Pathogenesis and toxins

Efficacy of *Clostridium botulinum* types C and D toxoid vaccination in Danish cows

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**A B S T R A C T**

In the present study the efficacy of botulism vaccine (formalised aluminium hydroxide gel adsorbed toxoid of *Clostridium botulinum* types C and D) was evaluated in four Danish dairy cows under field conditions. Other four dairy herds were unvaccinated. Blood serum of all animals was analyzed for specific *C. botulinum* types A, B, C, D and E antibodies using a developed ELISA. Feces of all animals were analyzed for botulinum neurotoxins (BoNTs) and *C. botulinum* spores. *C. botulinum* types C and D antibodies were significantly (*p < 0.05) increased in vaccinated animals. Vaccination with botulism vaccine significantly reduced (*p < 0.001) BoNTs and *C. botulinum* spores in cattle feces. Our findings represent that *C. botulinum* vaccination increases specific blood serum antibodies and reduces free BoNTs and *C. botulinum* spores in feces.

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1. Introduction

Botulism is caused by highly potent neurotoxins produced by *Clostridium* (C) *botulinum*, Gram-positive, anaerobic spore-forming bacteria. *Clostridium botulinum* is a ubiquitous Gram-positive, spore forming obligate anaerobic bacterium that primarily inhabits soil, dust and organic matter such as feces of animals and man, slaughterhouse wastes, biogas plant residues, and bio-compost [1-4]. Seven highly toxic neurotoxin isoforms (BoNTs) designated A-G are generated by *C. botulinum*. BoNTs are homologous proteins consisting of a 100-kDa heavy chain (HC) and a 50-kDa light chain (LC) of amino acids linked by an essential disulfide bridge that blocks the release of acetylcholine at the neuromuscular junction [5,6]. Human cases are mostly caused by types A, B, or E, while animal diseases are mostly caused by types C and D [7,8]. Acute intoxication caused by ingesting of preformed BoNTs in feed is well known [6]. In recent years, there has been an increasing incidence of a newly described form of bovine botulism which differs from regular food-borne botulism by its slow and chronic development and variety of non-specific symptoms. This protracted form may be developed when small sub-lethal amounts of BoNT are ingested or are produced in the gastrointestinal tract (GIT) and absorbed in small amounts over several days [8]. Recovery of diseased animals is difficult or impossible. In sub-acute cases, an immediate application of specific antibodies is helpful, but the costs of this therapy are often prohibitive [9]. There are two basic alternatives for prophylaxis of high risk individuals from *C. botulinum* intoxication; active immunization using a vaccine, or passive immunotherapy using immunoglobulin. The best known strategy to prevent *C. botulinum* types C and D infections in cows is vaccination. In endemic countries such as Australia, Brazil, South Africa and Israel, approved vaccines and vaccination programs exist to prevent acute and sub-acute *C. botulinum* types C and D intoxication [6]. In the present study the immunogenicity of a formalised aluminium hydroxide gel adsorbed toxoid of *C. botulinum* types C and D was evaluated in Danish dairy cattle using a developed ELISA system. Also the impact of vaccination on free BoNTs and *C. botulinum* spores in feces was studied.

2. Material and methods

2.1. Vaccine

Botulism vaccine, formalised aluminium hydroxide gel adsorbed toxoid of *C. botulinum* types C and D (Onderstepoort Biological Products, South Africa).
2.2. Animals and vaccination

The present experiment was performed in eight Danish farms designated W, K, R, V, S, T, B, and E. Animals kept in farms W, K, and V were vaccinated with botulism vaccines. Recommended doses of vaccine were injected subcutaneously. Vaccination regimen and time of sampling were described in Table 1. Animals kept in farms S, T, B, and E were not vaccinated. Random thirty cows (15 freshly calved and 15 highly yielding) from each farm were investigated for seroconversion as well as free BoNTs and C. botulinum spores in feces.

2.3. Analysis of humoral immune response using ELISA

2.3.1. Solid phase antigen for ELISAs

C. botulinum types A (7272), B (7273), C (2300), D (2301), and E (2302) obtained from National Collection of Type Cultures (NCTC) were used for preparation of ELISA antigens. Also culture supernatant from Clostridium sporogenes and Clostridium perfringens (Isolated and identified in Institute of Bacteriology and Mycology, Faculty of Veterinary Medicine, Leipzig University) were served as a control antigen to study the cross reactivity. All strains were cultured in reinforced clostridial medium (RCM; Sifin, Berlin, Germany) and incubated anaerobically at 37 °C for 7 days followed by freezing at −25 °C. Supernatants were checked for BoNT-type with type specific ELISA [9]. After thawing and mixing, the culture suspension was centrifuged at 10,000× g for 15 min and the clear supernatant was separated. 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). Data not shown. The antigen preparation was washed against PBS (pH 7.4) and concentrated with ultrafiltration at a molecular weight cut-off of 50 kDa (viva-tris, 20, Sartorius Stedim Biotech, Göttingen, Germany). The protein concentration was measured with a spectral photometer MBA 2000 and its integrated software (Perkin–Elmer, Norwalk, Connecticut, USA) and adjusted with PBS to 1 mg/ml.

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

ELISA plates were coated with 100 μl/well of detoxified antigen from C. botulinum (1 μg/ml in 0.1 M NaHCO₃) 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). After 30 min incubation, 15 μl 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 min at RT on a microtiter plate shaker. After 4 times washing, IgG from rabbits against bovine IgG (Fc) conjugated with horse radish peroxidase (HRP) (Dianova, Hamburg, Germany) diluted 1:20,000 in assay buffer (50 mM Tris 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. The HRP activity was determined by adding 100 μl/well of 3 mM H₂O₂ 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 H₂SO₄ (50 μl/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 on each sample (control OD). The control OD value was subtracted from each antigen specific OD value to calculate the Anti-C. botulinum IgG-levels relative to an internal laboratory standard (pooled blood samples from >3000 cows) that was defined as 100 percent.

2.4. Analysis of free BoNT/A–E and spores in feces

2.4.1. 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. The samples were 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-ELISAs.

2.4.2. Indirect detection of C. botulinum spores

Fecal specimens were diluted 1:10 (0.5 g in 4.5 ml) in RCM, 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.

2.4.3. BoNT-ELISA

The BoNT/A–E were determined by an ELISA developed in our institute [10]. 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-Immu-no-Washer 12 (Nunc, Wiesbaden, Germany). After coating the ELISA wells with capture antibodies (3 μg/ml, BoNT-immunoadfinity 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 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 μg/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 μg/ml

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Table 1

<table>
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<tr>
<th>Farm</th>
<th>Total number</th>
<th>Average milk yields kg/cow</th>
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<th>Samplingb</th>
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<td>2×</td>
<td>10.02/10.03.2012</td>
</tr>
<tr>
<td>K</td>
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<td>10.4</td>
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<td>15.03/15.04.2012</td>
</tr>
<tr>
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<td>1×</td>
<td>16.05.2012</td>
</tr>
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<td>13.06.2012</td>
</tr>
<tr>
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<td>11.2</td>
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<td>20.06.2012</td>
</tr>
<tr>
<td>B</td>
<td>400</td>
<td>10.8</td>
<td>Unvaccinated</td>
<td>20.06.2012</td>
</tr>
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<td>E</td>
<td>300</td>
<td>8.8</td>
<td>Unvaccinated</td>
<td>27.06.2012</td>
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</table>

a Botulinum vaccine, formalised aluminum hydroxide gel adsorbed toxoid of C. botulinum types C and D.

b Both blood and fecal samples were collected from 30 animals/farm (15 freshly calved and 15 highly yielding cows). Serum was used for testing of Clostridium botulinum types A, B, C, D and E antibodies using ELISA. Fecal samples were tested for BoNT types A, B, C, D and E and spores (indirectly).
IgG from horses against C. botulinum type E (WDT, Garbsen, Germany). After incubation at RT, the plates were washed four times with WS. The HRP activity was determined by adding 100 µl/well of 3 mM H2O2 and 1 mM 3', 5'-TMB. The substrate reaction was stopped with 1 M H2SO4 (50 µl/well) and the optical density (OD) was measured with an ELISA-reader at 450 nm. The sensitivity, specificity, precision, limit of detection, range of quantification and cross-reactivity with Clostridium tetani, C. perfringens, C. sporogenes, Clostridium sordellii, Clostridium novyi, Clostridium butyricum, Bacillus cereus, Streptococcus agalactiae, Streptococcus zooepidemicus, Staphylococcus aureus, Staphylococcus epidermidis, Escherichia coli, Proteus vulgaris, Proteus mirabilis, Pseudomonas aeruginosa, Candida albicans, Candida krusei were determined (Table 2).

2.4.4. Evaluation of BoNT-ELISA

The relative units (RU) were calculated with the measured OD-values as follow: (sample-OD minus twice the value of the control-OD [BoNT-negative sample of bovine feces]) multiplied by 1000 and dilution factors per minute substrate incubation time.

2.5. Statistical analysis

The statistical program SPSS 15 (SPSS Inc. Headquarters, 233 S. Wacker Drive, 11th floor, Chicago, Illinois 60606) was used for data analysis. The recorded parameters were tested for normal distribution by the Shapiro-Wilkinson Test. Because of a normal distribution, the arithmetic mean and standard deviation were calculated. Median and 1st and 3rd quartiles were calculated for normally distributed values. Significance tests using Fisher’s Exact Test and Mann–Whitney U Test were performed.

3. Results

3.1. Humoral immune response

C. botulinum types A–E antibodies were detected using ELISA plates coated with C. botulinum types A, B and E or types C and D antigens. No cross reactivity were observed with C. sporogenes and C. perfringens antigens. The average antibody titers of cows at the eight different farms as a percent of the normal blood serum pool from >3,000 cows (100%) are presented in Figs. 1 and 2. C. botulinum types C and D antibodies were significantly increased (p < 0.05) in animals kept in farms W, K and V (vaccinated twice) while animals kept in farm R showed no significant seroconversion (Figs. 1–3).

3.2. Free BoNT/A–E in animal feces

The free BoNT/A–E in animal feces were tested using BoNT-ELISA. The numbers of animals which were positive for BoNTs were significantly higher (p < 0.001) in unvaccinated compared with vaccinated animals (Fig. 4). BoNT/D was the predominant type in most of positive cases. Some samples were positive also for BoNT/A, C and E.

3.3. C. botulinum in animal feces

The numbers of animals which were positive for C. botulinum spores in feces analyzed indirectly by measurement of their spores in feces analyzed indirectly by measurement of their spore content measured by ELISA were significantly higher (p < 0.001) in unvaccinated compared with vaccinated animals (Fig. 1). BoNT/D was the predominant type in most of positive cases. Only one sample out of 30 tested samples from farm B (unvaccinated) was positive for C. botulinum type B. Interestingly, unvaccinated herds had significantly more free BoNT/D and C. botulinum type D positive cows than vaccinated animals (Fig. 4).

3.4. Relationship between antibody titres, free BoNTs and C. botulinum spores

As shown in Fig. 4, vaccinated animals which showed C. botulinum types C and D antibodies have significantly lower (p < 0.001)
free BoNT/D and botulinum spores. Vaccinated cows with detectable BoNTs, *C. botulinum* or BoNT/*C. botulinum* >10 RU/ml had significantly lower antibody titres. Significant antibody titres to *C. botulinum* types A, B and E (*p* = 0.002) also were seen between BoNTs positive and negative animals.

4. Discussion

Most botulinum toxoid vaccines, including the *C. botulinum* types C and D vaccine are crude extracts of BoNTs, progenitor toxins (PT) and other bacterial components as well as residual formalin in toxoid [6,11,12]. The accurate and sensitive measurement of antibodies against botulinum toxins is an important concept in vaccine development and clinical analysis. ELISA is considered an effective and suitable means for analyzing immunological responses to botulinum toxins [13] and has been used to evaluate the immune response to various botulinum vaccines in cattle [11,14] and humans [15].

For measuring of humoral immune response towards *C. botulinum* types C and D vaccination, ELISA was developed in the present study. The crude culture supernatants of *C. botulinum* types A-E were filtered with 50 kDa molecular weight cut-off filters to remove small proteins and formalin and to ensure that BoNTs, various haemagglutinins and their complexes are components of ELISA. It is well known that *C. botulinum* serotypes C and D possess five genes involved in the progenitor constituents and produce two types of progenitor toxins (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 [16–19]. The measured IgG antibodies in blood serum of animals are assumed to be antibodies resulting from the interaction with BoNTs, BoNTs complexes, PT and other proteins out of vaccine [20,21]. Gregory et al. [22] used a similar *C. botulinum* antigen preparation for development of ELISA system using crude culture supernatants of types C and D filtered with a 30 kDa molecular weight cut-off filter. An internal laboratory standard (pooled blood samples from >3000 cows) (defined as 100 percent) was used for calculation of the Anti-*C. botulinum* IgG–levels in vaccinated and unvaccinated animals. Our ELISA systems offer greater flexibility and economy technique for evaluation of humoral immune response towards *C. botulinum* vaccinations. *C. botulinum* types C and D antibodies

**Fig. 2.** Distribution of *C. botulinum* in unvaccinated farms (*n* = 30 per farm). Figures a, b, c and d represent farms S, T, B and E, respectively.

**Fig. 3.** *C. botulinum* types C and D antibodies in vaccinated (W, K, R and V) and unvaccinated (S, T, B, and E) farms. *C. botulinum* types C and D antibodies were significantly increased in vaccinated animals (*p* < 0.05). Cows vaccinated once with Botulism vaccine showed no significant seroconversion (Farm R).

**Fig. 4.** Influence of *C. botulinum* types C and D vaccination on the detection of BoNT type D and *C. botulinum* type D spores in feces of Danish cows. Asterisks indicate significant difference (*p* < 0.001). *N* unvaccinated = 120 *N* vaccinated = 120 (Fisher’s Exact Test).
were significantly increased in vaccinated animals. Cows which vaccinated once did not show significant seroconversion, however, previous studies demonstrated that either single or double immunizations with bivalent toxoid type D vaccines have provided good protection from type D toxin for 50 weeks [23] to 24 months [24]. In contrast, Steinman et al. [6] reported type D outbreaks 10–12 months after the last vaccination. These authors attributed these short protective times to exposure of the animals to very high BoNT/D levels in Israel.

As the used ELISA measures total neutralizing and non-neutralizing antibodies binding to a given antigen, but does not specifically measure the neutralizing antibody to the toxin [13], we have also tested the free BoNT/A-E and spores in animal feces. Vaccination against C. botulinum types C and D significantly reduced BoNT/D as well as C. botulinum type D spores in feces (Fig. 1). We assume that this reduction is a result of neutralizing antibodies to PT and BoNTs that interfere with gut motility. Botulism is not contagious by casual contact, but it can be transmitted between animals by predation or cannibalism. Contaminated foods usually contain spores as well as the toxin. Spores that are passing through the gastrointestinal tract may germinate and grow if the animal dies. It is believed that contamination of broiler litter with the carcasses of chickens that have died from various causes during animal dies. It is believed that contamination of broiler litter with animal feces, in areas where botulism is relatively common, vaccines may be used in animals including horses, cattle, sheep, goats, mink and birds to reduce the risk of botulism. Otherwise BoNTs and botulinum spores in animal feces can perpetuate the cycle, and may result in large outbreaks in birds or other species. Outbreaks of botulism can also contaminate the environment with spores, making future outbreaks more likely.

In conclusion, in the present study we have developed an ELISA system for evaluation of humoral immune response towards botulinum vaccination. C. botulinum vaccination increases specific blood serum antibodies and reduces free botulinum neurotoxins and C. botulinum spores in feces that reduce the risk of botulism.

References