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Short communication

Immunogenicity of *Clostridium perfringens* epsilon toxin recombinant bacterin in rabbit and ruminants

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1. Introduction

Clostridium perfringens's Epsilon toxin (ETX) is one of the most potent known microbial toxins [1]. ETX is a pore-forming β-toxin (β-PFT) produced in the small intestine as a 32.98 kDa protoxin and activated by trypsin, chymotrypsin, or λ-protease, among other carboxypeptidases [2]. ETX is the causative toxin of enterotoxaemia, caused by *C. perfringens* toxinotype D, which is the main infectious disease responsible for death in sheep all over the world [1,3]. It can also affect goats and, to a lesser extent, cattle [4]. ETX may also synergistically act with beta toxin (CPB) of *C. perfringens* toxinotype B and cause dysentery in sheep [3].

Enterotoxaemia frequently presents in outbreaks where the main risk factor is a drastic change to a high carbohydrate feed, which culminates in unbalanced gut flora, favouring *C. perfringens* growth and ETX production. After activation, ETX makes the intestinal mucosa more permeable and travels through the bloodstream affecting the kidneys, lungs, brain, and heart. ETX also causes increased vascular permeability that leads edema, neurological disorders, shock, and sudden death [1–3]. The highly lethal outcome of *C. perfringens* intoxications renders vaccination against its toxins the best prophylactic measure [3,5]. More than 150 million vaccine doses against clostridiosis are produced annually in Brazil [6]. The majority of vaccines currently in use are formaldehyde-inactivated toxins (toxoids) obtained from *C. perfringens* culture supernatant, a complex process with high biosafety risks. Therefore, recombinant vaccines have emerged as alternatives to conventional toxoids because they offer advantages in terms of both efficiency and production process safety [6–10,13,15].

In light of these issues, researchers have attempted to use non-purified recombinant proteins (i.e., inclusion bodies or CLS of recombinant *E. coli*) to vaccinate farm animals as a means of simplifying recombinant vaccines production process [6,7]. Seeking an even simpler strategy, our study describes the immunogenicity of inactivated recombinant *E. coli* expressing ETX (recombinant bacterin) in rabbits and ruminants.

2. Materials and methods

2.1. Ethical statement

This study was approved by the Ethical Committee for Animal Experimentation of the Federal University of Pelotas (Permit N 0102549/2017).

2.2. Production and characterization of recombinant ETX (rETX)

A synthetic gene encoding ETX (lacking the first 45 N-terminal residues signal sequence) was synthesized with optimal codon usage for *E. coli* (Epoch Life Science) and cloned into the pAE expression vector. *Escherichia coli* BL21 (DE3) Star™/pAE*etx* was used for expression and purification of rETX as previously described [9]. Purified recombinant protein was quantified by BCA kit (Thermo Scientific, USA), lyophilized, and stored at 4 °C until use.

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2.3. rETX antigenicity

rETX antigenicity was assessed by Western blot (WB) using anti-6xHis monoclonal antibody (Sigma-Aldrich, USA), pooled sera of rabbits vaccinated with either the recombinant bacterin or with the commercial vaccine. *E. coli* BL21 (DE3) Star™/pAE*etx* induced for 4 h protein extract was subjected to SDS-PAGE 12% and transferred to a nitrocellulose membrane (Bio-Rad). The membrane was blocked with 5% non-fat dry milk in PBS-T (0.05% Tween-20). Pooled rabbit sera diluted at 1:400 was used. Goat anti-rabbit IgG-peroxidase (Sigma Aldrich, USA) diluted at 1:5.000 was used as a secondary antibody. The reaction was revealed with 3,3′-diaminobenzidine. All incubation steps were performed at room temperature for 1 h under slight agitation followed by 3 washes with PBS-T.

2.4. Vaccine formulation and safety

After 4h expression, *E. coli* cells was harvested, washed twice with PBS (7.000*g*, 15 min, 4 °C), and incubated with 0.2% (v/v) formaldehyde (200 RPM, 24 h, 37 °C). The inactivated cells were harvested and washed twice with sterile PBS (7.000*g*, 15 min, 4 °C). Inactivation was confirmed by plating 100 μL of formaldehyde-treated cells on solid LB (18 h, 37 °C). rETX was quantified by SDS-PAGE 12% using BSA standards and TotalLab Quant software. Recombinant bacterins were then adsorbed onto 15% $Al(OH)_3$ (v/v) for 18 h at room temperature. The recombinant bacterin sterility and innocuity were evaluated as stipulated by the Brazilian Ministry of Agriculture, Livestock and Food Supply (MAPA) according to ministerial directive n. 49/1997 [14].

2.5. Potency test in rabbits

New Zealand rabbits (8 animals/group) weighting approximately 1.5 kg were inoculated subcutaneously with 2×10^8 CFU/ recombinant *E. coli* bacterin (50 μg rETX - Group 1) or 8×10^8 CFU/ (200 μg rETX – Group 2) in a final volume of 3 mL/dose at days 0 and 21. Group 3 was inoculated subcutaneously with 1 mL of commercial toxoid (Resguard Multi, Biovet, BRA) according to manufacturer's instructions. Group 4 was inoculated subcutaneously with 3 mL sterile PBS. The animals were anesthetized at day 35 with a ketamine-xilazine combination (30 and 10 mg/kg, respectively) and blood samples were drawn by cardiac puncture. The animals were subsequently euthanized with an overdose of the anaesthetic. The serum samples were obtained by centrifuging blood (3000*g*, 7 min), pooled, and stored at −20 °C until further use. Seroneutralization assays were performed in accordance with the ministerial directive n. 49/1997 [14]. This method has been previously used in other studies [6,9,16].

2.6. Recombinant bacterin immunogenicity in ruminants

Seven 6-month-old female Nelore cattle and seven adult Corriedale sheep were fed by grazing in pastures with controlled minerals, and *ad libitum* drinking water. Animals did not present any levels of anti-ETX antibodies in their sera prior to the beginning of this experiment. Sheep and cattle were vaccinated subcutaneously at days 0 and 28 with 8×10^8 CFU recombinant bacterin (200 μg of rETX) mixed with 15% $Al(OH)_3$ (v/v) in final volumes of 2 and 5 mL, respectively. Blood samples were drawn by puncturing the jugular vein at days 0 and 56 [9]. Sera were obtained by centrifuging blood samples (3000*g*, 7 min) and stored at −20 °C until further use. The antitoxin levels were determined by seroneutralization assay as described in the above section.

2.7. Statistical analysis

Data analysis was done using GraphPad Prism v7 (GraphPad Software, USA) using One-way ANOVA followed by Tukey's multiple comparisons test. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{****}P < 0.0001$.

3. Results

After confirming the rETX expression, the *E. coli* cells were successfully inactivated with 0.2% formaldehyde (v/v). The sterility tests exhibited no growth of either aerobic or anaerobic bacteria or fungi, and neither adverse nor unexpected reactions occurred in rabbits and ruminants subcutaneously vaccinated with twice the volume and dose used, thus indicating the innocuity of our vaccine formulation.

WB analysis against mAb anti-His6x exhibited a band of approximately 34 kDa, as expected for rETX. WB using the sera of rabbits vaccinated with either native or recombinant ETX revealed that rETX is recognized by both sets of sera (Fig. 1). Interestingly, multiple bands of high molecular mass (> 65 kDa) were observed on SDS-PAGE (Fig. 1a) and recognized by mAb anti-His6x (Fig. 1b), as well as by pooled sera from animals vaccinated with conventional toxoid (Fig. 1c) and rETX bacterin (Fig. 1d), suggesting the multimerization of rETX.

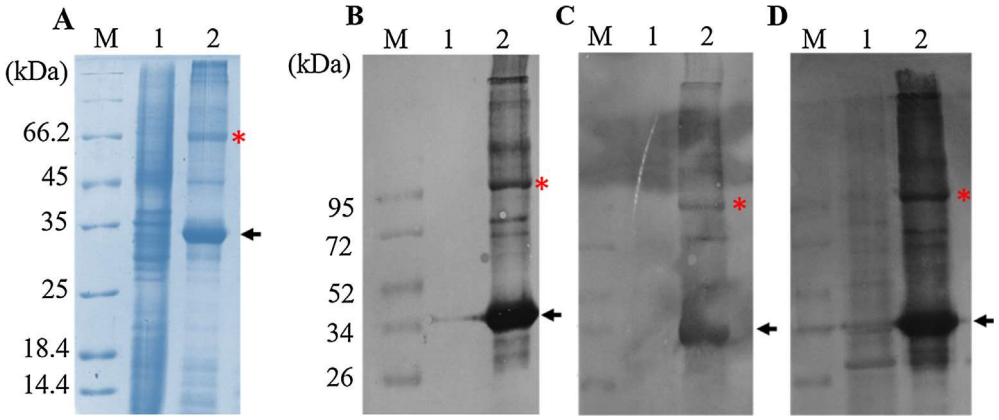

Fig. 1. SDS-PAGE 12% and Western bloting (WB) analysis to detect rETX. (A) SDS-PAGE for expression analysis of rETX (arrow, 34 kDa). (B) rETX expression confirmation by WB using mAb anti-His6x (Sigma-Aldrich). (C) Antigenicity analyses by WB with sera of animals vaccinated with commercial native toxoid, and (D) sera of animals vaccinated with bacterin rETX. M- Protein Ladder; 1- *E. coli* BL21 (DE3) Star™ wild type; 2- *E. coli* BL21 (DE3) Star™/pAE*etx* after 4 h expression. * Band of high molecular mass (> 65 kDa) suggesting the multimerization of rETX.

Table 1
Anti-toxin levels detected in cattle after immunization. The minimum value against epsilon toxin is 2 IU/mL according to MAPA.

ETX	Anti-toxin level per cattle (IU/mL)		
	Bacterin[a]	Purified[b]	Commercial[c]
	5.7	14.4	7.2
	4.8	14.4	6.0
	4.8	14.4	6.0
	4.8	12.0	6.0
	4.8	12.0	5.0
	4.0	12.0	5.0
	4.0	10.0	5.0
Mean ± SD	4.70 ± 0.58	12.74 ± 1.70	5.74 ± 0.81

SD, standard deviation.
[a] Nelore cattle immunized recombinant bacterin containing 200 μg of rETX.
[b] Girolando cattle immunized with 200 μg of each of the three purified recombinant antigens (rCPA, rCPB, and rETX).
[c] Commercial toxoid [9].

Table 2
Anti-toxin levels detected in sheep after immunization. The minimum value against epsilon toxin is 2 IU/mL according to MAPA.

ETX	Anti-toxin level per sheep (IU/mL)		
	Bacterin[a]	Purified[b]	Commercial[c]
	8.0	10.0	6.0
	8.0	10.0	5.0
	6.9	7.2	5.0
	6.9	7.2	5.0
	6.9	7.2	5.0
	6.9	6.0	5.0
	5.7	6.0	5.0
Mean ± SD	7.04 ± 0.79	7.66 ± 1.69	5.14 ± 0.38

SD, standard deviation.
[a] Corriedale sheep immunized recombinant bacterin containing 200 μg of rETX.
[b] Santa Ines sheep immunized with 200 μg of each of the three purified recombinant antigens (rCPA, rCPB, and rETX).
[c] Commercial toxoid [9].

The recombinant bacterin was capable of inducing up to 10 IU/mL of anti-ETX neutralizing antibodies in rabbits, similar to the amount the commercial toxoid did (positive control), and higher than the 2 IU/mL required by the CFR9/USDA [17]. No anti-ETX antibody titers were detected in the pooled sera of animals vaccinated with 50 μg rETX or PBS. Unlike the potency test in rabbit, sera of farm animals were not pooled, but individually analysed. The seroneutralizantion results, performed in mice, are described in Tables 1 and 2. Mean titers of anti-ETX neutralizing antibodies in cattle and sheep immunized with the recombinant bacterin were 4.7 ± 0.58 IU/mL and 7.04 ± 0.79 IU/mL, respectively. The results of a previous study by Moreira et al. [9] were added in the tables for better comparison of results between bacterin and purified vaccines. In this study, pre-immunization ruminants and negative control animals did not have any detectable anti-ETX neutralizing antibody levels.

According to the statistical analysis, the recombinant bacterin was able to induce significantly higher neutralizing antibody titres in cattle and sheep (Fig. 2). Compared to purified antigen, the recombinant bacterin induced similar titers of antitoxin in sheep (difference was not statistically significant), but lower titers in cattle ($P < 0.0001$). In sheep, the recombinant bacterin and purified rETX vaccines outperformed commercial vaccines ($P < 0.05$ and $P < 0.01$, respectively). Furthermore, recombinant vaccines induced higher titers than commercial vaccine in cattle as well (recombinant bacterin, $P < 0.01$; purified rETX, $P < 0.0001$).

4. Discussion

Vaccines against clostridiosis (native toxoids) present a low cost per dose (US$ 0.15–0.30) and are usually effective protecting against ETX. Non-purified recombinant vaccines are produced using a safe (no toxic activity), simpler and faster (matter of days instead of weeks) production process that poses no health threats [6,7,16]. As such, they may be economically competitive [11]. We demonstrate herein the production and evaluation of a recombinant *E. coli* bacterin containing rETX, which was capable of inducing neutralizing antibodies in rabbits, sheep, and cattle.

Many authors have already demonstrated the potential of rETX to prevent the diseases caused by *C. perfringens* toxinotypes B and D using purified recombinant proteins [6,7,9,12]. Lobato et al. [6] evaluated the potential of rETX inclusion bodies (IB) in rabbits, cattle, sheep, and goats; high levels of neutralizing antibodies were obtained in such study. Langroudi et al. [7] used the CLS of recombinant *E. coli* containing a chimera comprised of CPB and ETX to inoculate rabbits and obtained 10 and 6 IU/mL of anti-CPB and anti-ETX respectively. Even though the aforementioned vaccines do not require purification steps, cell lysis is still necessary. In the present study, both cell lysis and purification steps were dismissed. Recombinant *E. coli* cells after 4 h induction was inactivated, washed, and adsorbed onto aluminium hydroxide. This process is much simpler than those previously described, as it dismisses lysis, purification, and refolding steps [6,7]. This is the first study to demonstrate the immunogenicity of this novel vaccine production and formulation strategy in rabbits and ruminants to protect against ETX.

Several studies have proved rETX is efficient in inducing the production of neutralizing antibodies in rabbits [6,8,9], the animal model used in potency tests. Herein, we demonstrate the non-purified version of this same antigen (i.e., recombinant *E. coli*

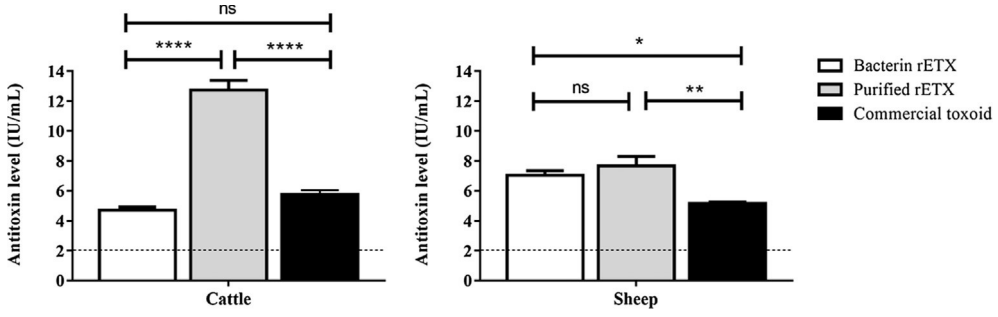

Fig. 2. Mean antitoxin level generated in cattle and sheep. All formulation vaccines surpassed the minimum level antitoxin required (dashed line; 2 IU/mL) against epsilon toxin. Statistical analysis was performed by two-way ANOVA followed by Tukey's multiple comparisons test. $^{*}P < 0.05$; $^{**}P < 0.01$ and $^{****}P < 0.0001$. The results of purified rETX and commercial toxoid are previous ours study by Moreira et al. [9] were used in graphic for comparison.

bacterin) is also immunogenic and capable of inducing up to 10 IU/mL of anti-ETX neutralizing antibodies in rabbits, as required by CFR9/USDA [17]. Our group has previously demonstrated that this strategy can effectively induce high titers of neutralizing antibodies against botulinum neurotoxins serotypes C and D when the antigen is presented either in a recombinant bacterin, as IB or in CLS [16].

Research has shown that the titer of neutralizing anti-ETX antibodies in rabbits is usually higher than the titer induced in farm animals. Moreira et al. [9] obtained 25 IU/mL of anti-rETX in rabbits and 12.74, 7.66, and 8.91 IU/mL in cattle, sheep, and goats respectively. Lobato et al. [6] evaluated the potential of rETX IB in rabbits, cattle, sheep, and goats, obtaining 40, 13, 26, and 14 IU/mL of anti-ETX neutralizing antibodies respectively. In this study, recombinant bacterin induced titers of 10, 4.7 and 7.04 IU/mL of anti-rETX in rabbits, cattle and sheep, respectively. This could, perhaps, be attributed to differences in size and weight of these animals, since all animal species were vaccinated with the same concentration of recombinant protein. This trend has also been observed for *C. perfringens* alpha and beta toxins [6,15].

The average antitoxins titers in sheep vaccinated with the recombinant bacterin was 7.04 IU/mL, higher than those induced by the commercial vaccine (5.14 IU/mL) and similar to those induced by purified rETX (7.66 IU/mL; $P < 0.05$). Cattle had similar antitoxin titers induced by the recombinant bacterin and the commercial vaccine (4.7 and 5.17 IU/mL, respectively; $P > 0.05$), but lower than those induced by purified rETX (12.7 IU/mL; $P < 0.001$). Despite the fact that the experiment carried out in this study had some differences (animal age, race, and location) in relation to those carried out by Moreira et al. [9], our results suggest that the recombinant bacterin is as effective as the commercial vaccines in sheep, and less effective than the purified rETX in cattle, even though both surpassed the minimum level required by MAPA [14]; differences in production costs and time should also been taken into consideration. The efficacy of the recombinant bacterin in sheep should also be highlighted considering that sheep are the species most affected by ETX [3]. Noteworthy, the recombinant bacterin appear to be stable and homogenous inducing anti-ETX neutralizing antibodies (i.e. animals had very similar titres), even when different batches were used in the experiments.

This study represents the first steps in developing a vaccine against *C. perfringens* ETX with a simple and safe production process that is capable of inducing protective immunity against epsilon toxin from *Clostridium perfringens* in rabbits and farm ruminants.

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Author contributions and conflict of interest

MF, CM-J, RD, and CM produced the vaccine. AR and FS performed the potency test in rabbits. MF, AR, FD, FS, and CM-J vaccinated the sheep and cattle. AR, CO, and FS performed the seroneutralization assay. FL, AM, FS, CO, JB, and FC supervised the study and reviewed of manuscript. MF, CC, FL, AM, FS, CO, JB, and FC designed the experiments, analysed and interpreted data, and wrote the manuscript. The authors declare no conflict of interest.

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