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Brief report

Vaccination of cattle with a recombinant bivalent toxoid against botulism serotypes C and D

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ABSTRACT

Cattle botulism is a fatal intoxication caused by botulinum neurotoxins (BoNTs) produced by *Clostridium botulinum* serotypes C and D resulting in economic losses. Vaccination is the most effective way to control botulism. However, the commercially available vaccines are difficult and hazardous to produce. Neutralizing antibodies against the C-terminal fragment of the BoNT heavy chain (H_C) are known to protect against lethal doses of BoNTs. We report the vaccination of cattle with a previously tested recombinant chimera consisting of *Escherichia coli* heat-labile enterotoxin B subunit and the H_C of BoNTs C and D. Vaccinated animals produced neutralizing antibodies against serotypes C and D averaging 5 ± 0 and 6.14 ± 1.06 IU/mL, respectively. For BoNT D, the titers were greater than those measured for the commercial vaccine, which induced titers of 5 ± 0 and 2.85 ± 1.35 against the respective serotypes, suggesting that this chimera is effective against cattle botulism.

1. Introduction

Clostridium botulinum is a Gram-positive, anaerobic, spore-forming bacillus that is ubiquitous in nature and capable of growing in decaying organic matter. Under anaerobic conditions, it produces botulinum neurotoxins (BoNTs), which are responsible for causing botulism, a fatal intoxication characterized by flaccid paralysis due the inhibition of acetylcholine release at the neuromuscular junction [1]. These toxins are classified into seven serotypes (A to G) according to their antigenic differences, although they present similar pharmacological mechanisms [2]. BoNT serotypes C and D are the most common toxins for outbreaks of cattle botulism in many countries, including Brazil [3–6], where it frequently presents fatality rates up to 100% [7]. Thus, bovine botulism is considered a cause of great economic loss.

The induction of neutralizing antibodies through vaccination is the best approach to control botulism [8]. Currently, vaccines against botulism are produced with formaldehyde-inactivated neurotoxins (toxoids) mixed with aluminum hydroxide as an adjuvant. Although efficient, this methodology presents some drawbacks: (1) the amount of native BoNT production *in vitro* is unpredictable and typically low; and (2) BoNTs are the most potent toxins known to mankind [9,10], requiring high levels of biosafety. Therefore, new approaches, such as the development of recombinant vaccines, appear to be promising to overcome these problems.

A recombinant chimera comprising LTB, a potent adjuvant of the humoral immune response, and the C-terminal fragments of BoNT serotypes C and D was previously produced and tested [11]. This construct was able to induce high levels of neutralizing antibodies (5 and 10 International Units per milliliter (IU/mL) against BoNTs C and D, respectively) in guinea pigs when administered with aluminum hydroxide as an adjuvant. The objective of the present work was to evaluate whether our construct was capable of inducing a protective immune response in cattle.

2. Materials and methods

2.1. Vaccine production, formulation and safety

The recombinant chimera comprising LTB and the C-terminal fragment of BoNT serotypes C and D was produced as previously described [11]. Vaccines were formulated by mixing recombinant protein with aluminum hydroxide [2.5–3.5% $Al(OH)_3$, pH 5.5–8] for 16 h under constant agitation [12]. Each vaccine dose contained 200 μg of purified chimeric protein and a final volume of 5 mL. The toxoid sterility and innocuity were evaluated as stipulated by the Brazilian Ministry of Agriculture, Livestock and Food Supply

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according to ministerial directive no. 23 [13]. The innocuity was also assessed in cattle that were not included in the experiment.

2.2. Cattle vaccination

Twenty-one seven-month-old male Nellore cattle, raised on pasture, that presented no detectable antibody levels against either BoNT C or D, were randomly segregated into three groups of seven animals each. Group one was vaccinated with the recombinant vaccine, group two with a bivalent commercial toxoid-based vaccine against serotypes C and D, and group three received 5 mL of sterile saline solution (NaCl 0.9% [w/v]). Immunization was performed subcutaneously on days 0 and 42. Fifty-six days after the first vaccination, blood samples were collected from the coccygeal vein to obtain serum samples to perform a neutralization assay in mice. Samples were stocked at −20 °C until further use.

2.3. Serum neutralization assay

Sera were titrated by a serum neutralization bioassay in mice as described by the Brazilian Ministry of Agriculture, Livestock and Food Supply in its ministerial directive no. 23 [13]. Briefly, sera dilutions were mixed with standard toxins at 37 °C for 30 min, and 0.2 mL of each dilution was inoculated intravenously in two Swiss Webster mice weighing between 18 and 22 g. The animals were observed for death or survival for three days. Retrotitration with standard anti-toxins C (5 IU/mL) and D (2 IU/mL) was performed to check the standardization of the toxins [14]. Animal experiments were carried out according to the guidelines of the Ethics Committee in Animal Experimentation of the Federal University of Pelotas (Permit No. 7542).

2.4. Statistical analysis

Statistix9 (Analytical Software) was used to perform ANOVA and Tukey's test to identify significant differences in antibody titers among the groups.

3. Results and discussion

Over the past decades, botulism has been a problem of major importance to cattle livestock due to its extremely high fatality rate [7]. The production of native toxoids is associated with many difficulties such as unstable production *in vitro* and biosafety issues due to the extremely high toxicity of the BoNTs [9]. In light of such problems, a recombinant chimera capable of inducing protective immunity against BoNT serotypes C and D that possesses no risk during the production process has been developed [11]. Furthermore, using this construct, a bivalent vaccine can be obtained through a single process. To date, this chimera has only been tested on guinea pigs, where it induced the production of high titers of neutralizing antibodies [11]. In this work, we report the immunogenicity assessment of this chimera in cattle.

No microbial growth was observed in any culture condition of the sterility test, and neither adverse nor unexpected reactions occurred in cattle subcutaneously vaccinated with twice the volume and dose used, thus indicating the innocuity of our vaccine formulation. These results were expected, as aluminum hydroxide is widely used as an adjuvant in cattle vaccines and, as demonstrated elsewhere, the H_C domains of BoNTs, alone or as a fusion protein, are non-toxic [11,15].

Animals vaccinated with the recombinant chimera were able to develop mean titers of 5 and 6.14 IU/mL against serotypes C and D, respectively, as determined in the serum neutralization bioassay in mice (Table 1). These titers are greater than the minimums of 5 and 2 IU/mL for serotypes C and D, respectively, established by the Brazilian Ministry of Agriculture, Livestock and Food Supply in its ministerial directive no. 23 [13]. The group vaccinated with the commercial bivalent vaccine presented mean titers of 5 ± 0 and 2.85 ± 1.35 IU/mL against BoNTs C and D, respectively (Table 2). Animals vaccinated with sterile saline solution presented no detectable antibodies against BoNT C or D. The standardization of BoNTs C and D was verified by retrotitration using standard antisera. As expected, 5 and 2 IU/mL of neutralizing antitoxins were detected against the respective toxins, indicating that the material used and the assay are reliable.

Table 1
Neutralizing antibodies titers against botulinum neurotoxin serotypes C and D after vaccination with the recombinant chimera tested in mouse neutralization assay.

Animals	BoNT C antitoxin (IU/mL)	BoNT D antitoxin (IU/mL)
Animal 1	5	5
Animal 2	5	5
Animal 3	5	7
Animal 4	5	7
Animal 5	5	5
Animal 6	5	7
Animal 7	5	7
Mean titer ± SD	5 ± 0	6.14 ± 1.06

Comparatively, animals vaccinated with the recombinant chimera generated the same amount of neutralizing antibodies against serotype C as those vaccinated with the commercial vaccine. As to serotype D, our vaccination strategy was able to induce more than twice the levels of neutralizing antibodies induced by the tested commercial toxoid. ANOVA and Tukey's test indicated that the difference was significant ($P < 0.001$). Although both strategies met the requirements established by Brazilian legislation [13], only animals vaccinated with the recombinant chimera presented levels of neutralizing antibodies against BoNT D that exceeded the minimum, whereas animals vaccinated with the commercial vaccine only presented the minimum accepted.

A study with Danish cows reported that a commercial vaccine containing formalin-inactivated BoNT serotypes C and D using $Al(OH)_3$ as an adjuvant could induce seroconversion and diminish the elimination of BoNTs and *C. botulinum* spores in animal feces [16]. Another study showed that the vaccination with recombinant H_C of BoNTs C and D, individually or co-administrated, can elicit protective immune response in horses, especially against BoNT D [17]. To our knowledge, this is the first report of cattle vaccination with a recombinant antigen to control botulism serotypes C and D. The present work suggests that our chimeric construct would be a good option as a vaccine for cattle against these botulinum serotypes. Notably, this chimera can be obtained in a high-yield process without biosafety risks that overcomes the problems of the current toxoid production method.

Table 2
Neutralizing antibodies titers against botulinum neurotoxins serotypes C and D after vaccination with a commercial bivalent vaccine tested in mouse neutralization assay.

Animals	BoNT C antitoxin (IU/mL)	BoNT D antitoxin (IU/mL)
Animal 8	5	2
Animal 9	5	2
Animal 10	5	5
Animal 11	5	5
Animal 12	5	2
Animal 13	5	2
Animal 14	5	2
Mean titer ± SD	5 ± 0	2.85 ± 1.35

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