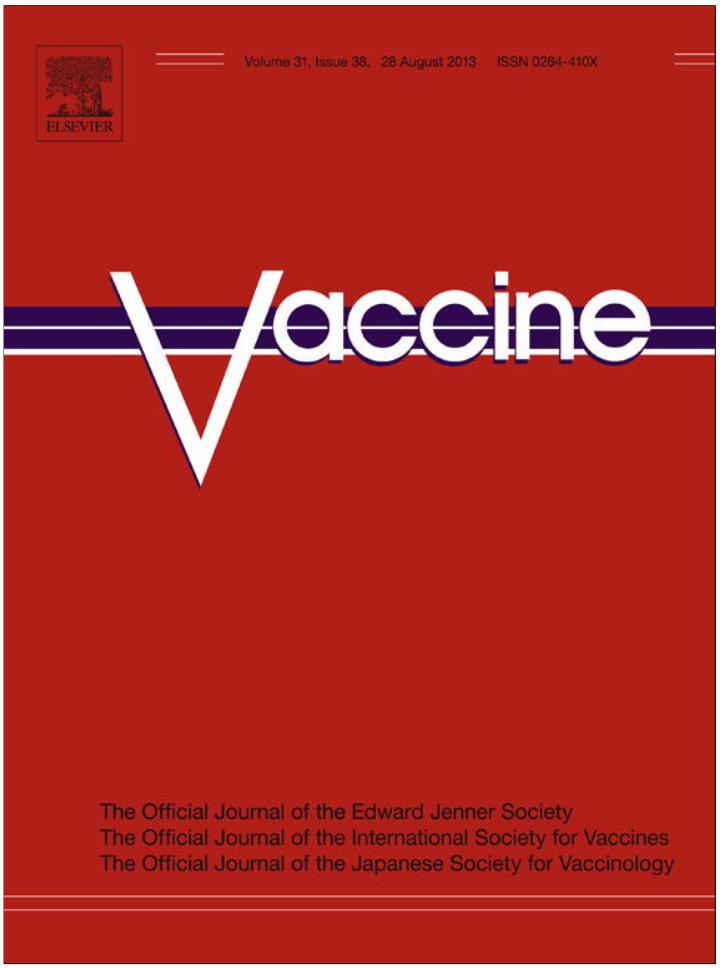

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Vaccination with recombinant *Clostridium perfringens* toxoids α and β promotes elevated antepartum and passive humoral immunity in swine

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ABSTRACT

Due to the increasingly restricted use of antimicrobials in animal production systems, the prevention and control of *Clostridium perfringens* type A- and C-induced diarrhea in piglets should be based on passive immunization via the prepartum vaccination of sows. Given the current obstacles in the production of conventional clostridial vaccines, the use of recombinant proteins has been considered to represent a promising alternative. In the present study, the neutralizing antibody response of immunized sows and their litters to a bivalent vaccine containing the *C. perfringens* recombinant toxoids alpha (rTA) and beta (rTB) produced in *Escherichia coli* was assessed. Rabbits ($n = 8$) and pregnant sows ($n = 7$) were immunized with 200 μg of each recombinant antigen using $Al(OH)_3$ as adjuvant. The alpha and beta antitoxin titer detected in the rabbits' serum pool was 9.6 and 20.4 IU/mL, respectively. The mean alpha and beta antitoxin titers in the sows' sera were 6.0 ± 0.9 IU/mL and 14.5 ± 2.2 IU/mL, and the corresponding individual coefficients of variation (CV) were 16.04% and 14.91%, respectively. The mean alpha and beta antitoxin titers in the litters' serum pools were 4.2 ± 0.4 IU/mL and 10.9 ± 1.7 IU/mL, and the CV between litters was 9.23% and 9.85%, respectively. The results showed that the rTA and rTB proteins produced and tested in the present study induced an immune response and can be regarded as candidates for the development of a commercial vaccine against *C. perfringens* type A- and C-induced diarrhea in pigs.

1. Introduction

Clostridium perfringens is a Gram-positive, anaerobic, spore-forming bacillus that is ubiquitous in nature as a commensal organism of the gastrointestinal tract of healthy humans and animals [1]. This bacterium is classified into five toxigenic types (A–E). Type C is the main diarrhea-causing enteropathogen in piglets up to seven days of age, and type A can also cause enteric disease in early life swine. Type A produces only the alpha toxin, while type C produces alpha and beta toxins, and both can induce diarrhea through the action of these toxins [2,3].

C. perfringens colonization of piglets occurs immediately after birth, and spores found in the environment and sow feces are the primary source of contamination. Considering the strong trend toward reducing or completely banning the use of antimicrobials in livestock, the prevention and control of *C. perfringens* infections in pigs has been increasingly based on the passive immunization of piglets [4]. However, the lack of specific immunobiologics complicates the control of infections by these agents and increases the rates of morbidity, mortality and culled animals, which results in losses to swine producers.

The development and production of conventional clostridial vaccines involves expensive, time-consuming and dangerous processes due to the necessary detoxification, purification and antigen concentration steps [5,6]. Furthermore, the continued selection of toxigenic strains that produce high titers of toxin is necessary [7]. Alternatively, the use of recombinant vaccines against clostridial infections has yielded promising results in other animal species [8–11] and is considered a more stable, high-yielding process with superior biosafety; thus, recombinant proteins may be an alternative for the prevention of diarrhea in newborn piglets. Therefore, the aim of the present study was to assess the neutralizing antibody

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response of immunized sows and their litters after the administration of a bivalent vaccine containing *C. perfringens* alpha (rTA) and beta (rTB) recombinant toxoids.

2. Materials and methods

2.1. Native toxin

Beta toxin from *C. perfringens* type C (ATCC 3638), which was used as a standard in test level L+, and phospholipase C (alpha toxin) from *C. perfringens* (Sigma Aldrich) were generously donated by the National Agricultural Laboratory of Minas Gerais (LANAGRO/MG) for the sera neutralization tests.

2.2. Vaccine formulation and safety

The rTA and rTB were produced in *Escherichia coli* according to the method proposed by Milach et al. [11]. Briefly, the recombinant plasmids pAE-*ta* and pAE-*tb* were transformed into *E. coli* BL21 (DE3) strain pLysS by heat-shock. The transformed bacteria were grown in 50 mL LB containing ampicillin (100 μg/mL) and chloramphenicol (25 μg/mL) at 37 °C for 16 h. Then, this volume was transferred to 500 mL of the same medium and induced with IPTG to a final concentration of 0.5 mM for 5 h under the same conditions when culture reached OD_{600} = 0.5–0.8. The fractions containing the expressed proteins were verified by anti-His Western blot, purified by Ni-affinity chromatography and quantified by BCA protein assay kit (Thermo Scientific). Both purified rTA and rTB were lyophilized until the use. The 50% lethal dose (LD_{50}) of the proteins was estimated in six-week old *Balb/c* mice, which were inoculated intravenously with four quantities of the toxin (0.1, 1, 10 and 100 μg).

Recombinant toxoids were emulsified in a 1:2 ratio with an aluminum hydroxide suspension [2.5–3.5% $Al(OH)_3$, pH 5.5–8] and were stored at room temperature for 16 h under constant agitation [9]. The toxoid sterility and innocuity were evaluated as stipulated by the Brazilian Ministry of Agriculture, Livestock and Food Supply according to ministerial directive No. 49 [12] and the European Pharmacopoeia [13], respectively.

2.3. Potency test

The potency tests for rTA and rTB were performed according to the Code of Federal Regulations (CFR9) of the United States Department of Agriculture [16]. Eight New Zealand rabbits weighing between 1.8 and 2.6 kg were inoculated subcutaneously with 200 μg of each recombinant antigen using $Al(OH)_3$ as adjuvant [9]. Twenty-one days after the first dose, the group received a second dose of the same vaccine formulation. Thirty-five days after the first administration, the animals were anesthetized with Zoletil® (20 mg/kg) for carotid artery cannulation and blood samples were collected. The animals were euthanized with an overdose of the anesthetic. The serum was separated by centrifugation and was pooled and stored at −20 °C until further use.

2.4. Passive immunity assessment

Seven pregnant sows were subcutaneously vaccinated twice with the vaccine (200 μg of each recombinant antigen with $Al(OH)_3$) at weeks five and two before parturition. Another seven pregnant sows were inoculated with a sterile saline solution (NaCl 0.9%, w/v) as a negative control group. Twenty-four hours after colostrum consumption, blood samples were collected from the sows and their piglets by venipuncture. The serum of each individual sow and the pooled sera of litters were collected and stored at −20 °C until further use.

2.5. Assisted colostrum intake

Piglets were submitted to assisted colostrum intake [14,15]. Briefly, it was assured that each animal was capable of reaching a teat and consuming colostrum just after birth. Once each piglet has stopped suckling, it was marked and separated from the others to increase colostrum intake of the remaining neonates, so that each animal would ingest similar amounts of colostrum. Weaker piglets incapable of reaching a teat received at least 10 mL of colostrum by a syringe directly into the mouth.

2.6. Serum neutralization assay

The pooled sera of litters, rabbit serum and the serum of each individual sow were titrated by serum neutralization in mice according to the method suggested by the United States Department of Agriculture (USDA) [17] for alpha antitoxin and the European Pharmacopoeia [13] for beta antitoxin. Briefly, sera dilutions and standard toxin mixes were incubated at 37 °C for 30 min, and 0.2 mL of each dilution was inoculated intravenously in 10 Swiss Webster mice weighing between 18 and 22 g. The animals were observed every 24 h for three days, and the number of dead and surviving animals was counted. The 50% neutralization (IC_{50}) titer was estimated according to the method proposed by Reed and Müench [17] and was expressed in international units per milliliter (IU/mL). Animal experiments were carried out according to the guidelines of the Ethics Committee in Animal Experimentation of the Federal University of Minas Gerais (CETEA UFMG 102/10).

3. Results and discussion

Microbial growth was not detected in the sterility tests of the bivalent recombinant vaccine during the 21 days of incubation. In the safety test, local or systemic reactions were also not observed in sows subcutaneously inoculated with two times the test dose. Although native *C. perfringens* alpha and beta toxins show DL_{50} of 3.0 and 0.4 μg/kg, respectively, in mice inoculated intravenously [18], animals inoculated with 100 μg of rTA or rTB showed no clinical signs of toxicity or death in the current study, demonstrating the safety of the recombinants at the tested doses.

The alpha antitoxin titer detected in the rabbits' pooled sera was 9.6 IU/mL, which is more than twice the minimum limit (4 IU/mL) established by the USDA [16]. A titer of 20.4 IU/mL was obtained for beta antitoxin, which was more than twice the minimum limit (10 IU/mL) determined by Brazilian legislation based on the European Pharmacopoeia [13]. The rTB-induced antibody titer was significantly higher than that induced by conventional, commercial, polyvalent vaccines containing a beta toxoid native to Brazil [19].

England's National Institute for Biological Standards and Control (NIBSC), a reference institution for the production and distribution of immunogens, recommends using 5.6 mg of native beta toxoid. In the present study, by inoculating only 200 μg of rTB, which is 28 times less than the indicated by NIBSC, it was possible to induce high neutralizing antibody titers in sows. This finding allows the development of a high throughput production and a reduction in the amount of required antigen for vaccination. The use of 200 μg of rTA and rTB was based on the study of Lobato et al. [9], where the same dose was used to immunize ruminants with recombinant epsilon toxoid, resulting in an satisfactory immune response. In our previous study [11], we tested 100 μg of rTB in rabbits, which resulted in 14 IU/mL of neutralizing antibodies, which is more than the minimum established by Brazilian legislation (10 IU/mL). Since

Table 1
Alpha and beta antitoxin levels in rTA- and rTB-immunized sows' sera.

Animals	Alpha antitoxin (IU/mL)	Beta antitoxin (IU/mL)
Sow 1	6.9	17.3
Sow 2	4.8	12
Sow 3	4.8	12
Sow 4	5.7	14.4
Sow 5	6.9	17.3
Sow 6	5.7	14.4
Sow 7	6.9	14.4
Mean titer ± SD	**6.0 ± 0.9**	**14.5 ± 2.2**
Sows 8–14 (negative control)	ND	ND

ND, not detected.

Table 2
Alpha and beta antitoxin levels in the serum pool of rTA- and rTB-immunized sows' litters.

Animals	Alpha antitoxin (IU/mL)	Beta antitoxin (IU/mL)
Litter 1	4	12
Litter 2	4	10
Litter 3	4	10
Litter 4	4	12
Litter 5	4.8	12
Litter 6	4	10
Litter 7	4.8	10
Mean titer ± SD	**4.2 ± 0.4**	**10.9 ± 1.7**
Litters 8–14 (negative control)	ND	ND

ND, not detected.

our previous results were slightly better than the presented here, further studies on the dose-response relationship should be performed to determine the best vaccine formulation.

C. perfringens alpha and beta antitoxin neutralizing titers in immunized sows' sera and their litters' pooled sera are outlined in Tables 1 and 2, respectively. Prior to immunization, none of the sows used in the experiment had detectable alpha and beta antitoxin serum titers, according to the results of serum neutralization tests performed in mice (data not shown). The mean alpha and beta antitoxin titers in the sows' serum were 6.0 ± 0.9 IU/mL and 14.5 ± 2.2 IU/mL, and the individual coefficient of variation (CV) was 16.04% and 14.91%, respectively. The mean beta antitoxin value was similar to that reported by Kelneric et al. [20], who used a vaccine consisting of a mixture of bacterin and *C. perfringens* type C and D toxoids but did not specify the protein concentration of antigens. However, the aforementioned researchers noted greater individual variation among vaccinated sows, with titers ranging from 9 to 26 IU/mL. In the current study, the titers of neutralizing antibodies detected in immunized sows' sera were more homogeneous than those obtained in previous reports [20,21], suggesting the potential application of recombinant toxoids in bivalent vaccines for pregnant sows.

The mean alpha and beta antitoxin titers in the litters' serum pools was 4.2 ± 0.4 IU/mL and 10.9 ± 1.7 IU/mL, and the corresponding CV between litters was 9.23% and 9.85%, respectively. The mean value of the beta antitoxin was higher than that noted by Kelneric et al. [20] of 8.7 IU/mL and ranged from 2.25 to 15 IU/mL. However, the mean beta antitoxin titer presented in the current study was lower than that (23 IU/mL) obtained by Mastisheck and McGinley [22] and ranged from 0.5 to 75.5 IU/mL. The observed differences may be attributed to the composition of the immunogen. Namely, in the study performed by Mastisheck and McGinley [22], a monovalent native beta toxoid was used; however, the antigen concentration was not reported. The wide range of beta antitoxin titers among litters observed in the studies by Mastisheck and McGinley [22] and Kelneric et al. [20] may be due to issues with animal colostrum intake, which is a key step for the passive immunization of newborn piglets. Assisted colostrum intake [14,15] was performed in the present study, ensuring that all of the animals had access to colostrum immediately after birth, which is reflected in the homogeneous alpha and beta antitoxin titers and the low coefficient of variation among different litters.

An effective vaccine should be capable of eliciting an immune response against the main virulence factors of a pathogen. In the case of bacteria of the genus *Clostridium*, this response should be primarily based on specific neutralizing antibodies against clostridial toxins [10]. Animal immunization with vaccines of chimeric recombinant antigens or a mixture of recombinant proteins, as used in the present study, induce higher titers of alpha and beta antitoxins compared to monovalent or bivalent conventional vaccines [20,22], as well as to bivalent recombinant chimeric vaccines [10]. Despite methodological differences between studies on recombinant clostridial toxoids, the results unanimously suggest that recombinant proteins are effective in inducing neutralizing antibody levels. The present study was the first to evaluate the immune response against rTA and rTB proteins in rabbits, sows and piglets. Furthermore, a limited number of articles are available in the literature concerning this topic, especially regarding the detection of neutralizing antibodies in piglets against *C. perfringens* alpha and beta toxins [10,20–22].

The data described above also suggests that the structures of the neutralizing epitopes of toxins were maintained in rTA and rTB because the antibodies generated after immunization with these antigens were capable of recognizing native alpha and beta toxins used in serum neutralization tests performed on mice. Additionally, the recombinant toxins showed no toxicity at the concentration used for animal vaccination. The administration of two doses of vaccine rTA and rTB toxins induced the production of high titers of neutralizing antibodies in sows' serum, which were passively transferred to their litters via colostrum intake. This evidence suggests that the proposed vaccine and immunization strategy stimulated a maternal immune response. Thus, recombinant antigens can be considered candidates for the development of a vaccine against *C. perfringens* types A and C in pigs.

The results of the current study indicate that *C. perfringens* rTA and rTB toxoids induce a satisfactory immune response in vaccinated animals and their offspring. Such immunogens are less laborious and faster to produce, thereby reducing costs and facilitating the manufacturing of vaccines, although necessitating an increase in production.

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Conflict of interest statement: The authors declare that they have no competing interests.

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