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Inactivated recombinant *Escherichia coli* as a candidate vaccine against *Clostridium perfringens* alpha toxin in sheep

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ABSTRACT

Clostridium perfringens type A is the causative agent of gas gangrene and gastroenteric ("yellow lamb disease") disease in ruminants, with *C. perfringens* alpha toxin (CPA) being the main virulence factor in the pathogenesis of these illnesses. In the present study, we have developed recombinant *Escherichia coli* bacterins expressing rCPA and used it to vaccinate rabbits and sheep. Doses of up to 200 μg of rCPA used for inoculation, induced 13.82 IU.mL^{-1} of neutralizing antitoxin in rabbits, which is three times higher than that recommended by the USDA (4 IU.mL^{-1}). In sheep, recombinant bacterins induced antitoxin titers of 4 IU.mL^{-1}, 56 days after the first dose. rCPA which was expressed, mainly, in inclusion bodies, was not found to influence the immunogenicity of the vaccine. The recombinant *Escherichia coli* bacterin, produced simply and safely, is capable of affording protection against diseases caused by *C. perfringens* CPA. The current findings represent a novel production method for CPA vaccines potentially applicable to veterinary medicine.

1. Introduction

Alpha toxin (CPA) of *Clostridium perfringens* is a phospholipase C (PLC), approximately 43 kDa, encoded by chromosomal gene *plc* [1], expressed in all toxinotypes (A-E) [2]. Structurally, CPA is divided into an N-terminal domain and a C-terminal domain. The N-terminal domain possesses the enzymatic activity of phospholipase C, responsible for the hydrolysis of cell membrane phospholipids, causing a lethal, necrotizing and/or bleeding effect. The C-terminal domain, which is bound to the calcium ion, is responsible for cell surface binding [3]. CPA is mainly produced by *C. perfringens* toxinotype A, which can also produce other toxins such as enterotoxin (CPE) and beta 2 toxin (CPB2) [4].

Gas gangrene, one of the major myotoxic diseases of ruminants, is mainly related to CPA (80% of cases). It is characterized by severe necrosis and accumulation of gas in the tissues (muscles, fascia, and subcutaneous), progressing to shock and often death, generating significant economic losses in dairy industry [5–9]. Although *C. perfringens* type A is mostly isolated from the environmental sources and from the gastrointestinal tract of clinically healthy domestic animals, this pathogen is also responsible for a rare form of acute enterotoxemia in lambs, known as yellow lamb disease [10,11]. The pathogenesis of this disease is controversial, but it is assumed that the lesions and clinical signs are primarily due to CPA activity [12,13]. Previous studies have shown the potential of purified recombinant CPA (rCPA) in the control of gas gangrene [14,15] and gastrointestinal diseases, such as necrotic avian enteritis [16], necrohemorrhagic enteritis [17] and diarrhea in piglets [18]. Recently, our group also demonstrated the protective potential of purified rCPA in rabbits, cattle, sheep, and goats [19]. However, the production of purified recombinant antigens is laborious and cost-ineffective for animal vaccination. Therefore, the objective of this study was to evaluate the efficacy of a simple and easily produced, inactivated *Escherichia coli* vaccine expressing rCPA (recombinant bacterin) adsorbed on aluminum hydroxide, for immunization in sheep.

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2. Material and methods

2.1. Ethics

All animal experiments were performed in accordance with the guidelines of the National Council for Animal Experimentation Control (CONCEA). This study was approved by the Ethical Committee for Animal Experimentation of the Federal University of Pelotas (Permit N° 0102549/2017).

2.2. Subcloning and expression of rCPA

The full length of CPA coding sequence was amplified (Epoch, Life, Science) and subcloned into the expression vector pET28a (Invitrogen, CA, USA). Briefly, CPA was amplified by PCR using primers containing sites for *Nhe*I and *Hind*III, respectively: *cpa*-F (5′-CTAGCTAGCTGGGATGGTAAGATTGAT-3′) and *cpa*-R (5′-CCCAAGCTTTCATTTGATGTTGTAGGTAGA-3′). The expression, characterization, and purification of rCPA was performed as described by Moreira et al. [19]. The *E. coli* strain BL21 (DE3) Star ™ was then transformed with the pET28a/*cpa* vector followed by culture of the pre-inoculum at 37 °C for 18 h at 180 rpm. Subsequently, the inoculum was prepared and the expression was induced with 1 mM isopropyl-β-D-1-thiogalactopyranoside during the logarithmic growth phase ($OD_{600nm} = 0.6–0.8$). The expression and purification steps was performed rCPA expression was characterized by SDS-PAGE and Western blot with anti-His6x mAb (Invitrogen, CA, USA).

2.3. Production of the vaccine

After expression of rCPA, the cells were collected by centrifugation and suspended in phosphate buffered saline (PBS) supplemented with 0.2% (v/v) formaldehyde and maintained at 37 °C for 18 h at 180 rpm. The inactivated cells were washed twice in PBS (7000×*g*, 10 min, 4 °C). Inactivation was confirmed by inoculating 100 μL of sample into 10 mL of Luria-Bertani broth (LB) and plating on solid LB, grown at 37 °C for 18 h rCPA was quantified by 12% SDS-PAGE using a BSA curve and analyzed in the TotalLab Quant software.

The sterility and innocuousness of the recombinant bacterin, containing rCPA, was evaluated as recommended by ordinance N. 49 of the Ministry of Agriculture, Livestock, and Supply (MAPA, 1997). The dose of 200 μg rCPA, at approximately 8×10^8 CFU mL^{-1}, in the form of recombinant *E. coli* bacterin adsorbed on 15% (v/v) aluminum hydroxide was used. Innocuity was assessed by inoculating two sheeps with twice the vaccine dose (400 μg) and observing for side effects for 72 h. These protocols are further described on Moreira et al. study (2016) [19].

2.4. Potency test of the recombinant bacterin

The recombinant bacterin potency test was performed according to the Code of Federal Regulations (CFR9) of the United States Department of Agriculture (USDA). Eight New Zealand rabbits, weighing between 1.8 and 2.6 kg, were vaccinated, subcutaneously, with two doses of 200 μg of rCPA, at a 21-day interval. Fifteen days after the second dose, the animals were anesthetized with a combination of ketamine (80–100 mg kg^{-1}) and xylazine (8–10 mg kg^{-1}). Blood was then collected by cardiac puncture. Sera were obtained by centrifugation of blood (3000×*g*, 10 min) before being mixed and stored at −20 °C until further use.

Titration of CPA antitoxin was performed using USDA methodology (USDA, 2002), wherein 1 mL of serum was incubated with $10 \times LD_{50}$ of CPA toxin (Sigma-Aldrich) for 30 min at 37 °C. Five mice were inoculated intravenously with 0.2 mL of the homogenate and observed for 72 h. A subsequent protection of 80% of the animals corresponds to the CPA antitoxin titre of 4 IU.mL−1. This method has been previously used in other studies [17–21].

2.5. Immunogenicity of recombinant bacterin in sheep

Fourteen adult sheep of Texel breed were fed *ad libitum* with pasture, mineral supplementation, and water. Seven sheep were vaccinated with 8×10^8 CFU mL^{-1} recombinant bacterins (200 μg rCPA) mixed with 15% $Al(OH)_3$ (v/v) to a 2 mL final volume (Group 1). As a positive control, seven sheep were vaccinated with commercial toxoid (Covexin® 9, Schering-Plough– Group 2). As a negative control, sheep of day 0 was used because there was no titter prior to the study. All animals were vaccinated subcutaneously on days 0 and 28. Blood samples were collected via jugular vein puncture on days 0, 28, and 56 [19]. Sera were obtained by centrifugation of blood samples (3000×*g*, 7 min) and stored at −20 °C until further use. Antitoxin levels in pooled sera of animals were determined by the serum neutralization assay as described in the previous section.

2.6. Detection of anti-rCPA antibodies by indirect ELISA

Indirect ELISA was used to evaluate *anti*-rCPA antibody levels in individual sheep sera at days 0, 28 and 56. Nunc-Immuno MicroWell MaxiSorp™ plates were coated with 100 ng/well purified rCPA diluted in buffer carbonate/bicarbonate (TCB) at pH 9.6 incubated for 18 h at 4 °C). Plates were washed three times with PBS-T between each step. Post-coating incubations were performed for 1 h at 37 °C in a final volume of 100 μl/well. Plates were blocked with 5% skimmed milk powder in PBS-T. Serum samples were diluted 1:300 in PBS-T and added to the duplicate plate. The peroxidase-conjugated sheep antiIgG (Sigma-Aldrich, USA) was diluted 1:5000 and added to the plate. The reaction was developed using Ortho-phenylenediamine (OPD; Sigma-Aldrich, USA) in 0.2 M citrate-phosphate buffer (TPS), pH 4.0 and 0.02% H_2O_2. The plates were incubated for 15 min at room temperature and OD_{492nm} was measured using the Biochrom EZ Read 400 microplate reader.

2.7. Statistical analysis

Data analysis was done with GraphPad Prism v7 (GraphPad Software, USA) using *t*-test and One-way ANOVA followed by Tukey's multiple comparisons test ($P < 0.05$).

3. Results

Using a seroneutralization assay, we found that rabbits vaccinated with inactivated *E. coli* cells expressing rCPA exhibited 13.82 IU.mL^{-1} titer of *anti*-CPA neutralizing antibodies. Sheep vaccinated with rCPA recombinant bacterins showed no sign of toxicity or behavior change and showed antitoxin-CPA titre of 4 IU.mL^{-1}, whereas the animals vaccinated with commercial toxoid presented less than 4 IU.mL^{-1}, as the five inoculated mouse from the titration of CPA antitoxin assay, 2 died nearly 72 h post challenge. Using the indirect ELISA assay, we show, in Table 1, the *anti*-CPA total antibody levels (OD_{492nm}) in sera from sheep vaccinated with *E. coli* bacterins expressing rCPA and commercial toxoid. With respect to the antibody titers obtained in the sera, from animals vaccinated with the recombinant rCPA bacterins, there was an increase in antibody titers ($p = 0.0088$) 56 days after vaccination whereas no difference was observed ($p = 0.7494$) between antibody titres of the animals vaccinated with the commercial toxoid.

Table 1
Anti-CPA antibody levels (OD_{492nm}) in sheep serum vaccinated with recombinant bacterin and commercial toxoid.

CPA	*Anti*-CPA antibody levels (OD_{492nm})					
	rCPA Bacterin			Commercial Toxoid		
	0	28	56	0	28	56
Animal 1	0.485	0.289	0.723	0.347	0.454	0.423
Animal 2	0.327	0.604	0.630	0.287	0.449	0.440
Animal 3	0.291	0.218	0.333	0.339	0.325	0.459
Animal 4	0.254	0.361	0.489	0.258	0.275	0.507
Animal 5	0.262	0.601	0.452	0.291	0.218	0.333
Animal 6	0.311	0.488	0.401	0.254	0.361	0.489
Animal 7	0.296	0.371	0.338	0.531	0.339	0.346
Mean ± SD	**0.318 ± 0.07**	**0.419 ± 0.15**	**0.486 ± 0.148**	**0.329 ± 0.09**	**0.346 ± 0.08**	**0.428 ± 0.067**

4. Discussion

In Brazil, the actual course of clostridia in sheep is difficult to determine due to clandestine slaughtering and the breeding system, which is still mostly carried out in a secondary way and with little technicalities [22,23]. Among the major clostridiosis, gas gangrene has been found to occur in isolated and usually fatal form. The protocol followed for vaccination against clostridiosis in ruminants involves a primary vaccination at 3–4 months of age, followed by two doses at a 28-day interval, warranted by annual revaccination. The absence of booster doses after the first dose is common in Brazil which drastically affects its protective efficacy against clostridiosis [24]. In addition, native CPA toxoids from *C. perfringens* type A are not monitored by MAPA, which may produce a negative impact on the induction of protective immune responses in animals, since the quality of the antigens used is unknown.

The present study is the first attempt to demonstrate the immunogenicity of an *E. coli* bacterin containing rCPA inclusion bodies, which proved to be innocuous, since the animals tested showed no sign of toxicity or behavior change, and efficient, being able to induce antitoxin-CPA titre of 13.82 $IU.mL^{-1}$in rabbit serum, which is three times higher than that recommended by USDA (4 $IU.mL^{-1}$).

The use of purified rCPA has been proposed as a promising alternative to conventional toxoids in the prophylaxis of gas gangrene, necrohemorrhagic enteritis in cattle, and necrotic enteritis in birds and newborn pigs [14–19,25]. Recently, our group showed that a purified, and not treated with formaldehyde, rCPA vaccine induced antitoxin titer of 9.6 $IU.mL^{-1}$ in rabbits, which is 4 $IU.mL^{-1}$ less than the antitoxin titer induced by the recombinant bacterin of the present study. In pigs immunized with purified rCPA, in the final third of gestation, the vaccine induced average antitoxin titres of 6 and 4 $IU.mL^{-1}$ in serum and colostrum, respectively. This vaccine was also able to induce antitoxin titres of 5.19, 4.34, and 4.7 $IU.mL^{-1}$ in cattle, sheep, and goats, respectively [19].

The rCPA used in the present study was aggregated in the form of inclusion bodies, which did not influence the immunogenicity of the antigen. This could be concluded from the fact that the titers obtained in the present study were higher (13.84 $IU.mL^{-1}$) than those obtained in rabbits immunized with the same dose of 200 μg purified soluble rCPA (9.6 $IU.mL^{-1}$). Although the titres are higher than those recommended in sheep, the titre is three times lower (4 $IU.mL^{-1}$) than what is obtained in rabbits. It is noteworthy, that the animals did not show any *anti*-rCPA neutralizing antibody titers in the sera prior to the start of the experiment. The higher titers in the model species, compared to the target species, have already been demonstrated by Lobato et al. [20] in which the vaccination of rabbits, goats, sheep and cattle with 200 μg inclusion bodies of recombinant epsilon toxin (rETX) induced 40, 14.3, 26 and 13.1 IU mL^{-1} of neutralizing antitoxins. Also, Moreira et al. [19] induced higher titers in rabbits compared to sheep, cattle, and goats by using 200 μg of purified rCPA, rCPB, and rETX. These results point to the risk that the antitoxin titers generated in the model species are overestimated and do not represent the response induced in the target species.

The use of unpurified recombinant vaccines against clostridiosis has been proposed in the previous studies [20,21,26]. Langroudi et al. [26] used inactivated cell lysate (toxoid) containing a recombinant chimera composed of beta and epsilon toxins and, respectively, obtained antitoxin titers of 10 and 6 $IU.mL^{-1}$ in rabbits. The values were higher as compared to those recommended by the USDA and antitoxin was capable of protecting 90% of mice post challenge. Although unpurified vaccines (inclusion bodies and lysis supernatant) have shown efficiency in inducing the protective immune response in animals and have the advantages of not requiring purification and refolding steps, cell lysis is still required. In the present study, recombinant *E. coli* cells were inactivated after 4 h of expression and utilized in the preparation of the vaccines. The formulation was further used in the immunization of the animals, where it was found to induce protective immunity in both rabbits and sheep. Thus, the production process of the recombinant bacterin was greatly simplified, eliminating not only the steps of purification but also cell lysis.

The present study is a step towards advancement in the method of producing vaccines against *Clostridium perfringens* CPA. The methodology proposed here is simple, safe and effective, eliminating the purification steps. Inactivated *Escherichia coli* cells expressing CPA were found to be a potent candidate for the induction of neutralizing antibodies in both rabbits and sheep.

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Conflicts of interest

No conflict of interests of any kind is declared by the authors.

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