RCM without *C. botulinum* antigen served as a control antigen to determine the degree of non-specific solid phase binding of immunoglobulin in each sample (control OD). The control OD value was subtracted from each antigen specific OD value to calculate the

Anti- *C. botulinum* IgG relative to an internal laboratory standard (pooled blood samples from >3,000 cows) that was defined as 100 percent.

Analysis of free BoNT/A-E and *C. botulinum* spores in rumen fluid and faeces

Preparation of rumen fluid samples and faeces for detection of BoNT/A-E

Rumen fluid and faecal samples were diluted 1:3 in PBS (Dulbecco, pH 7.4) containing 0.1% Triton X-100, 0.1% Tween 20 and 10 mM EDTA. The samples were thoroughly mixed and frozen at -20°C. After thawing, the diluted samples were centrifuged at 7,000 x g for 15 min and the clarified supernatants were analyzed with BoNT-ELISAs.

Indirect detection of *C. botulinum* spores

Rumen fluid and faecal samples were diluted 1:10 (0.5 g in 4.5 ml) in RCM, vigorously mixed, heated at 80°C for 10 min, incubated at 37°C for 7 d under anaerobic conditions and subsequently stored at -20°C until tested. After thawing, the culture sample was centrifuged at 7,000 x g for 15 min and the clear supernatant was analyzed for type-specific soluble antigens of *C. botulinum* types A-E by ELISA.

BoNT-ELISA

BoNT/A-E were determined by an ELISA developed in our institute (Krüger et al. 2012). 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 NaHCO$_3$ and the wash solution (WS) was 0.9% NaCl with 0.05 % Tween20 (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 46°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 1M HCl] containing 0.9% NaCl, 5 mM EDTA, 1% gelatin from cold water fish skin, 0.2% bovine serum albumin, 0.1 mg/ml rabbit IgG from normal serum and 0.2% Tween 20 (chemicals from Sigma-Aldrich or Fluka, Taufkirchen, Germany). After incubation, the wells were washed five times with WS and loaded with the detection antibodies conjugated with HRP (diluted in assay buffer). *C. botulinum* Types A and B were detected with 2.5 ug/ml horse [Fab]2 from IgG against *C. botulinum* A and B (Novartis Vaccines and Diagnostics Co, Marburg, Germany). Types C and D were detected with 0.1 µg/ml of IgG from rabbits developed against BoNT/C and D (Institute of Bacteriology and Mycology, University of Leipzig). Type E was detected with 2.5 mg/ml IgG from horses against *C. botulinum* type E (WDT, Garbsen, Germany). After incubation at RT, the plates were washed four times with WS. HRP activity was determined by adding 100 µl/ml well of 3 mM H$_2$O$_2$ and 1 mM 3, 3', 5,5'-TMB. The substrate reaction was stopped with 1 M H$_2$SO$_4$ (50 ml/well) and the OD was measured with an ELISA-reader at 450 nm.

Evaluation of BoNT-ELISA

Relative units (RU) were calculated from the measured OD values as follows: (sample OD minus twice the value of the control-OD [BoNT-negative bovine feces sample]) multiplied by 1,000 and the dilution factor per minute substrate incubation time.

Statistical analysis

The statistical analysis was carried out with GraphPad Prism 4 (GaphPad Software, La Jolla, USA). A two-way analysis of variance followed by unpaired Student t-test was used to identify significant differences between means.

Results

Clinical history of tested animals

In the present study, a total of 52 fresh calving downer cows and 206 apparently healthy cows at 14 dairy farms were investigated. The percentage of DCS cows was between 2-15% in the farms investigated in this study (Table 1) and mineral treatment was not sufficient to compensate and prevent high losses. DCS cows did not respond for treatment with 500 ml Sorbivert® and C-B-Gluconat 24%®. The animals were injected again intravenously with 500 ml Sorbivert®, C-B-Gluconat 24%® and 20 ml Metaphosol® (Toldimfosnatrium-Trihydrat) without showing any progress.

Table 2. *Clostridium botulinum* and BoNT in faeces.

Farm No	BONT		C. botulinum	
	Positive	Suspected	Positive	Suspected positive
2	1/5	3/5	0/5	1/5
4	2/5	0/5	0/5	1/5
7	2/5	2/5	0/5	0/5
13	2/5	0/5	1/5	1/5
14	0/5	0/5	0/5	0/5

Botulinum neurotoxin (BoNT) and *C. botulinum* in rumen fluid and faeces

All rumen fluids were negative for BoNT and *C. botulinum*. BoNT/D and *C. botulinum* type D spores were detected (Table 2) in faecal samples from five cows (not simultaneously taken from DCS cows) on farms 2, 4, 7, 13, and 14. The number of BoNT positive samples in faeces of periparturient cows of farms were 3/5, 2/5, 4/5, and 2/5 for farms numbers 2, 4, 7, 13, and 14, respectively.

Detection of *C. botulinum* IgG antibodies in blood serum

The downer cows had higher IgG titres for *C. botulinum* ABE and CD than the healthy cows (Fig. 1).

Discussion

DCS is an important disease in dairy farms and most often is the cause of early loss of cows. The imbalance of minerals such as Ca, Mg and P are mentioned as the main causes of this disease (Barlet and Davicco 1992, Fürll 2005). Normally, infusion of these minerals is enough to facilitate recovery. The percentage of DCS cows was between 2-15% in the farms investigated in this study (Table 1) and mineral treatment has not been sufficient to compensate and prevent high losses. One hypothesis is that the hypocalcemic state, or ischemia, due to prolonged recumbency may increase cell membrane permeability of muscle fibers and allows the loss of potassium from cells to cause the myotonia as the physiological cause of DCS (Goff 2004).

The insufficiently higher levels of AST, CK, urea, Cu and Zn and significantly lower levels of cholesterol in downer cows were found compared with the healthy animals (data not shown). However Co, Mn and Se levels were similar for the two groups (data not shown). Since these results were obtained from 14 different farms, it is implausible that feeding had influenced them. Downer cows did not respond for treatment with Sorbivert® and C-B-Gluconat 24%® as well as intravenously injection of Sorbivert®, C-B-Gluconat 24%® and 20 ml Metaphosol®. Documentation about the success of treatment and about the future life does not happen.

ELISA is considered an effective and suitable means for analyzing immunological responses to botulinum toxins (Lindsey et al. 2003) and has been used to evaluate the immune response to various botulinum vaccines in cattle (Brown et al. 1999, Fujinaga et al. 2009). For measuring of *C. botulinum* ABE and CD antibody titers, a previously developed ELISA was used (Krüger et al. 2013). Our ELISA systems offer greater flexibility and economic technique for evaluation of humoral immune response towards *C. botulinum* vaccinations. DCS cows exhibit significantly higher *C. botulinum* ABE ($p<0.001$) and CD ($p<0.0001$) antibody titers than healthy cows on the same farm. Recumbency is also a clinical sign of chronic botulism (Krüger et al. 2012). The *C. botulinum* antibody levels and the detection of BoNT/D in faeces of periparturient cows of farms 2, 4, 7, and 19 show that this pathogenic spore forming bacterium occurs on these farms. The measured IgG antibodies in blood serum of animals are assumed to be antibodies resulting from the interaction with BoNTs, BoNTs complexes and progenitor toxins (PT) (Nakamura et al. 2007, Fujinaga 2010) hence *C. botulinum* serotypes C and D possess five genes involved in the progenitor constituents and produce two types of PT, M-PT (a complex of BoNT and NTNHA) and L-PT (a complex of M-PT and HAs). The L-TP containing HA-33 component transport across the intestinal epithelial cell monolayer is more effective than pure BoNTs (Fujinaga et al. 1997, Inui et al. 2010, Sagane et al. 2012). Antagonistic bacteria (enterococci) are very important in preventing germination and proliferation of C. *botulinum* in the gastrointestinal tract (Shehata et al. 2013). Herbicide Roundup®, with its active component glyphosate is a highly effective and important herbicidal chemical that
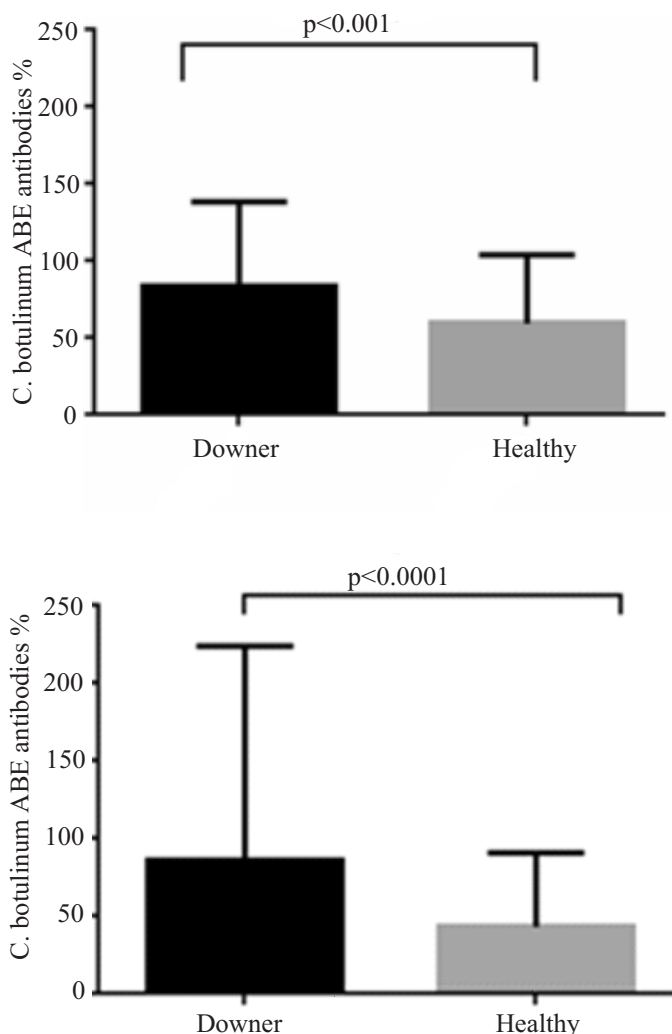
Fig. 1. *C. botulinum* ABE and CD antibody levels in downer (N= 52) and healthy cows (N= 206).

inhibits 5-enolpyruvyl shikimate 3-phosphate synthase (EPSPS), an enzyme of the shikimate pathway in plants, bacteria, fungi, algae and protozoa (Barry and Padgette 1992). This enzyme converts phosphoenol-pyruvic acid and 3-phosphoshikimic acid to 5-enol-pyruvyl-3-phoshoshikimic acid. Inhibition of this enzyme (EPSPS) shuts down the shikimate pathway to inhibit aromatic amino acid biosynthesis (Cerdeira and Duke 2006). Since glyphosate herbicides inhibit aromatic amino acid biosynthesis, they are toxic to many bacterial cells. Kruger et al. (2013) reported that glyphosate reduces antagonistic enterococci and supports *C. botulinum* because it is resistant to glyphosate.

In the present study, BoNT were tested in animal feces and rumen fluid using a simple and rapid enrichment method to detect toxigenic *C. botulinum*. This ELISA showed a high sensitivity (100%) and specificity (100%) (Krüger et al. 2013). The limits of detection were 4, 17, 4, 2 and 37 pg/mL for BoNT types A, B, C, D and E, respectively (Krüger et al. 2013).

Interestingly, *C. botulinum* was not detected in the rumen fluid indicating that proliferation takes place in the hind gut. *C. botulinum* occurs in feed, and grass silages are often contaminated (Krüger et al. 2012). In particular, silages from pastures fertilized with biogas plant residues are highly contaminated and we have documented that biogas plant residues harbor *C. botulinum* spores (Neuhaus et al. 2015).

In conclusion, the presence of a significantly higher *C. botulinum* ABE and CD antibody levels in DCS cows than the healthy animals suggests that the chronic *C. botulinum* toxico-infection could be a predisposing factor for DCS.

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