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Recombinant vaccine against botulism in buffaloes: Evaluation of the humoral immune response over 12 months

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ABSTRACT

Botulism is a neuroparalytic intoxication, usually fatal, caused by the botulinum toxins (BoNTs). Vaccination is the best-known strategy to prevent this disease in ruminants. Serotypes C and D and their variants CD and DC are the main types responsible for botulism in bovine and buffaloes in Brazil and cattle in Japan and Europe. Brazil has a herd of approximately 1.39 million buffaloes and is the largest producer in the Western world. This study aimed to assess the humoral immune response of buffaloes during the 12-month period after vaccination against BoNT serotypes C and D with a recombinant vaccine in three different concentrations (100, 200, and 400 μg) of non-purified recombinant proteins (Vrec) and also with a bivalent commercial toxoid (Vcom). Vrec400 was the best vaccine among those tested because it induced higher levels of antibodies and maintained higher levels of antibodies for the longest time, while Vrec200 could be considered the most cost-effective vaccine for large-scale production. None of the vaccines were able to promote continuous immunological protection within the timeframe proposed by the current Brazilian vaccination protocol. Further studies should focus on vaccine adjustments to ensure continued humoral protection against botulism.

1. Introduction

Botulism is a neuroparalytic intoxication, usually fatal, caused by botulinum toxins (BoNTs). BoNTs, the most toxic biological substance known, are mostly produced by *Clostridium botulinum,* a Gram-positive spore forming, rod-shaped, strict anaerobe bacterium that is omnipresent in nature and under harmful environmental conditions it can sporulate and survive for long periods, but when conditions are suitable, it develops into vegetative forms and may produce one or more than one of the seven serotypes of BoNTs (A to G), which, although antigenically distinct, have an analogue mechanism of action: inhibition of acetylcholine release at the myoneural junction [1–6]. Serotypes C and D are the main types responsible for botulism in bovines and buffaloes in Brazil [6,7], and their mosaic variants CD and DC are reported in bovine botulism in Japan [8] and in Europe [9]. So far, there are no studies in Brazil subtyping BoNT in livestock outbreaks of botulism [10], however, five strains of *Clostridium botulinum* group III type DC, all originated from Brazil, have been reported [11] and therefore it cannot be ruled out that mosaic types may be involved in Brazilian outbreaks of botulism. The disease in livestock is considered endemic in Australia, South Africa, Israel and Brazil [2,12], and sporadic cases are reported in Europe [9,13]. In Brazil, the botulism outbreaks in ruminants are mainly caused by ingesting preformed BoNTs [14] and intoxication is often associated with osteophagy owing to mineral deficiency (particularly phosphorus), consumption of poorly produced silage, and contaminated food or water [6,13,15]. In buffaloes, the waterborne spread of BoNTs seems to play an important epidemiological role in the occurrence of the disease, since it is described in all cases of botulism in buffaloes reported in Brazil [6,7,16].

Brazil has a herd of approximately 1.39 million animals and is the largest buffalo producer in the Western world [17]. Due to the

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severe nature of the disease, which is associated with limited treatment options and consequently high mortality rates, botulism is a serious economic concern [18]. The best-known strategy to prevent botulism types C and D in large ruminants is vaccination since protection is entirely dependent on the presence of specific neutralizing antibodies upon absorption of the toxin [2,19]. Commercially available vaccines against botulism in animals are produced based on a conventional method that involves culture of *C. botulinum*, further purification and inactivation of the neurotoxins to obtain the toxoids. This production method has some disadvantages: *C. botulinum* requires special components on its culture medium, strains show variable yield of toxin production between different batches, and its manipulation offers intoxication risks to workers and therefore involves costs with high levels of biosafety [20]. There is currently no specific vaccine commercially available nor specific protocol of vaccination for buffaloes, so buffalo breeders resort to bovine vaccines and replicate the protocol of vaccination recommended to this species of a priming dose in 4-month-old calves, booster dose 4 weeks after the first dose, and doses every 12 months thereafter.

Recombinant technology allows the production of highly purified, effective, and non-toxic antigens produced in suitable amounts with no need to cultivate and manipulate *C. botulinum* and its neurotoxins [20]. Thus, there are already studies that report evaluation of recombinant vaccines against botulism in animals such as guinea-pigs, cattle and buffaloes [1,3,5,14,21], but none of them reported the evaluation of the humoral immune response to a recombinant vaccine against botulism serotypes C and D after achieving the minimum requirements established by Brazilian legislation [22] which requires, besides the sterility testing and control of innocuity the control of vaccine efficiency. Vaccine efficacy control or potency test consists, in brief, of inoculating guinea pigs from 350 to 450 g subcutaneously with two doses of 5.0 mL, the booster dose being applied 21 days after the first vaccination. The animals are bled by cardiac puncture on day 42 (forty-two) and the sera obtained are titrated to the level of Botulinum Antitoxin C and D in international units per milliliter by serum neutralization in mice. The vaccine is approved when the result is at least 5.0 IU/mL and 2.0 IU/mL for antitoxins C and D, respectively.

This study aimed to assess the humoral immune response of buffaloes during the 12-month period after vaccination against BoNT serotypes C and D with a recombinant vaccine in three different concentrations (100, 200, and 400 μg) of non-purified recombinant proteins (Vrec) and also with a bivalent commercial toxoid (Vcom).

2. Materials and methods

2.1. Ethics statement

The study was conducted in accordance with the Brazilian National Council for Animal Experimentation (CONCEA). It was submitted to the Animal Use Ethics Committee of the Federal University of Pará (CEUA/UFPA) and approved under license number 9668220616.

2.2. Vaccines

Recombinant vaccine formulations containing the C-terminal fragment of botulinum neurotoxin (H_CBoNT) serotypes C and and D were produced according to the protocol described by Moreira Jr. et al. [4]. Recombinant botulism antigens were expressed in an *Escherichia coli* system and the recombinant vaccines formulated containing 100, 200, or 400 μg of non-purified H_CBoNT/C and H_CBoNT/D per dose (5 mL) and aluminum hydroxide as adjuvant. The sterility test was performed in thioglycolate and Sabouraud broths at a temperature of 37 °C and 25 °C, respectively, with a daily reading for a period of three weeks to verify microbiological growth. The lack of toxicity was evaluated by inoculating two buffaloes with double concentration of the vaccine with the highest concentration used in the experiment (800 μg) and the animals were observed for side effects for a period of 72 h.

A commercial bivalent botulinum toxoid (Botulina – Vallée® - lot number 002/16) containing toxins C and D with concentration of botulinum toxoid not described by the manufacturer was purchased in the local commerce and used according to the manufacturer's instructions.

2.3. Vaccination of buffaloes

Fifty Murrah crossbreed buffaloes of both genders with initial ages between two and six months, with no detectable antibody titres against BoNTs C or D, were randomly divided into groups of ten animals. The first three groups Vrec100, Vrec200 and Vrec400 were inoculated with the recombinant vaccine formulations at concentrations of 100, 200, and 400 μg of recombinant proteins, respectively. The fourth group (Vcom) was inoculated with the commercial vaccine, while group five, the negative control, received 5 mL of sterile solution of NaCl 0.9% (w/v). Vaccination was performed subcutaneously in the neck on days 0 and 28. Blood samples were collected monthly over 10 months (days 56, 90, 120, 150, 180, 210, 238, 270, 310, 330, and 365) by venopunction of the jugular vein and centrifuged (3000×*g*, 7 min) to obtain serum samples and stocked in microtubes of 2.0 mL at −20 °C until further use.

2.4. Humoral immune response evaluation

Sera samples were individually evaluated by the serum neutralization bioassay in mice according to normative instruction number 23 (NI 23) of the Brazilian Ministry of Agriculture, Livestock, and Food Supply (MAPA) [22]. In short, sera dilutions and standard toxins were mixed at 37 °C for 1 h and after that 0.2 mL of each dilution was inoculated intravenously in two mice (Swiss Webster weighing 20 ± 2 g). The animals were observed for 72 h to check if they were dead or alive. The titer was obtained as the inverse of the lowest dilution in which all mice died.

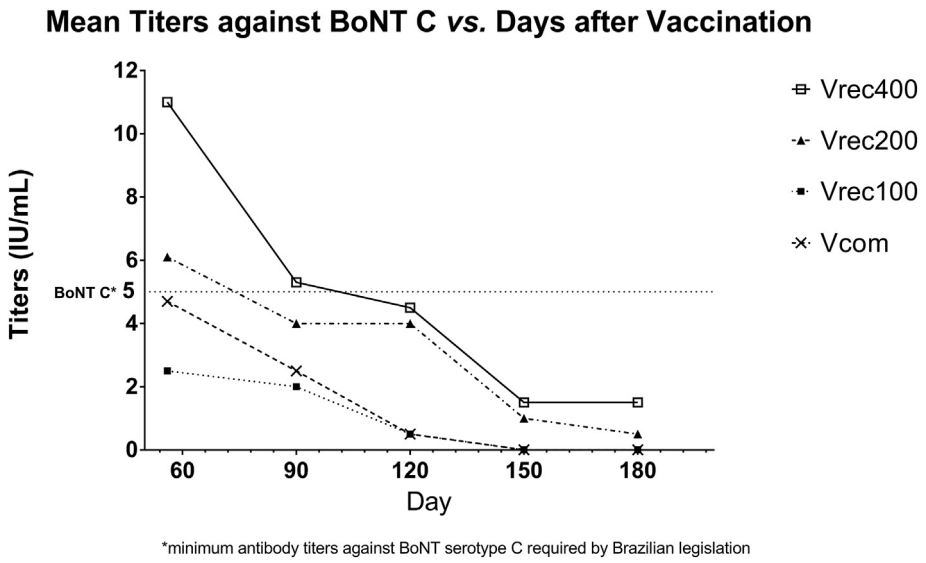
2.5. Statistical analysis

Differences in means of antibody titers between groups were analyzed statistically by two-way repeated measures analysis of variance (ANOVA) and SIDAK (*post hoc* test) using IBM® SPSS Statistics 25. For all tests, only data resulting in P values < 0.001 were regarded as statistically significant.

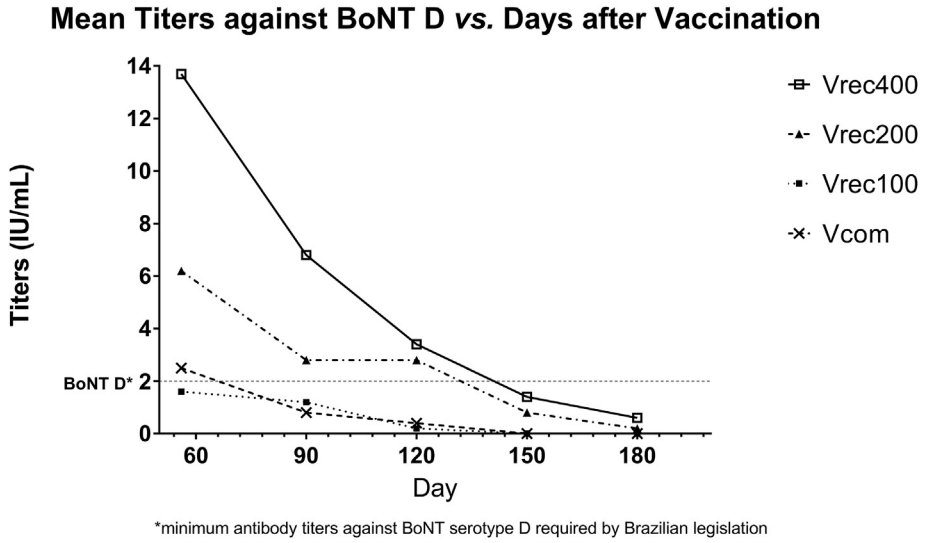
3. Results

The sterility test resulted in no growth of fungi or bacteria after a 21-day observation period. In the innocuity test, as well as during the 12 months of the study no adverse reactions were observed in the animals that received the recombinant vaccines. The negative control group (G5) did not presented detectable titers against BoNTs C or D, and none of the animals in the farm presented clinical signs compatible with botulism during the study.

Different vaccine formulations induced varying levels of antibody titers against BoNTs C and D throughout the study. At day 56, all vaccines were able to induce immune response against both BoNTs C and D, and all groups showed some level of antibody titers, but detected only up to day 180 (Graphs 1 and 2).



Graph 1. Mean titers against botulinum neurotoxin (BoNT) serotype C *vs.* days after vaccination of buffaloes inoculated with two doses of commercial toxoid (Vcom) and two doses of recombinant vaccine in different protein concentrations (Vrec100; Vrec200; Vrec400) tested by the serum neutralization bioassay in mice on days 56, 90, 120, 150, and 180.



Graph 2. Mean titers against botulinum neurotoxin (BoNT) serotype D *vs.* days after Vaccination of buffaloes vaccinated with two doses of commercial toxoid (Vcom) and two doses of recombinant vaccine in different protein concentrations (Vrec100; Vrec200; Vrec400) tested by the serum neutralization bioassay in mice on days 56, 90, 120, 150, and 180.

The samples of days 210, 240, 270, 300, 330, and 365 in all experimental groups did not present detectable titers to the seroneutralization technique in mice for both serotypes C and D (the technique is limited to detecting values of antibody titers only equal to or greater than 5 and 2 IU/mL, respectively).

The percentage of animals that reached and remained with minimal antibody titers against serotypes C and D required by Brazilian legislation also varied significantly throughout the study (Tables 1 and 2).

There was no effect of the gender (male *vs.* female) on antibody titers for both BoNTs C and D: [$F(1, 24) = 0.047$; $p > 0.001$] and [$F(1, 24) = 0.713$; $p > 0.001$], respectively. Likewise, there was no effect of age (older or younger than 4 months) on antibody titers for both BoNTs C and D: [$F(1, 24) = 0.360$; $p > 0.01$] and [$F(1, 24) = 0.0179$; $p > 0.01$], respectively.

ANOVA showed that there was an interaction effect between time *vs.* vaccine formulation on means of antibody titers for both BoNTs C and D: [$F(7.938, 95.262) = 7.814$; $p < 0.001$] and [$F(4.985, 59.815) = 53.964$; $p < 0.001$] respectively.

At first (day 56), all vaccines were able to induce at least the minimum level of neutralizing antibodies required by law (IN 23) for BoNT D, whereas for BoNT C, only Vrec400 and Vrec200 were able to satisfy this requirement. Vrec400 induced the best immune humoral response when compared to the other vaccines, as it induced higher mean titers for the longest period for both BoNT's. More details of the performance comparison between BoNT concentration and day after vaccination are shown in Tables 3 and 4.

It should be noted that Vrec400 and Vrec200 were the vaccines that were able to induce the longstanding immune responses against BoNT C and D. It was possible to detect neutralizing antibodies, statistically different from zero, until day 120, while Vcom and Vrec100 stimulated immune response antibody titers only up to day 90, and with lower mean antibody titers. The main difference between Vrec400 and Vrec200 was the highest mean antibody

Table 1
Percentage of animals with minimal antibody titers against serotype C required by Brazilian legislation on days 56, 90, 120, 150, and 180. Samples of days 210, 240, 270, 300, 330, and 365 in all experimental groups did not present detectable titers.

Seroconversion rate* BoNT C					
Vaccine	Day				
	56	90	120	150	180
Vrec100	50%	40%	10%	0%	0%
Vrec200	100%	80%	80%	20%	10%
Vrec400	100%	100%	90%	30%	30%
Vcom	90%	50%	10%	0%	0%

* with consideration of the minimum antibody titers required by NI 23 MAPA (5 IU/mL for BoNT C).

Table 2
Percentage of animals with minimal antibody titers against serotype D required by Brazilian legislation on days 56, 90, 120, 150, and 180. Samples of days 210, 240, 270, 300, 330, and 365 in all experimental groups did not present detectable titers.

Seroconversion rate* BoNT D					
Vaccine	Day				
	56	90	120	150	180
Vrec100	70%	60%	10%	0%	0%
Vrec200	100%	80%	80%	40%	10%
Vrec400	100%	100%	100%	60%	30%
Vcom	80%	40%	20%	0%	0%

* With consideration of the minimum antibody titers required by NI 23 MAPA (2 IU/mL for BoNT D).

Table 3
Estimated mean titers (IU/mL) compared by SIDAK (*post hoc*) in the interaction of time *vs.* vaccine formulation for BoNT C.

Estimated means compared by SIDAK (*post hoc*)				
Day	Vaccine Formulation - BoNT C			
	Vrec400	Vrec200	Vcom	Vrec100
56	$11^{a,A}$	$6.1^{a,B}$	$4.7^{a,B}$	$2.5^{a,C}$
90	$5.3^{b,A}$	$4^{a,A}$	$2.5^{a,A}$	$2^{a,B}$
120	$4.5^{b,A}$	$4^{a,A}$	$*0.5^{b,B}$	$*0.5^{a,b,B}$
150	$*1.5^{c,A}$	$*1^{b,A}$	$*0^{b,A}$	$*0^{b,A}$
180	$*1.5^{c,A}$	$*0.5^{b,A}$	$*0^{b,A}$	$*0^{b,A}$

Letters were used to point out means titers that were statistically equal or different. Small letters were used to compare BoNT C mean titers in the same vaccine formulation by time (columns), and capital letters were used to compare BoNT C mean titers by different vaccine formulations on the same day (lines). Means that do not share a letter are statistically different.
* Values statistically equivalent to zero.

Table 4
Estimated mean titers (IU/mL) compared by SIDAK (*post hoc*) in the interaction of time *vs.* vaccine formulation for BoNT D.

Estimated means compared by SIDAK (*post hoc*)				
Day	Vaccine Formulation - BoNT D			
	Vrec400	Vrec200	Vcom	Vrec100
56	$13.7^{a,A}$	$6.2^{,B}$	$2.5^{a,C}$	$1.6^{a,C}$
90	$6.8^{b,A}$	$2.8^{b,B}$	$0.8^{b,B}$	$1.2^{a,B}$
120	$3.4^{c,A}$	$2.8^{b,A}$	$*0.4^{b,B}$	$*0.2^{b,B}$
150	$*1.4^{d,A}$	$*0.8^{c,A}$	$*0^{b,A}$	$*0^{b,A}$
180	$*0.6^{d,A}$	$*0.2^{c,A}$	$*0^{b,A}$	$*0^{b,A}$

Letters were used to point out mean titers that were statistically equal or different. Small letters were used to compare BoNT D mean titers in the same vaccine formulation by time (columns) and capital letters were used to compare BoNT D mean titers by different vaccines formulations in the same day (lines). Means that do not share a letter are statistically different.
* Values statistically equivalent to zero.

titers induced by Vrec400 at day 56, since on days 90 and 120, the performances of both formulations were statistically equivalent. Vcom was better than Vrec100, as it induced higher antibody titers against BoNT C and equivalent antibody titers against BoNT D at day 56. On day 90, the performances of both formulations were statistically equivalent.

4. Discussion and conclusions

Botulism represents a real economic concern for animal production in view of the fatal nature of the disease associated with extreme limitation of treatment options for intoxicated animals [10,18]. It should be considered that the etiological agent of botulism, *C. botulinum,* is an ubiquitous microorganism, a natural inhabitant of the environment and also able to survive in adverse conditions for long periods in its sporulated form [1–6]. These characteristics make the control of the disease by means of eradication of the agent theoretically impossible, making prophylaxis through vaccination the main measure for the control of the disease. Thus, it is desirable that the vaccines induce the highest titres of immune response for the longest possible period [23]. Despite the importance of this disease in buffaloes, only one study on the vaccine against botulism in buffaloes has been published to date [14]. To our knowledge, this is the first study to test an immunogen in buffaloes, evaluating its protection curve of antibodies for one year to compare different formulations of recombinant vaccines and a commercial vaccine against botulism.

The neutralizing antibody titers and the longevity of the immune response were shown to be dependent on the concentration of recombinant proteins C and D used in the vaccine, since Vrec400 induced the highest initial mean antibody titers (11 and 13.7, respectively), and it was also the formulation that maintained higher mean antibody titers (1.5 and 0.6, respectively) for the longest time (180 days) for both BoNT C and BoNT D (Tables 3 and 4), being the best formulation within these requirements. This positive linear correlation between the concentrations of recombinant proteins in the vaccines *vs.* antibody titers were also observed in bovine by Moreira et al. (2018) [21] using the same recombinant vaccine. In fact, the results described in cattle by this publication in relation to the vaccine potency test are very similar to those reported in this study. On the other hand, from the point of view of industrial scale production, Vrec200 was the most cost-effective formulation because even inducing lower antibody titers (6.1 and 6.2, respectively) than Vrec400, it was still in accordance with the Brazilian legislation and achieved the same longevity of immune response as Vrec400, with the advantage that Vrec200 required only half of the amount of recombinant proteins to be produced. These results associated with poor performance of the commercial vaccine turn Vrec200 and Vrec400 into candidates to replace conventional commercial vaccines.

In a scenario in which vaccine protocols against botulism in ruminants recommend annual booster doses, it is desirable that the longevity of the immune response stimulated by these vaccines persists for at least 12 months. However, we must highlight that even the best formulation (Vrec400) was unable to induce antibody titers, statistically different from zero, against BoNT C and BoNT D further than day 120. This low immune response longevity seems to be the Achilles tendon of the tested botulism vaccines. In 2006, Steinman et al. [12] analyzed serological and epidemiological data of outbreaks of botulism type D in cattle systematically vaccinated against botulism C and D in Israel to evaluate the different reasons for vaccine failure. They concluded that vaccination protocols (2 vaccinations with 4 weeks between doses starting at 2 months of age of the calves, followed by an annual booster) used on dairy farms were insufficient to ensure immunological protection for the entire period between the last vaccine dose and the next annual booster dose. In 2007, Steinman et al. [19] conducted a field vaccination study to evaluate three different immunization protocols and the effect of maternal anti-BoNT/D antibodies, at the priming dose in two-month-old calves. They concluded that the current vaccination strategy of using a priming dose in two-month-old calves followed by booster doses after four weeks and annually thereafter did not result in continuous protective levels of anti-BoNT/D antibodies, and to solve this lack of continue immune protection they recommended adding a six-month booster dose to the current protocol. The same protocol could be applied to the recombinant vaccines evaluated in this study in an attempt to increase the longevity of the immune response.

In the present study, the effect of the gender on the mean titers of antibodies against BoNTs C or D was not observed, a result that was already expected. However, the non-effect of age on the average antibody titers against BoNTs C or D was a surprise since in Brazil, the recommended vaccination protocol for commercial vaccines against botulism is for animals to be vaccinated from four months of age. It is believed that the presence of blocking levels of maternally derived antibodies might be an obstacle to a successful vaccination in young animals due to a suppressive effect on active immunization [24]. However, Steinman et al. (2007) [19] compared serum samples of calves (born from cows routinely vaccinated against botulism) for anti-BoNT/D antibodies before vaccination (maternally derived anti-BoNT/D antibodies) and after vaccination protocols starting at two months of age, and they concluded that maternally derived antibodies did not interfere with the immune response of the calves. Two facts could explain why there was no difference between the mean antibody titers among groups (younger *vs.* older than 4 months): first, there is actually no influence of maternal antibodies on the immune response of calves vaccinated with the formulations tested, and second, the property where the study was conducted had no history of occurrence nor vaccination against botulism. Thus, the cows of this farm were not challenged naturally or vaccinated to produce antibodies against BoNTs C or D, and consequently, there was no transfer of passive immunity from cows to calves to interfere in the development of the immune response of the calves. Thus, it appears that the vaccine protocol against botulism can be adjusted for vaccination of animals younger than four months without impairment of the immune response, especially in herds that do not have a history of vaccination against botulism.

In conclusion, Vrec400 was the best vaccine among those tested for having induced higher levels of antibodies and the best longevity of the immune response and Vrec200 could be considered the most cost-effective vaccine for large-scale production. None of the vaccines were able to promote continuous immunological protection within the timeframe proposed by the current Brazilian vaccination protocol. The poor performance of all vaccines in longevity immune response indicates that future studies may adjust vaccine protocols adding a booster dose between annual doses to the current vaccination protocol, adjusting the binomial concentration of recombinant proteins *vs.* period between booster doses or use other types of adjuvants in the formulation of vaccines to ensure continued humoral protection against botulism.

Declaration of competing interest

None.

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