Production and Evaluation of a Recombinant Chimeric Vaccine against *Clostridium botulinum* Neurotoxin Types C and D

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Abstract

Bovine botulism is a fatal disease that is caused by botulinum neurotoxins (BoNTs) produced by *Clostridium botulinum* serotypes C and D and that causes great economic losses, with nearly 100% lethality during outbreaks. It has also been considered a potential source of human food-borne illness in many countries. Vaccination has been reported to be the most effective way to control bovine botulism. However, the commercially available toxoid-based vaccines are difficult and hazardous to produce. Neutralizing antibodies targeted against the C-terminal fragment of the BoNT heavy chain (H₃) are known to confer efficient protection against lethal doses of BoNTs. In this study, a novel recombinant chimera, consisting of *Escherichia coli* heat-labile enterotoxin B subunit (LTB), a strong adjuvant of the humoral immune response, fused to the H₃ of BoNT serotypes C and D, was produced in *E. coli*. Mice vaccinated with the chimera containing LTB and an equivalent molar ratio of the chimera without LTB plus aluminum hydroxide (Al(OH)₃) developed 2 IU/mL of antitoxins for both serotypes. Guinea pigs immunized with the recombinant chimera with LTB plus Al(OH)₃ developed a protective immune response against both BoNT/C (5 IU/mL) and BoNT/D (10 IU/mL), as determined by a mouse neutralization bioassay with pooled sera. The results achieved with guinea pig sera fulfilled the requirements of commercial vaccines for prevention of botulism, as determined by the Brazilian Ministry of Agriculture, Livestock and Food, Supply. The presence of LTB was essential for the development of a strong humoral immune response, as it acted in synergism with Al(OH)₃. Thus, the vaccine described in this study is a strong candidate for the control of botulism in cattle.

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Introduction

Botulism is a severe and fatal disease characterized by flaccid paralysis due to the inhibition of acetylcholine release at the neuromuscular junction caused by the botulinum neurotoxins (BoNTs) produced by *Clostridium botulinum* serotypes C and D [1], a Gram-positive anaerobe rod and spore-forming bacteria present in soil, water and decaying organic matter. There are seven serotypes of BoNTs (A-G) that vary antigenically, though they have the same pharmacological activity [2].

In many countries, including Brazil, BoNTs serotypes C and D are responsible for causing botulism in cattle [3–5]. Cattle with calcium and phosphorus deficiencies often resort to bone chewing to supplement their lack of minerals, which is the main cause of endemic botulism [6]. Dutra et al. [7] reported seven outbreaks of bovine botulism in Brazil, which were associated with contaminated water and resulted in 99.92% lethality. In another study [8], the same authors reported more than seven outbreaks of bovine botulism due to contaminated bedding for poultry, resulting in 3,299 dead animals. More recently, Costa et al. [5] reported an outbreak of bovine botulism caused by serotypes C and D present in contaminated food, with 100% lethality in a dairy-producing property. Outbreaks of botulism have also been reported in Europe [9,10] and North America [11], where there is also great concern that infected animals will become a source of food-borne botulism for humans. Therefore, this disease is one of the main causes of cattle...
neuronal receptor, H\textsubscript{C} prototoxins and are activated by enzymatic cleavage by BoNT poisoning [12,13]. Current commercial vaccines are solve the problems inherent to toxoid vaccine production.

In this study, using only one fermentative (LTB), a potent adjuvant of the humoral immune response and downstream process, we developed a chimeric vaccine, recombinant subunit vaccines are interesting strategies to solve the problems inherent to toxoid vaccine production.

Chimeric proteins carrying epitopes from different pathogens, linkers, or adjuvant sequences offer increased immunogenicity for recombinant antigens and can also elicit broad immune responses [22–24]. In this study, using only one fermentative and downstream process, we developed a chimeric vaccine, consisting of the H\textsubscript{C} region of BoNTs serotypes C and D fused to the B subunit of the Escherichia coli heat-labile enterotoxin (LTB), a potent adjuvant of the humoral immune response [25,26]. We then further evaluated the immunogenicity of this vaccine in animal models.

**Material and Methods**

**Ethics statement**

This study was carried out in strict accordance with the recommendations of the Conselho Nacional de Controle de Experimentação Animal (CONCEA). The protocol was approved by the Committee on the Ethics of Animal Experiments of the Federal University of Pelotas (Permit No. 9286). All efforts were made to minimize animal suffering.

**Native botulinum neurotoxins and standard sera against botulinum neurotoxins serotypes C and D**

Native botulinum neurotoxins were produced by Clostridium botulinum serotypes C strain Onderstepoort Veterinary Institute 01/1992 and D strain Onderstepoort Veterinary Institute 02/1992 obtained from the Onderstepoort Veterinary Institute (South Africa) standardized to 1 L+/mL. Standard sera against BoNTs serotypes C and D were obtained from the Center for Diseases Control (CDC) lot 76.0342 catalog number BS0612 and lot 76.0338 catalog number BS0611, respectively.

**Gene synthesis and cloning**

A synthetic gene encoding the fused H\textsubscript{C} of BoNT serotypes C and D (H\textsubscript{C}/H\textsubscript{D}) was synthesized by Epoch Biolabs, Inc. (USA) with optimal codon usage for E. coli. The protein sequences of H\textsubscript{C} (gi:115185) and H\textsubscript{D} (gi:115188) were used as reference to design the gene. Restriction sites were added to allow cloning into the pAE and pAE/ltb expression vectors [25] for E. coli (Table 1). A three-glycine linker (3xGly) was added between the two H\textsubscript{C}S to enable the proper folding of each H\textsubscript{C}. DNA manipulation was performed according to the protocols previously described by Sambrook and Russel [27]. Briefly, after digestion, electrophoresis on an agarose gel was performed. Bands representing the fragments encoding proteins of interest and expression vectors were purified from the gel or digestion reactions, respectively, using the Gel Band Purification Kit (GE Healthcare) and illustra GFX PCR DNA. After purification, the inserts and vectors were quantified and ligated with T4 DNA ligase (New England Biolabs). The ligation products were used to transform E. coli TOP10, which were cultured overnight on LB agar plates with 100 µg/mL ampicillin.

**Expression and purification of the recombinant chimeras**

The recombinant vectors were transformed into E. coli BL21 (DE3) Star by heat shock method [27]. Bacteria transformed with each recombinant vector were grown in Luria-Bertani (LB) broth supplemented with 100 µg/mL of ampicillin in a shaker (37 °C, 150 RPM) until the mid-log growth phase (OD\textsubscript{600}=0.6-0.8). Heterologous protein expression was induced for 3 h by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM. The cells were harvested by centrifugation (16,000 x g, 10 min, 4 °C), suspended in lysis buffer (0.2 M NaH\textsubscript{2}PO\textsubscript{4}, 0.5 M NaCl, 10 mM imidazole), incubated with 50 µg/mL of lysozyme for 1 h at room temperature, disrupted by sonication and centrifuged again (10,000 x g, 30 min, 4 °C). The pellets with inclusion

| Table 1. Specifications regarding restriction enzymes, gene fragments and chimera characteristics. |

| Recombinant chimera | Restriction enzymes\(^a\) | Expression vector | Insert | Fragment length (pb) | Molecular mass (kDa)\(^b\) |
|---------------------|-----------------------------|-------------------|--------|----------------------|--------------------------|
| rLTB/H\textsubscript{C}/H\textsubscript{D} | XhoI and HindIII | pAE/ltb | h\textsubscript{c}/h\textsubscript{d} | 2562 | 112.4 |
| rH\textsubscript{C}/H\textsubscript{D} | KpnI and HindIII | pAE | h\textsubscript{c}/h\textsubscript{d} | 2568 | 100.0 |

\(^a\) Purchased from New England Biolabs
\(^b\) Predicted by Vector NTI Advance 11 (Invitrogen)
Figure 1. Gene sequence and translation of the synthesized ltb/hC/C/H/d construct. Representation of the synthetic gene which encodes rLTB/H_C/C/H/D protein. The first codon of each subunit is in bold and identified by an arrow. The 3xGly linker codons are underlined. Stop codon is indicated by an asterisk (*).

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bodies were washed three times with lysis buffer and incubated overnight at 4 °C with lysis buffer supplemented with 0.2% or 0.4% (w/v) Na-laurylsarcosine (NLS) and centrifuged again (10,000 x g, 30 min, 4 °C). The recombinant proteins were purified by affinity chromatography, using HisTrap™ HP 1 ml columns Ni Sepharose™ on the ÄKTAprime™ automated liquid chromatography system (GE Healthcare), according to the manufacturer’s instructions. Purified fractions were dialyzed against phosphate buffered saline (PBS, pH 7.4) with 0.05% (v/v) Triton X-100 overnight and lyophilized until further use. Protein quantification was performed using the BCA™ Protein Assay (Pierce). The purity and integrity of the recombinant antigens were evaluated by 12% SDS-PAGE.

**Characterization of the recombinant chimeras**

Approximately 5 µg of each of the purified proteins were run in SDS-PAGE 12% and then transferred to a Hybond-ECL nitrocellulose membrane (GE Healthcare) using Tris-Glycine transfer buffer (Tris 25 mM, Glycine 0.2 M, methanol 20% [v/v]) for 1 h under 100 V and approximately 400 mA. The membrane was blocked for 1 h at room temperature with non-fat dry milk 5% (w/v) diluted in PBS with 0.05% (v/v) Tween 20 (PBS-T). The membrane was incubated with mouse monoclonal anti-polyHistidine antibody (Sigma-Aldrich) diluted 1:6,000 in PBS-T for 1 h at room temperature. Rabbit anti-mouse IgG antibody conjugated with peroxidase (Sigma Aldrich) was diluted 1:4,000 in PBS-T and used as secondary antibody. The membrane was incubated the same way as before and revealed by chemiluminescent assay using ECL Western blotting substrate (Thermo Scientific) according to manufacturer’s instructions. After each incubation step, three washes were performed with PBS-T in order to eliminate residual reagents.

The ELISA was performed to evaluate the antigenicity of the chimeras. At each step, plates were incubated for 1 h at 37 °C. Flat-bottom 96-well plates (Nunc) were coated with 200 ng per well of rLTB/H2C/H3D diluted in coating buffer (carbonate-bicarbonate 0.2 M, pH 9.7). The other antigens were added at the same molar ratio. Plates were blocked with non-fat dry milk 5% (w/v) diluted in PBS-T. The standardized anti-toxin C or D sera were diluted in PBS-T to 1 IU/mL and rabbit anti-choleria toxin antibody (Sigma Aldrich) was diluted 1:4,000 in PBS-T and used as secondary antibody. The membrane was incubated the same way as before and revealed by chemiluminescent assay using ECL Western blotting substrate (Thermo Scientific) according to manufacturer’s instructions. After each incubation step, three washes were performed with PBS-T in order to eliminate residual reagents.

**Mice and guinea pig vaccination**

Female Swiss Webster mice, 6 to 8 weeks old, were randomly segregated into 5 groups of 10 animals each. Group one was comprised of animals vaccinated with 50 µg of rLTB/H2C/H3D without conventional adjuvant to evaluate the potential of rLTB as a humoral immune response adjuvant. Group two was comprised of animals vaccinated with 50 µg of rLTB/H2C/H3D plus a 15% aluminum hydroxide suspension (Al(OH)3). Group three was comprised of animals vaccinated with the same molar ratio of rH2C/H3D plus 15% Al(OH)3. Group four was comprised of animals vaccinated with a commercial toxoid vaccine; the dose used was one-twentieth of the dose recommended for cattle. Group five (control group) was comprised of animals vaccinated with 200 µL sterile PBS. Three doses were administered intraperitoneally on days 0, 14, and 28. Blood samples were collected on day 35 from the retro-orbital plexus, and sera were separated by centrifugation (7 min; 3,000 x g), pooled and frozen until further use.

Guinea pigs were vaccinated subcutaneously on days 0 and 21 with 200 µg of each chimera. The groups, each with 10 randomly sorted animals, were the same as those described above for the experiments in mice. Blood samples were collected directly from the heart on day 42, and sera were separated as previously described and used for a mouse neutralization bioassay.

**Evaluation of the humoral immune response**

The humoral immune responses of the mice and guinea pigs were evaluated by a mouse neutralization bioassay performed in accordance with the Brazilian Ministry of Agriculture, Livestock and Food Supply in its ministerial directive n. 23 [28]. Briefly, 1 L+ of each standardized native toxin was incubated for 60 min at 37 °C with 1 mL of undiluted and diluted (1:2, 1:3, 1:5, 1:10 and 1:20) pooled sera containing neutralizing antibodies. 1 L+ is the minimal dose of toxin that, when incubated with 1 IU/mL of standard antisera, is still able to kill the whole mice population. After the incubation period, two mice, weighting 18 to 22 g, were inoculated intravenously and observed for 72 h, with survival checked every 24 h. The titters, expressed in international units per mL (IU/mL), were directly calculated as the minor dilution of serum that, even after incubation with 1L+ of BoNT, is still capable of killing the inoculated mice.

**Statistical analyses**

Statistix9 (Analytical Software) was used to perform ANOVA and Tukey’s test to analyze antigenicity and establish significant differences in the ELISA results.

**Results**

**Vector construction and the expression and purification of recombinant chimeras**

The synthetic gene encoding for H3C and H3D (Fig. 2A), cloned by Epoch Biolabs Inc. (USA) into the pUC19 cloning vector, was digested with endonucleases to release a fragment encoding both chimeras together (Figure 2B and 2C). These genes were cloned into either the pAE/ltb or pAE expression vectors, resulting in two different constructs: pAE/ltb-h_c-h_d and pAE/h_c-h_d. Digestion with endonucleases confirmed the correct cloning of each construct. Thereafter, E. coli BL21 (DE3) Star transformed with pAE/ltb-h_c-h_d or pAE/h_c-h_d...
expressed recombinant proteins of approximately 112 and 100 kDa, respectively, which were the expected molecular weights; however, additional bands with lower molecular weights than expected were also present (Figure 3A and 3B). The additional bands are likely to be truncated proteins because they all reacted with anti-polyHistidine in a Western blot analysis (Figure 3C). Both chimeras were obtained as inclusion bodies and were efficiently solubilized in lysis buffer containing 0.4% and 0.2% NLS, respectively. The one-step refolding process using PBS with 0.05% Triton X-100 did not interfere with protein solubility, since aggregates were not observed. Using this process, it was possible to achieve up to 100 mg of purified recombinant antigen per liter of culture.

Recombinant chimeras are recognized by standard sera

Recombinant chimeras were characterized with ELISAs using standard sera containing anti-BoNTs serotypes C and D and anti-cholera toxin. The ELISAs showed positive reactions of anti-BoNT/C, anti-BoNT/D and anti-CT against rLTB/H

C/H

D. As expected, rH

C/H

D did not react only with anti-CT. Furthermore, the negative control (crude protein extract of E. coli BL21 (DE3) Star) did not react with any sera. Additionally, the standard sera anti-BoNT/C and anti-BoNT/D did not react with rLTB (Figure 4). These results indicate that all domains of the recombinant chimeras are antigenic because they were recognized by standard sera and were significantly different from the negative controls (p < 0.001).

Recombinant chimeras are innocuous and elicit neutralizing antitoxins

Neither mice nor guinea pigs vaccinated with recombinant chimeras presented abnormal responses or lesions at their vaccination sites, indicating the safety of both recombinant chimeras. Sera samples collected from vaccinated animals were used to evaluate the humoral immune response. The mouse neutralization bioassay results from both mice and guinea pigs are presented in Table 2. In both experiments, rLTB/H

C/H

D plus Al(OH)

3 resulted in the best vaccination strategy. Pooled sera from the mice contained 2 IU/mL antibodies against both C and D toxins. Pooled sera from the guinea pigs contained 5 IU/mL and 10 IU/mL antibodies against toxins C and D, respectively. In mice, the other constructs elicited less than 2 and 1 IU/mL antibodies against C and D, respectively. Similarly, in guinea pigs, the other antigens elicited less than 1 IU/mL antibodies against both toxins. The commercial vaccine tested in mice was only effective against BoNT serotype C, whereas in guinea pigs, it elicited the expected antibody levels for both serotypes. The vaccination of guinea pigs with rLTB/H

C/H

D plus Al(OH)

3 met the requirements of the Brazilian Ministry of Agriculture, Livestock and Food Supply in its ministerial directive N° 28 and induced at least two times more antitoxins against BoNT serotype D than the commercial vaccine.

The formulations with only rLTB or Al(OH)

3 as single adjuvants induced low levels of neutralizing antibodies. In contrast, vaccination using both rLTB and Al(OH)

3 induced high
levels of antitoxins. Clearly, there is a synergism between rLTB and Al(OH)$_3$, which is important for the induction of a stronger humoral immune response against the two analyzed BoNTs.

Discussion

Botulism in cattle has been the cause of great economic losses in the past years [5,7,8], with reports indicating that it is a potential source of food-borne botulism in humans [9,10]. This disease is therefore considered a widespread problem for both livestock production and human health. Currently, toxoids used to vaccinate animals are produced by C. botulinum fermentation and the further inactivation of produced toxins by formaldehyde. These procedures result in a non-predictable process that involves high biological risks because the native BoNTs are the most potent toxins known to humans [29]. Additionally, residual formaldehyde may affect the vaccine’s safety [17]. The strategy utilized by our group overcomes these problems because heterologous protein expression in E. coli can be highly regulated, and the H$_C$ domain of the BoNTs is non-toxic, thus eliminating risks to workers during vaccine production.

We developed a bivalent recombinant vaccine with only one fermentative and downstream process, thereby facilitating vaccine production by avoiding additional fermentation steps. Most studies using E. coli to produce recombinant H$_C$ domains yield an average of 40 mg/L, except when a production optimization procedure is performed [30]. When only H$_C$ and H$_D$ production is considered, the yield average is higher, reaching 70 mg/L-culture [13,18,31,32]. The expression strategy we employed was able to produce up to 100 mg of recombinant protein per liter of culture, indicating high expression efficiency. Thus, considering the previously reported expression yield for individual H$_S$s, our chimeras exhibit a more effective production system for vaccines, with only one bioprocess needed to produce what would normally require at least 4 different fermentation steps. An attempt to optimize the expression of rLTB/H$_C$/H$_D$ using the same parameters as described by Yari et al. [33] in modified M9 medium did not interfere with the protein yield or the protein solubility (data not shown).

Our mouse and guinea pig vaccination results demonstrated that both fused constructs did not cause adverse side effects and are capable of inducing neutralizing antibodies (Table 2). These results were expected because the H$_C$ domains of BoNTs are non-toxic and capable of inducing protection against botulism [18]. Although the fusion of H$_C$/H$_D$ and LTB might eliminate a few important protective epitopes, the immune responses of the mice and guinea pigs suggest that some epitopes were still present, thereby allowing the generation of immunity to botulism. Although a linker consisting on 3xGly was added between rH$_C$ and rH$_D$, it is possible that the conformational epitopes of rH$_C$ could not fold properly when compared to rH$_D$, which is flanked only by rH$_C$. This is possible because H$_D$ elicited higher neutralization titers than H$_C$ in guinea pigs. Three glycine residues were chosen because this amino acid has no side chain and thus confers flexibility between the domains, allowing the correct protein folding of each one. In fact, previous studies have described the effective use of glycine-rich linkers, such as (Gly$_3$Ser)$_n$, and (Gly–Ser–Gly)$_n$, even in vaccine studies due to the flexibility of glycine [34–39]. These linkers are larger than the
3xGly used in this work, supporting the hypothesis that it is too short to allow the proper folding of H\textsubscript{C}C, and thus explaining the difference in the production of antibodies against toxins C and D in guinea pigs. Sakamoto et al. [38] described the influence of linker length and flexibility upon functionality of each domain of a recombinant chimera. Thus, it is possible that using a \((\text{Gly}_4\text{Ser})_3\) linker instead of only 3xGly may result in a stronger humoral immune response.

Despite different vaccination schedules, a remarkable discrepancy was observed between the results of the two animal models. While the chimeric vaccine induced high neutralizing antibodies levels in guinea pigs, the mice did not show a similar result. Although neutralization assays are not commonly performed with mouse sera, our data are not in accordance with the literature, as previous studies have shown that BALB/c and ICR mice can generate high levels of protection against several BoNTs [18,40]. One possible explanation for these experimental differences is the mouse strain that we used (Swiss Webster). This type of mouse is genetically heterogeneous and thus has the advantage of representing a varied population. In addition, it is not an enhanced T\textsubscript{H}2 responder strain, such as the BALB/c strain, which could explain the low antibody production against the antigens that were used. In contrast, guinea pigs are ideal model organisms for immunology tests against botulism, and, thus, it is expected that high levels of immunological responses could be generated [41,42].

The rLTB/H\textsubscript{C}C/H\textsubscript{C}D chimera plus Al(OH)\textsubscript{3} induced high levels of neutralizing antibodies against both serotypes in guinea pigs. It is noteworthy that the Brazilian Ministry of Agriculture, Livestock and Food Supply determined that a vaccine against bovine botulism must induce minimum of 5 IU/mL and 2 IU/mL of anti-BoNT C and D neutralizing antibodies, respectively, to be approved [28]. In our results from the mouse neutralization bioassay, the chimera rLTB/H\textsubscript{C}C/H\textsubscript{C}D plus Al(OH)\textsubscript{3} induced 5 IU/mL and 10 IU/mL for serotypes C and D, respectively, which is in accordance with the government’s mandate. These values were comparable to the commercial vaccine and much higher than the other tested constructs. Takeda et al. [43] vaccinated ducks with a C/D mosaic toxoid (the H\textsubscript{C}C portion consisted of serotype D) and obtained an average of 6 IU/mL, as determined by the mouse neutralization bioassay. Moreover, in our study, sera samples were obtained from guinea pigs three weeks after the last vaccination dose, in a manner similar to that of Takeda [43]. Nonetheless, we obtained an average of 7.5 IU/mL when considering both serotypes, which highlights the potential of our chimera. However, guinea pigs vaccinated with either rLTB/H\textsubscript{C}C/H\textsubscript{C}D alone or rH\textsubscript{C}C/H\textsubscript{C}D plus Al(OH)\textsubscript{3} did not display an appropriate humoral immune response.

### Table 2. Levels of neutralizing antibodies against BoNTs serotypes C and D in vaccinated mice and guinea pigs.

| Vaccine formulation | Mice* | Guinea pigs* |
|---------------------|-------|--------------|
|                     | Serotype C | Serotype D | Serotype C | Serotype D |
| rLTB/H\textsubscript{C}C/H\textsubscript{C}D | 2 IU/mL | 1 IU/mL | ≤ 1 IU/mL | ≤ 1 IU/mL |
| rLTB/H\textsubscript{C}C/H\textsubscript{C}D + Al(OH)\textsubscript{3} | 2 IU/mL | 2 IU/mL | 5 IU/mL | 10 IU/mL |
| rH\textsubscript{C}C/H\textsubscript{C}D + Al(OH)\textsubscript{3} | 2 IU/mL | 1 IU/mL | ≤ 1 IU/mL | ≤ 1 IU/mL |
| Commercial vaccine  | 5 IU/mL | 1 IU/mL | 5 IU/mL | 3 IU/mL |
| PBS                 | ND\textsuperscript{a} | ND\textsuperscript{a} | ND\textsuperscript{a} | ND\textsuperscript{a} |

* Values obtained by mouse neutralization bioassay.

\textsuperscript{a} ND, not detectable.

**Figure 4. Antigenicity evaluation of the recombinant chimeras by ELISA using standard anti-BoNT C and D sera.** Anti-BoNT C serum (A) showed that the rLTB/H\textsubscript{C}C/H\textsubscript{C}D antigen has more antigenic epitopes than rH\textsubscript{C}C/H\textsubscript{C}D, while anti-BoNT D serum (B) shows no difference between these two antigens. Anti-cholera toxin serum (C) was used to evaluate the antigenicity of the rLTB domain. Crude protein extract from \textit{E. coli} BL21 (DE3) Star and purified rLTB were used as controls for the specificity of the three sera. Different letters above the bars (A, B, or C) indicate significant differences (p < 0.001) according to Tukey’s test. Absorbances (Abs) shown were measured at 450 nm wavelength.

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The best results were obtained using the constructs containing LTB, a powerful adjuvant of humoral immune response [22,23,25,26]. The presence of LTB was essential for the development of a strong humoral immune response when it acted in synergism with Al(OH)₃. Thus far, there have not been any works reporting a bivalent vaccine against botulism using a single polypeptide chain containing antigens and adjuvant. Additionally, there have not been any works evaluating LTB as an adjuvant in vaccines for preventing botulism. Conceição et al. [22] used LTB fused to the R1 antigen of Mycoplasma hyopneumoniae P97 adhesion to immunize mice without conventional adjuvants. This treatment induced the production of strong humoral and cellular immune responses, highlighting the use of LTB as an adjuvant of the immune response with antigens fused to its C-terminal portion. Indeed, the fusion of other antigens to the C-terminal end of LTB does not impair its biological activity, as was already reported [44,45].

Zeng et al. [46] published a similar work with a trivalent recombinant chimera against C. perfringens toxins, in which vaccines with fused toxins or co-administered antigens were demonstrated to have higher immunogenicity than antigens alone, eliciting a high titer of neutralizing antibodies. These results corroborate ours, in which synergism between LTB and Al(OH)₃, together with vaccination with fused antigens, induced elevated titers of neutralizing antibodies. The large difference between the levels of neutralizing antibodies generated by vaccination of guinea pigs with Al(OH)₃ and either rLTB/H₃C/H₃D or rH₁C/H₁D support these findings and indicate that LTB indeed works as a systemic humoral immune response adjuvant, even though it was originally described to be a mucosal adjuvant [47]. Additionally, animals vaccinated with rLTB/H₃C/H₃D without conventional adjuvant developed low levels of neutralizing antibodies, suggesting that synergism between Al(OH)₃ and LTB is essential for the induction of an appropriate humoral immune response.

Both LTB and Al(OH)₃ are capable of inducing Tₐ2-type responses [26,48], which in turn activate B cells through secretion of IL-4 and IL-5 [49], resulting in B cell proliferation and a strong humoral response consisting of the production of both s-IgA and IgG [50], as well as other isotypes. Aluminum hydroxide also creates a deposit of antigens, which extends the antigen exposure time to the immune cells, resulting in a stronger and more specific systemic response. Although the mechanism of action of LTB remains unclear, it has been shown that its activity stems from its ability to bind to GM1 ganglioside receptors [26,47], which are present on almost all mammalian cells, including antigen-presenting cells [51]. In this manner, LTB plays a key role by easing antigen uptake and presentation by dendritic cells, macrophages and, most importantly, B cells. Furthermore, LTB has been shown to up-regulate the expression of essential molecules for the development of an appropriate and strong immune response, such as the B7.1 and B7.2 co-stimulatory molecules. It also augments the expression of chemokine receptors and MHC class II on antigen-presenting cells [52]. Consequently, the adjuvant effects induced by both LTB and Al(OH)₃ act synergistically and complement each other, resulting in a stronger systemic humoral response. Thus, the rLTB/H₃C/H₃D molecule can be considered a “3 in 1” product, as it contains (1) a vaccine against cattle botulism serotype C, (2) a vaccine against cattle botulism serotype D, and (3) an adjuvant molecule, all in a single polypeptide chain.

In conclusion, we describe in this study the potential of the recombinant chimera rLTB/H₃C/H₃D as a novel strategy to prevent botulism in cattle by vaccination. The use of rLTB/H₃C/H₃D plus Al(OH)₃ could be considered a potential commercial product, although large-scale production must still be established. We also corroborated the results from other studies [22,25] establishing the potential of LTB as a humoral immune response adjuvant. Furthermore, our study also provides insights for studies with respect to other bacterial toxins.

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Author Contributions

Conceived and designed the experiments: LG CEC GM FC. Performed the experiments: LG CEC GM FS RA MM FL FC. Analyzed the data: LG CEC GM FS RA MM FL FC. Contributed reagents/materials/analysis tools: FC FL OD. Wrote the manuscript: LG CEC GM FS MM OD FC.

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