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Immune response of horses to vaccination with the recombinant Hc domain of botulinum neurotoxin types C and D

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ABSTRACT

Botulinum neurotoxins, predominantly serotypes C and D, cause equine botulism through forage poisoning. The C-terminal part of the heavy chain of botulinum neurotoxin types C and D (HcBoNT/C and D) was expressed in *Escherichia coli* and evaluated as a recombinant mono- and bivalent vaccine in twelve horses in comparison to a commercially available toxoid vaccine. A three-dose subcutaneous immunization of adult horses elicited robust serum antibody response in an ELISA using the immunogen as a capture antigen. Immune sera showed dose-dependent high potency in neutralizing specifically the active BoNT/C and D in the mouse protection assay. The aluminium hydroxide based mono- and bivalent recombinant HcBoNT/C and D vaccines were characterized by good compatibility and the ability to elicit protective antibody titers similar or superior to the commercially available toxoid vaccine.

1. Introduction

Botulinum neurotoxins (BoNT), produced by *Clostridium botulinum*, are the most poisonous substances known. Although divided into seven serotypes according to their distinct antigenicity, all serotypes share a similar structure. BoNT are proteins of approximately 150 kDa, with a heavy chain (100 kDa) and a light chain (50 kDa) linked by a disulfide bond. They act at the neuromuscular junction blocking the release of acetylcholin, interfering within the exocytotic mechanism (cleavage of a SNARE protein) thus leading to a flaccid paralysis [1]. Classical botulism affects all mammals, birds, and fish. Outbreaks in animals are characterized by the involvement of several to all animals of a group, a high mortality and possibly important financial losses.

The most common form of equine botulism is due to forage poisoning, an emerging problem in Europe often related to feeding of grass silage [2,3]. Horses are considered to be very sensitive to BoNT [4] and serotypes C and D are most common in horses [5]. Worldwide, only two commercial toxoid vaccines for protection against botulism are licensed for use in horses: type B (BotVax B®; Neogen Corporation, Lexington, KY, USA) and types C and D (Botulism Vaccine®; Onderstepoort Biological Products, Onderstepoort, RSA). Shortcomings associated with the pentavalent toxoid vaccine for use in humans are well known and similar to the toxoid products for horses. The toxoid product consists of a crude extract of clostridial protein containing residual formalin, which may influence the immunogenicity and reactogenicity of the vaccine. Because of safety measures and the relatively low yields of toxin production from *C. botulinum*, production costs of toxoid vaccines are high [6].

The carboxy-terminal 50 kDa domain of the heavy chain (Hc) of BoNT, which alone is non-toxigenic, mediates receptor binding to target neurons [1]. The Hc domain of the serotypes A–F, produced in recombinant *Escherichia coli* or *Pichia pastoris*, has been demonstrated to elicit protective immunity in laboratory animals [6–10]. The Hc domain elicits similar protective immunity compared to the whole heavy chain, in contrast to the amino terminal domain of the heavy chain (Hn), which is much less immunogenic [11].

Studies on subunit vaccines for serotypes predominantly affecting animals, like types C and D, have been performed using mice and duck [7,11–14]. In a preceding study in our laboratory, HcBoNT/C in combination with an adjuvant based on solid lipid cationic microparticles and glycopeptides derived from *Lactobacillus bulgaricus* was tested in two adult horses and compared to the commercially available type C and D toxoid vaccine [15]. It was concluded that the recombinant vaccine showed fewer adverse reactions compared to the only commercially available vaccine but induced a similar protective level of neutralizing antibodies. There was no correlation between the serological response to HcBoNT/C and the neutralizing capacity of serum [15]. Based on these results, the study was conducted including HcBoNT/D and investigating the immune response to mono- and bivalent recombinant vaccine formulated either with the microparticle based adjuvant or aluminium hydroxide in adult horses. Sera of the four horses were

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tested again using refined protocols for ELISA and mouse bioassay. For this purpose the recombinant Hc domains of BoNT/C and D were formulated as mono- and bivalent vaccines with two types of adjuvants, and investigated in adult horses. The immune response was tested serologically by an indirect ELISA for total IgG against recombinant HcBoNT/C and D as well as against BoNT/C and D holotoxin used as capture antigens. Neutralizing antibodies were quantified in the mouse bioassay. Adverse reactions to the vaccine were monitored.

2. Materials and methods

2.1. Recombinant vaccine

HcBoNT/C was produced in *E. coli* BL21 (DE3) carrying plasmid pET28a (Novagen, Gibbstown, NJ, USA), with the gene fragment of *C. botulinum* type C strain 468 [16], which encodes the 425 C-terminal amino acids of the heavy chain of BoNT/C. HcBoNT/D was produced similarly from *C. botulinum* type D strain 1873 [17], including the 413 C-terminal amino acids. The recombinant plasmids were constructed and the recombinant proteins were expressed and purified by cobalt chelate affinity purification (Clonetech, Takara Bio Inc., Shiga, Japan) as described for HcBoNT/A [18] and dialyzed against PBS buffer. Purity of the recombinant protein was confirmed by visual inspection of coomassie-blue stained SDS-PAGE in comparison to a molecular weight marker.

Two types of adjuvants were used for the formulation of the experimental vaccine: GERBU Pharma Adjuvant® (GPA) (Gerbu Biotechnik GmbH, Gaiberg, Germany), which is an adjuvant based on solid lipid cationic microparticles and glycopeptides derived from *L. bulgaricus*, and aluminium hydroxide (Alu Gel-S®; SERVA Electrophoresis GmbH, Heidelberg, Germany). Antigen stocks of HcBoNT/C and D were diluted in PBS with either Gerbu Pharma Adjuvants 80% or Alu-Gel-S SERVA (aluminium hydroxide 0.25%) as an adjuvant under aseptic conditions to a final volume of 2.5 ml.

2.2. Immunization of horses

Ten adult Swiss Warmblood horses were vaccinated subcutaneously in the brisket three times at a one- and three-month interval with either 100 μg of HcBoNT/D using GPA as an adjuvant (nr. 5 and 6) or a combination of 100 μg each of HcBoNT/C and D using GPA (nr. 7) or aluminium hydroxide (nr. 8–10) as an adjuvant, or a combination of 10 μg each of both HcBoNT/C and D using aluminium hydroxide as an adjuvant (nr. 11–14) in a total volume of 2.5 ml. Sera of four adult Swiss Warmblood horses of a previous vaccination study were re-evaluated. Two horses (nr. 1 and 2) had been vaccinated intramuscularly with a commercially available BoNT type C and D toxoid vaccine (Botulism Vaccine®; Onderstepoort Biological Products, Onderstepoort, RSA) and two horses (nr. 3 and 4) had been vaccinated subcutaneously with 100 μg of HcBoNT/C using GPA as an adjuvant thrice at a one- and seven-month interval.

All horses were in good general condition and did not show any signs of illness. During the whole vaccination period, the animals were clinically supervised and pathological symptoms, in particular local reactions at the injection site, were recorded. Blood samples from horses were collected by jugular venipuncture into serum separator tubes on the day of vaccination prior to the injection of the vaccine and two weeks after. Following clot formation at room temperature, they were centrifuged at 2500 × *g* for 15 min; individual serum aliquots were prepared and stored frozen at −20 °C. All procedures applied were in accordance with Swiss guidelines of animal welfare and had been approved by the Federal Veterinary Office of Switzerland (approval number 155/02 and 9/06).

2.3. Measurement of serum antibody titers

2.3.1. Indirect ELISA for the detection of IgG antibodies against HcBoNT/C or D

Maxisorp microtitration plates (Nunc GmbH & Co. KG, Langenselbold, Germany) were coated with 100 ng/well of HcBoNT/C or HcBoNT/D in 100 μl carbonate buffer (0.05 M, pH 9.6) at 4 °C overnight. After three washings with PBS-Tween (0.1%) buffer, non-specific binding sites were blocked with 200 μl carbonate buffer containing 2% bovine serum albumin (BSA) for 45 min at room temperature on a microplate shaker (600 rpm). Three washings were followed by the addition of equine sera diluted (1:100) in PBS-Tween (0.1%) in quadruplicate, each at 100 μl/well for 1.15 h at room temperature on the shaker. The optimal serum dilution (1:100) was determined beforehand by serial dilutions to endpoint (as an example see supplementary data, Figure S1). For background control values no serum was added.

After a further washing step, 200 μl of peroxydase-labelled rabbit anti-horse IgG antibody (1:20,000) (Sigma–Aldrich Corp., St. Louis, MO, USA) or 200 μl of peroxydase-labelled anti-rabbit IgG antibody (1:20,000) (Kirkegaard & Perry Laboratories Inc., Gaithersbury, MD, USA) diluted in PBS-Tween (0.1%) was added to the corresponding wells and plates were kept 1.5 h at room temperature on the shaker. After a new cycle of washings, enzymatic activity was shown by the addition of 200 μl/well orthophenylene-diamine substrate (0.4 mg/ml) (Sigma–Aldrich Corp., St. Louis, MO, USA) in citrate buffer (0.05 M, pH 5) containing 0.06% hydrogen peroxide. Plates were kept in darkness and the reaction was read after 10 min at 450 nm (MRX Microplate Reader, Dynex Technologies, Chantilly, VA, USA). Results are expressed as the mean values of four measurements of the OD given at a dilution of 1:100 after subtraction of the OD of the background controls.

2.3.2. Indirect ELISA for the detection of IgG antibodies against BoNT/C or D holotoxin

Microtitration plates coated with purified BoNT/C (*C. botulinum* type C) or D (*C. botulinum* type D) (Metabiologics Inc., Madison, WI, USA) were used as described above to determine total IgG against holotoxin. As secondary antibody, 200 μl/well alkaline phosphatase-labelled anti-horse IgG in PBS-Tween (1:30,000) (Sigma–Aldrich Corp., St. Louis, MO, USA) was used and incubated for 2 h at room temperature. The bound antibody was detected after incubation with p-nitrophenylphosphate in Tris buffer (Sigma–Aldrich Corp., St. Louis, MO, USA) for 30 min in the dark. Absorbance values were immediately read at 405 nm (MRX Microplate Reader). Results are expressed as the OD given at a dilution of 1:100 after subtraction of the OD of the background controls.

2.3.3. Mouse protection assay

Neutralizing antibody titers to BoNT/C and D, were measured by the sera's ability to neutralize the neurotoxin in vitro in combination with the mouse lethality assay according to the European Pharmacopoeia [19]. For the mouse protection assay, male mice EOPS, Charles River, weighing 16–18 g, were used. Botulinum toxin was prepared from culture of *C. botulinum* type C strain 468 and *C. botulinum* type D strain 1873 grown in TGYH medium for four days under anaerobic conditions at 37 °C. The culture was centrifuged at 10,000 × *g* for 15 min at 4 °C to separate bacteria and bacterial debris from the culture supernatant that was subsequently acidified to pH 3.5, stabilized with 5% sterile glycerol, and stored at 4 °C. Neutralizing antibodies were assayed by the recommended method from the European Pharmacopeia known as the "L+" test. The test dose determines the relationship between a toxin concentration and a reference antitoxin serum. By definition, one L+ toxin dose is the smallest quantity of toxin, when mixed with one international unit (IU) of reference serum, that kills 100% of injected mice by intra-

peritoneal route within 96 h. International standards for botulinum type C (NIBSC code 01/508) and type D (NIBSC code 61/001) were obtained from NIBSC. The test dose, which is the smallest amount of toxin in 0.5 ml volume when incubated with standard serum (representing 0.5 IU/ml for type C and 5 IU/ml for type D preparations used in this study) causing the death of a group of four mice (0.5 ml injected intra-peritoneally into each mice), was 5L+10 (40,000 LD_{100}/ml) for type C and 5L+ (10,000 LD_{100}/ml) for type D. Variable volumes of horse serums diluted in phosphate buffer (pH 6.3) containing 0.2% gelatine were mixed with the test dose (2 ml) and the final volume was adjusted to 5 ml with buffer. The mixtures were homogenized and incubated at room temperature for 1 h. 0.5 ml of each mixture was injected by intra-peritoneal route at each mice of a group of 4. The mice were observed for 96 h. The mixture that contains the largest volume of antitoxin that fails to protect the mice from death contains 5 IU for type D and 0.5 IU for type C. The detection limit of this assay was 0.25 IU/ml for BoNT/D and 0.25 IU/ml for BoNT/C. Neutralizing antibodies titers are given as international units per ml serum, one international unit (IU) of antitoxin being defined as neutralizing 10^4 mouse IP LD_{50} [20].

3. Results

3.1. Safety of the recombinant vaccine based on aluminium hydroxide is superior

All seven horses vaccinated with the aluminium hydroxide based recombinant vaccine tolerated the vaccine very well, and showed only minor painless local swelling which resolved completely after two days. The Botulism Vaccine® caused more adverse reactions in the two horses vaccinated with this product. Both horses showed formation of painful oedema, which completely resolved after one to two weeks. With the GPA based recombinant vaccine an inter-individual variation of the local adverse reaction was recorded. Three of the five horses (nr. 3, 4 and 5) showed only minor oedema, which resolved after up to five days. One horse (nr. 7) developed extensive painful swelling that lasted up to two weeks after each injection, and one horse (nr. 6) showed an increase in the adverse reaction with minor non-painful swelling after the first and extensive painful swelling after the third vaccination.

3.2. Induction of anti-BoNT/C and D IgG in vaccinated horses

A rise of total IgG in response to the vaccinations is seen in all horses when using the immunogen as a capture antigen (Figs. 1–4). Sera of the horses showed a weak positive reaction on day 0, prior to vaccination. In horses vaccinated with the toxoid vaccine (nr. 1 and 2) a marked rise in total IgG against BoNT holotoxin (Figs. 3 and 4), but a weak or no rise in IgG against the Hc fragment is recorded (Figs. 1 and 2). Vice versa, the holotoxin based capture ELISA measure almost no IgG sero-conversion in horses vaccinated with the Hc fragment (Figs. 3 and 4, horses nr. 3–14), in contrast to the ELISA using the Hc fragment as a capture antigen (Figs. 1 and 2). No cross-reactive IgG response between HcBoNT/C and D is seen in horses vaccinated with the monovalent vaccines (Figs. 2 and 3, horses nr. 3–6). Between the horses vaccinated with the mono-

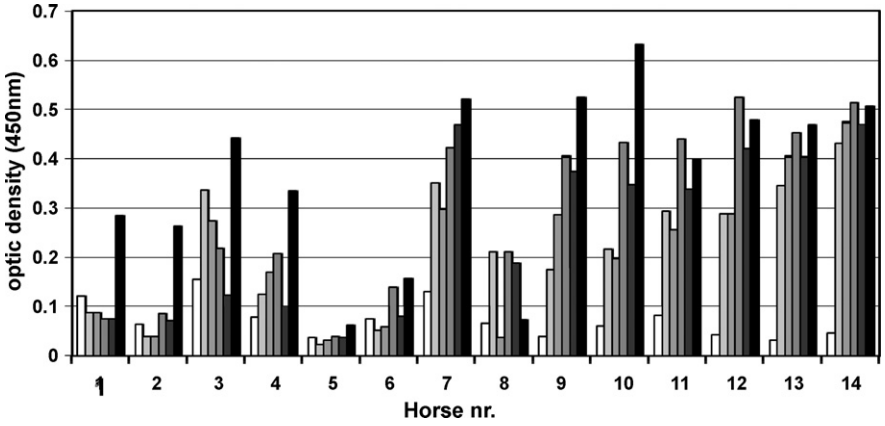

Fig. 1. Total IgG antibody response against HcBoNT/C of horses (nr. 1–14) before and after the three consecutive vaccinations, measured by indirect ELISA using HcBoNT/C as a capture antigen, pre-vaccination (white bars), post-1st vaccination, pre-2nd vaccination, post-2nd vaccination, pre-3rd vaccination (grey bars with increasing darkness), and post-3rd vaccination (black bars). Standard deviations were below 5% of the mean values of four independent measurements and are not shown in the figures.

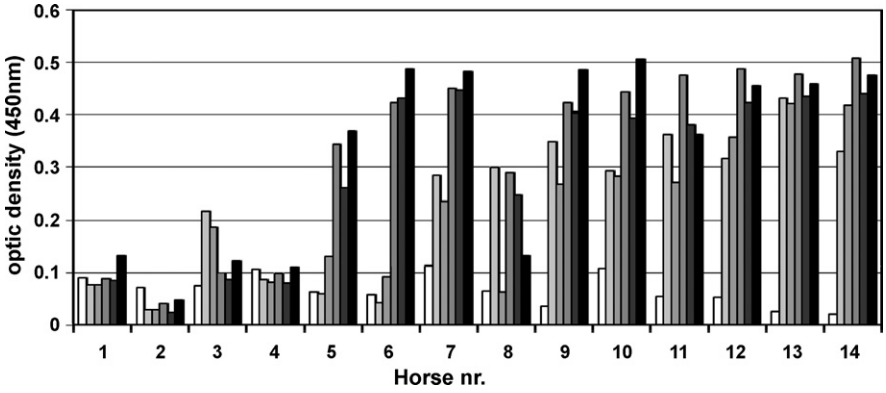

Fig. 2. Total IgG antibody response against HcBoNT/D of horses (nr. 1–14) before and after the three consecutive vaccinations, measured by indirect ELISA using HcBoNT/D as a capture antigen, pre-vaccination (white bars), post-1st vaccination, pre-2nd vaccination, post-2nd vaccination, pre-3rd vaccination (grey bars with increasing darkness), and post-3rd vaccination (black bars). Standard deviations were below 5% of the mean values of four independent measurements and are not shown in the figures.

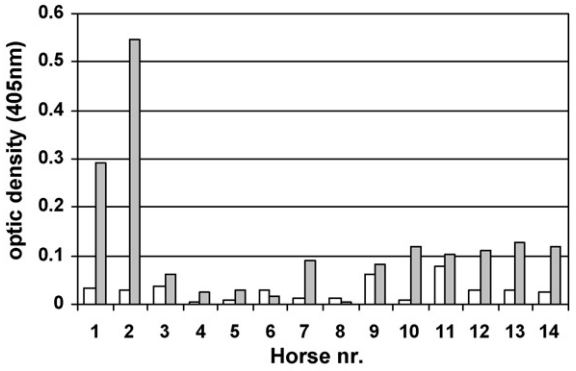

Fig. 3. Total IgG antibody response against BoNT/C of horses (nr. 1–14) before the first vaccination (white bars) and after the third vaccination (grey bars), measured by indirect ELISA using BoNT/C as a capture antigen. Standard deviations were below 5% of the mean values of four independent measurements and are not shown in the figures.

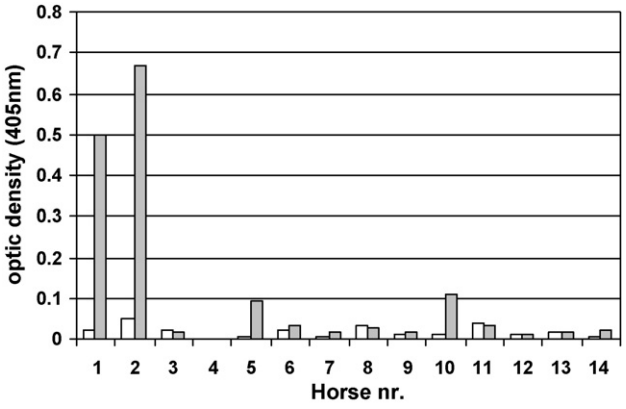

Fig. 4. Total IgG antibody response against BoNT/D of horses (nr. 1–14) before the first vaccination (white bars) and after the third vaccination (grey bars), measured by indirect ELISA using BoNT/D as a capture antigen. Standard deviations were below 5% of the mean values of four independent measurements and are not shown in the figures.

or bivalent vaccine, as well as with the higher or lower dose of antigen no quantitative difference in total IgG is observed. Horse nr. 8 shows a much weaker antibody response compared to the other horses.

3.3. Induction of botulinum toxin-neutralizing antibodies in vaccinated horses

All horses except for horse 8 show a rise in neutralizing antibody titers after the third vaccination (Table 1). No cross-protective potential is seen in the sera of the two horses vaccinated with HcBoNT/C only (nr. 3 and 4) and with HcBoNT/D only (nr. 5 and 6).

The neutralizing antibody titers against serotype D are markedly higher than against serotype C independent of the use of the toxoid or recombinant antigen. Horses vaccinated with the higher dose of recombinant immunogen show somewhat higher neutralizing antibody titers than horses vaccinated with the lower dose. No difference in response to the mono- or bivalent experimental vaccine can be observed. Neutralizing titers of horses vaccinated with the recombinant vaccine are similar or higher compared to the two horses vaccinated with the commercially available toxoid vaccine. Horse nr. 8 does not show a distinct rise in neutralizing antibody titers against BoNT/D, neither did it show neutralizing capacity against BoNT/C.

4. Discussion

Prevention of botulism can be efficiently achieved by vaccination, which generates neutralizing antibodies against botulinum neurotoxin. Commercially available vaccines against botulinum toxin for equines are formalin-inactivated toxoids (serotypes B and C/D). However, toxoid vaccines are associated with several drawbacks, a potential hazard to laboratory workers during detoxification, expensive and time-consuming production [6] and marked side effects. Recombinant subunit vaccines have been shown to circumvent these drawbacks and elicit protective immunity in animals. In our study the experimental recombinant vaccine was characterized by good compatibility and the ability to elicit antibody titers similar or superior to the commercially available toxoid vaccine.

Horses are assumed to be among the most sensitive animals to botulism [4]. However, the exact dose–response relationship, the amount of toxin ingested in a natural outbreak as well as the correlation between the level of neutralizing antibody and the ability to withstand an exposure to botulinum neurotoxin are unknown. While the efficacy of the currently available commercial equine vaccine, Botulism Vaccine®, appears satisfactory, this has not been documented in recent studies. Since its development and introduction in 1938, the toxoid vaccine has been successfully used in the control of botulism in different animal species [21,22]. Horses vaccinated with the recombinant vaccine induced neutralizing antibody

Table 1
Neutralizing serum titers of horses against BoNT/C and D prior to vaccination (pre) and after the third vaccination (post) tested in the mouse protection assay.

Horse	Vaccine	Days of vaccination	Neutralizing serum titers (IU/ml) against			
			BoNT/C		BoNT/D	
			pre	post	pre	post
1	BoNT/C and D toxoid	0; 21; 266	<0.9	3.75–12.5	>1.6	50–65
2	BoNT/C and D toxoid	0; 21; 266	<0.375	3.75–12.5	ND	15–65
3	HcBoNT/C 100 μg + GPA	0; 30; 231	<0.375	3.75–12.5	<0.5	<0.5
4	HcBoNT/C 100 μg + GPA	0; 30; 231	<0.375	0.5–3.75	<0.5	<0.5
5	HcBoNT/D 100 μg + GPA	0; 30; 90	<0.375	<0.5	<1.25	20–40
6	HcBoNT/D 100 μg + GPA	0; 30; 90	<0.375	<0.5	<1.25	1000–2000
7	HcBoNT/C and D 100 μg + GPA	0; 30; 90	<0.375	3.75–12.5	<1.6	500–1000
8	HcBoNT/C and D 100 μg + Alu	0; 30; 107	<0.7	<0.25	<1.6	0–6.5
9	HcBoNT/C and D 100 μg + Alu	0; 30; 107	<0.375	3.75–12.5	<1.6	200–400
10	HcBoNT/C and D 100 μg + Alu	0; 30; 107	<0.375	3.75–12.5	<5.4	500–667
11	HcBoNT/C and D 10 μg + Alu	0; 30; 90	ND	2–4	ND	100–200
12	HcBoNT/C and D 10 μg + Alu	0; 30; 90	ND	2–4	ND	25–50
13	HcBoNT/C and D 10 μg + Alu	0; 30; 90	ND	4–5	ND	50–100
14	HcBoNT/C and D 10 μg + Alu	0; 30; 90	ND	4–5	ND	10–200

titers similar or superior to the two horses vaccinated with the toxoid vaccine. The recombinant vaccine can therefore be regarded as protective.

Natural exposure to sub-clinical as well as sub-lethal doses of BoNT resulted in a specific antibody response in cattle shown by ELISA and mouse protection assay [23] and has been detected by ELISA in unvaccinated horses in endemic areas of grass sickness in Israel [24] and Great Britain [25]. The detectable IgG titers on day 0, prior to the first vaccination, in our study, are therefore most likely due to a previous exposure to sub-clinical doses of BoNT/C and D or antigenetically similar antigens.

The neutralizing antibody titers showed to depend on the dose of HcBoNT/C and D used in the vaccine (Table 1), corresponding to dose-dependent immune response to HcBoNT/C and D as observed in mice [12]. With identical doses of HcBoNT/C and D, markedly higher neutralizing antibody titers were seen against serotype D. Significantly different antibody response depending on the toxoid serotype was also observed in man. Neither the Lf (flocculated toxoid) nor the lethal dose equivalent is a precise measure of the antigenicity of botulinum toxoids [26]. This is supported by the finding that in mice for full protection in a BoNT challenge, half of the vaccination dose of HcBoNT/D is needed in comparison to HcBoNT/C [12]. We show that bivalent formulation has an equally good immunogenicity compared to the monovalent vaccine. No cross-protection between types C and D has been observed (Table 1), which is in agreement with results from vaccinated mice [11,12].

One horse (nr. 8) generally showed a weak immune response to vaccination with HcBoNT/C and D, both for total IgG measured by ELISA and neutralizing antibodies measured by the mouse protection assay. A highly variable immune response and the occurrence of non-vaccine-responsive individuals however are known, and were reported after vaccination with botulinum toxoid. It has been reported in cattle and humans as well [23,27–29]. While the study does not intend to correlate total IgG titers from ELISA and neutralizing antibody titers, it has to be noted that thirteen out of the fourteen horses show rise in ELISA titers as well as in the neutralizing antibody titers, whereas one horse (nr. 8) shows a very weak immune response as measured by ELISA and the mouse protection assay (Table 1 and Figs. 1–4). ELISA systems were considered an effective method to analyze the immune response to botulinum toxins and correlated well with protection in toxin challenge studies, in mouse protection assay and in natural outbreaks [24]. Anti-Hc-ELISA antibody titers appeared to correlate closely with survival following toxin challenge in mice [12]. Serum ELISA titers, using the complexed or pure corresponding toxin as a capture antigen, reflected survival results in mice vaccinated with the Hc fragment after challenge with BoNT/C and D toxin [14].

Using the Hc fragment of BoNT/B as an antigen for vaccination in rhesus monkeys and the Hc fragment as well as BoNT/B holotoxin as capture antigen in ELISA, a good correlation between ELISA titers and mouse protection assay was seen [30]. Evaluating sera of heavy chain subunit vaccinated individuals by ELISA using the recombinant part as well as the purified or complexed toxin has been described and generally found to be correlating well [12,30]. In contrast, we only found strong signal in ELISA when the corresponding vaccination immunogen was used as a capture antigen. Thus horses vaccinated with recombinant HcBoNT proteins serologically only reacted with the corresponding Hc-ELISA containing recombinant HcBoNT as a capture antigen and not with the "holotoxin" ELISA containing as an antigen detoxified *C. botulinum* supernatants in which HcBoNT is contained only in minute amounts. Reversely, horses vaccinated with detoxified *C. botulinum* supernatants strongly reacted with the "holotoxin" ELISA but barely with the Hc-ELISA, since their immune reaction was directed against detoxified "holotoxin" and other components of *C. botulinum* supernatants present in the vaccine preparation. This phenomenon may be expected from the nature of the recombinant purified protein as a vaccine, which is represented in minute amounts on ELISA plates coated with whole BoNT/C or BoNT/D preparations only. This has also been described [11] but not as pronounced as in our study.

Undesirable local reactions due to relatively crude toxoid antigen in botulinum vaccines have been described in humans [31], and were observed by us in the two horses vaccinated with the toxoid vaccine. The local adverse reactions in horses after injection of the GPA based recombinant vaccine were inter-individually variable. The aluminium hydroxide based vaccine, however, was well tolerated by all vaccines.

Our data demonstrate that recombinant HcBoNT/C and D are able to produce neutralizing antibodies against botulinum neurotoxin types C and D in the horse, and hence fulfil the basic requirement for the development of a potent and clinically well-tolerated vaccine to protect horses against equine botulism.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vaccine.2009.07.021.

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