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Clostridium perfringens α and β recombinant toxoids in equine immunization[1]

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ABSTRACT.- Freitas N.F.Q.R., Barbosa J.D., Otaka D.Y., Ferreira M.R.A., Rodrigues R.R., Moreira Jr C., Conceição F.R. & Salvarani F.M. 2020. ***Clostridium perfringens* α and β recombinant toxoids in equine immunization**. *Pesquisa Veterinária Brasileira 40(10):776-780*. Instituto de Medicina Veterinária, Universidade Federal do Pará, BR-316 Km 61, Saudade II, Cristo Redentor, Castanhal, PA 68740-910, Brazil. E-mail: felipems@ufpa.br

Clostridium perfringens is considered one of the main causative agents of superacute enterocolitis, usually fatal in the equine species, due to the action of the β toxin, and is responsible for causing severe myonecrosis, by the action of the α toxin. The great importance of this agent in the equine economy is due to high mortality and lack of vaccines, which are the main form of prevention, which guarantee the immunization of this animal species. The aim of this study was to evaluate three different concentrations (100, 200 and 400μg) of *C. perfringens* α and β recombinant toxoids in equine immunization and to compare with a group vaccinated with a commercial toxoid. The commercial vaccine was not able to stimulate an immune response and the recombinant vaccine was able to induce satisfactory humoral immune response in vaccinated horses, proving to be an alternative prophylactic for *C. perfringens* infection.

INDEX TERMS: *Clostridium perfringens*, alpha toxin, beta toxin, recombinant toxoids, equine, immunization, vaccination, myonecrosis, enterocolitis, horses.

RESUMO.- [**Toxóides recombinantes α e β de *Clostridium perfringens* na imunização de equinos.**] *Clostridium perfringens* é considerado um dos principais agentes causadores de enterocolites superagudas, geralmente fatais na espécie equina, devido à ação da toxina β, além de ser responsável por causar quadros graves de mionecrose, pela ação da toxina α. A grande importância desses agentes na equinocultura, deve-se a elevada mortalidade e a inexistência de vacinas, principal forma de prevenção, que garantam a imunização dessa espécie animal. O objetivo deste trabalho foi avaliar três diferentes concentrações (100, 200 e 400μg) dos toxóides recombinantes α e β de *C. perfringens* na imunização de equinos, bem como comparar com um grupo vacinado com um toxóide comercial. A vacina comercial não se mostrou capaz de estimular uma resposta imune e a vacina recombinante foi capaz de induzir resposta imune humoral satisfatória em equinos vacinados, provando ser uma alternativa profilática para infecção por *C. Perfringens*.

TERMOS DE INDEXAÇÃO: *Clostridium perfringens,* toxina alfa, toxina beta,toxóides recombinantes, equinos, imunização, vacinação, mionecroses, entecolites.

INTRODUCTION

Clostridium perfringens is an anaerobic Gram-positive bacterium (Zaragoza et al. 2019), considered to be na important gastrointestinal pathogen in neonate foals of up to 10 days old (East et al. 1998). Types A and C are considered the most epidemiologically important for equine specie (Choi et al. 2003, Diab et al. 2012, Gohari et al. 2014, Olivo et al. 2016).

In equine species, *C. perfringens* type C is associated with enterocolitis and diarrhea, with or without hemorrhage, and acute progression that manifests as colic, abdominal distension, fever, dehydration, tachycardia, tachypnea and circulatory shock, for which the β toxin is responsible (Sayeed et al. 2008, Gohari et al. 2014). The death of animals results mainly from the high absorption of this toxin by the gut (Songer 1996, Songer & Uzal 2005, Uzal & Songer 2008). In a study conducted by Weese et al. (2001), *C. perfringens* type

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A was isolated in 19% of fecal samples from adult horses with diarrhea, demonstrating the association of this agent with enteric problems in adult animals also.

Myonecrosis in horses is mainly caused by *C. perfringens* type A through the action of the alpha toxin (Choi et al. 2003). The disease is characterized by rapid progression, with muscle liquefaction necrosis and gas formation, and may be associated with signs of toxemia. Most cases of myonecrosis are associated with the presence of solution of continuity of the skin, however *C. perfringens* is also able to penetrate healthy skeletal muscle (Peek et al. 2003).

The sporulation capacity of the genus *Clostridium*, and the fact that it may be a commensal microorganism of the intestinal microbiota of healthy animals, favors its presence in the soil for long periods, making eradication difficult (Freedman et al. 2016). The clinical conditions caused by the bacteria are superacute in character, and associated with, the absence of commercial vaccines that guarantee the immunization and prevention of these clostridioses in the equine species. The actions of these agents consequently lead to high mortality (Vengust et al. 2002), demonstrating the extreme importance of *C. perfringens* types A and C for the equine economy. Therefore, the objective of this study was to evaluate the humoral immune response of horses vaccinated with different concentrations of *C. perfringens* α and β recombinant toxoids.

MATERIALS AND METHODS

Ethics statement. The study was conducted in accordance with the "Conselho Nacional de Controle de Experimentação Animal" (CONCEA). It was submitted to the Animal Use Ethics Committee of the "Unviersidade Federal do Pará" (CEUA-UFPA) and approved under license number 7310201016.

Commercial vaccine. We used the commercial VISION 10® vaccine which contained the α and β *Clostridium perfringens* toxoids. The manufacturer did not specify the concentrations of either antigen. This commercial vaccine is not specific to horses, as there are no species-specific vaccines available. However, this does not make their use unfeasible, since the antigens are the same as those contained in the recombinant vaccine. The manufacturer's recommendations for use in bovine species were followed.

Recombinant vaccine. The production of recombinant toxins α and β was performed according to the methodology previously described by Milach et al. (2012) and Salvarani et al. (2013).

Recombinant toxoids at concentrations of 100, 200 and 400μg were dissolved into aluminum hydroxide suspension (2.5-3.5% $Al(OH)_3$), from Omega Produtos Ltda, in a final volume of 2mL per dose, and stirred at room temperature for 24h.

In order to verify sterility, 0.5mL of each of the vaccine formulations were sown in four tubes containing 20mL of thioglycolate broth, and four tubes containing 20mL of Sabouraud broth. Two thioglycolate broth tubes were incubated under anaerobic conditions, and the other Sabouraud and thioglycolate broth tubes were incubated under aerobic conditions. All tubes were incubated at 37°C for 21 days with daily readings (Brasil 1997).

For the safety test (European Pharmacopoeia 1998) four horses were inoculated, two with the commercial vaccine and two with the recombinant vaccine, containing twice the highest concentration to be tested (800μg), by subcutaneous and intramuscular routes. The animals were observed for seven days for the occurrence of local or systemic reactions.

Immunization of animals and titration of neutralizing antibodies. Fifty male and female Mangalarga Marchador animals, aged between one and 12 years, with no history of vaccination against *C. perfringens* were used in this study. The animals were randomly divided into five groups: 100μg Recombinant Vaccine Group (G1), 200μg Recombinant Vaccine Group (G2), 400μg Recombinant Vaccine Group (G3), Commercial Vaccine Group (G4) and Negative Control Group (G5), which received the administration of sterile saline (NaCl 0.9%). All animals received two doses (2mL), intramuscularly, on the neck board, on days 0 and 28 after the first dose.

On day 56 after the first vaccine dose, moment in which the vaccine efficiency is evaluated, through the potency test in the target species according to the literature (Moreira et al. 2016, 2018, Otaka et al. 2017), blood samples were collected by jugular venipuncture. After clot formation, centrifugation was performed at 3000g for 5 minutes to obtain serum, which was stored at -20°C until further use.

Serum titration was performed using the mouse neutralization technique; for β toxin the methodology described by the European Pharmacopoeia (1998), and for alpha toxin the methodology according to the USDA (2002). Serum dilutions were mixed with standard α and β toxins at 37°C for 30 minutes and 0.2mL of each dilution was intravenously inoculated into two Swiss Webster strain mice weighing between 18 and 22g. The animals were observed for 72 hours for death or survival, with results recorded every 24 hours. The neutralization titer of each dilution was calculated by the method of Reed and Muench (1938) and expressed in international units per milliliter (IU/mL).

Statistical analysis. We performed analysis of variance and Tukey tests to identify statistically significant differences in antibody titers between groups, using Statview statistical software version 5.0.0.0, adopting a statistical significance interval set at 95% ($p<0.05$).

RESULTS

No microbiological growth was observed in the sterility tests of the recombinant vaccine. In the innocuity test, the animals that were inoculated subcutaneously with the commercial vaccine showed intense local reaction, characterized by pain on palpation and edema, which disappeared after seven days. The animals did not show local or systemic alterations after inoculation using intramuscular administration routes for both vaccines.

The horses inoculated with Saline Group G5 did not develop antibodies against α and β toxins. The neutralizing antibody titers after 56 days of inoculation with Recombinant Vaccine Groups G1, G2, G3, and Commercial Vaccine Group G4 were obtained by the seroneutralization technique and are shown in Table 1.

When evaluating mean anti-α and anti-β (Fig.1) neutralizing antibody titers, no significant differences ($p<0.05$) were found between groups inoculated with recombinant vaccines at a concentration of 100μg (G1) and commercial (G4). No significant differences were found between the animals vaccinated with 200μg (G2) and 400μg (G3) concentrations of the recombinant vaccine. However, there was a significant difference between groups G2 and G3 compared to G4.

As the concentration of recombinant proteins increased, there was a linear increase in the humoral immune response in horses for both anti-α and anti-β antibodies. Regression analysis was performed, showing significant results ($p<0.001$), and is shown in Figure 2.

Table 1. Anti-α and anti-β neutralizing antibody titers, means and standard deviations (SD) obtained at 56 days after the first vaccination of horses with the recombinant vaccines 100 (G1), 200(G2) and 400µg(G3) and commercial vaccine (G4)

Vaccines	G1		G2		G3		G4	
Samples	Anti-α	Anti-β	Anti-α	Anti-β	Anti-α	Anti-β	Anti-α	Anti-β
1	0	10	5.7	10	5.7	14.4	0	0
2	4	0	6.9	12	4	14.4	0	10
3	0	10	5.7	14.4	4.8	17.3	0	10
4	4	10	4.8	14.4	5.7	14.4	4	10
5	4	0	4	10	6.9	12	0	10
6	0	10	6.9	14.4	6.9	12	4	0
7	0	0	6.9	14.4	6.9	12	4	10
8	4	0	4	12	5.7	10	0	10
9	4	10	4.8	12	6.9	14.4	0	0
10	0	0	5.7	17.3	8	17.3	0	10
Seroconversion rate	50%	50%	100%	100%	100%	100%	30%	70%
Means ± SD	2±2.11	5±5.27	5.54±1.13	13.09±2.30	6.15±1.19	13.82±2.40	1.2±1.93	7±4.83

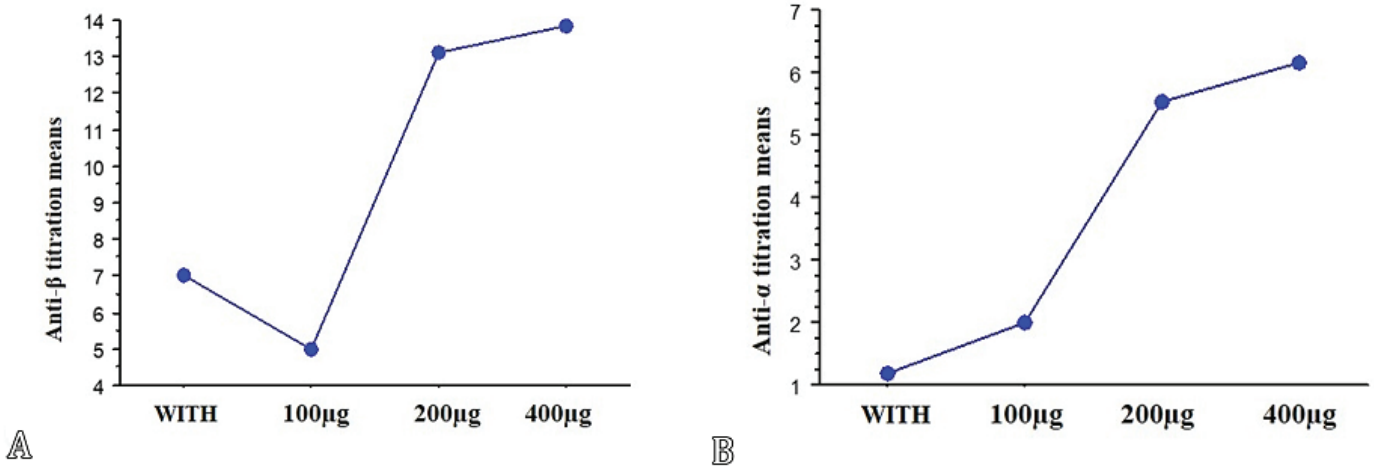

Fig.1. (**A**) Mean titers of anti-α neutralizing antibodies induced by the commercial vaccine (COM) and the different concentrations of the recombinant vaccine. (**B**) Mean titers of neutralizing anti-β antibodies induced by the commercial (COM) and the different concentrations of the recombinant vaccine.

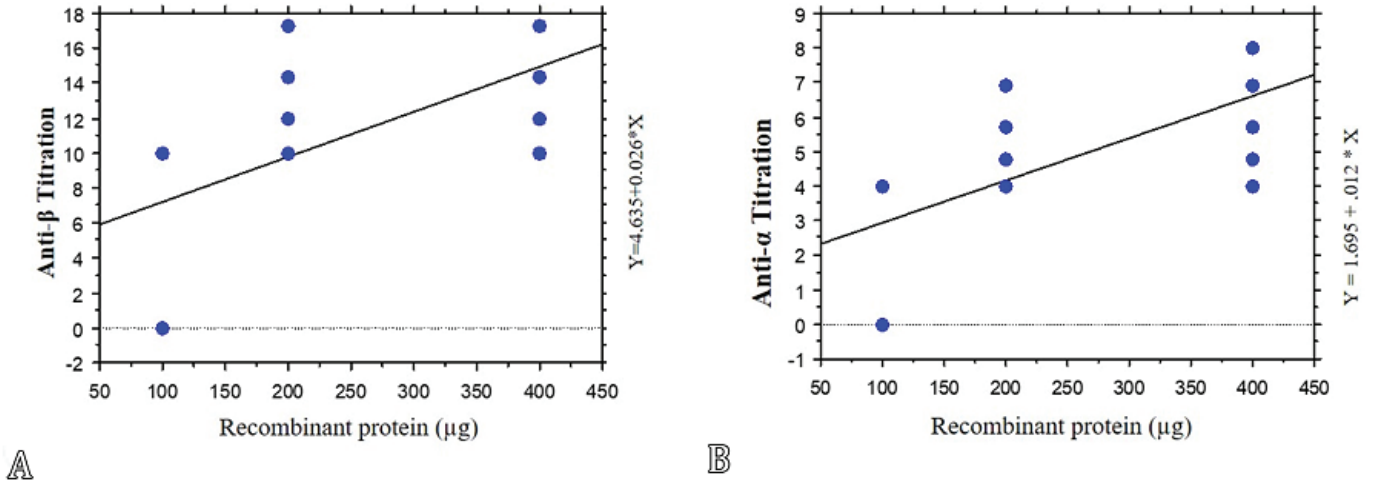

Fig.2. (**A**) Regression equation for anti-α titrations comparing different concentrations of recombinant proteins. (**B**) Regression equation for anti-β titrations comparing different concentrations of recombinant proteins.

DISCUSSION

Enterocolitis is an important cause of death in adult and neonate horses, as it usually presents as superacute changes in toxemia, which result in acute diarrhea, colic, dehydration, tachypnea, cardiovascular collapse and intestinal necrosis (Diab et al. 2012), due to the action of *Clostridium perfringens* β toxin, which is present in 40% of the intestinal content samples of horses with enterocolitis (Gohari et al. 2014). *C. perfringens* type A is also associated with severe myositis in the equine species, being the most prevalent agent in this type of infection (Peek et al. 2003), which is characterised by the presence of acute malignant edema, fever, depression, cutaneous emphysema, muscle liquefaction and toxemia, and other changes related to α toxin action (Horner 1982). The present study establishes a possible alternative to be used as prophylaxis against these superacute toxi-infections caused by the action of *C. perfringens* α and β toxins.

There is no vaccine against *C. perfringens* infections in the equine species currently available. The commercial vaccine available in Brazil, besides not being species-specific, was also not able to stimulate a humoral response with minimum antibody levels required for α and β toxins, in all inoculated animals of this study. Our work is the first to develop and test recombinant vaccines in the immunization of horses against *C. perfringens* toxins α and β.

The use of recombinant vaccines in animal immunization has been studied, and has shown excellent seroconversion results in swine (Salvarani et al. 2013), bovine, ovine, caprine (Moreira et al. 2016) and bubaline species (Otaka et al. 2017). This study tested different concentrations of α and β toxin recombinant vaccines at 100, 200, and 400μg, however neutralizing antibody levels above 4IU/mL are the USDA (2002) guidelines' minimum recommendation for toxin α, and 10IU/mL, for β toxin is required by Brazilian legislation, which is based on the European Pharmacopoeia (1998). These values were obtained only in groups immunised with formulations containing concentrations starting at 200μg, with no significant difference between the titers obtained from groups G2 and G3. None of the inoculated animals developed clinical signs of toxicity or death, demonstrating the safety of the studied recombinants.

There was a linear increase in the immune response between the amount of antigen per vaccine dose and the titers of neutralizing antibodies to α and β toxins. This correlation was similar to that found by Otaka et al. (2017) when testing concentrations of 100, 200 and 400μg of recombinant *Clostridium botulinum* neurotoxins C and D in buffalo, and also by Moreira et al. (2018) in bovine species.

As in our study, the mean titers obtained from swines (Salvarani et al. 2013) from cattles, goats and sheeps (Moreira et al. 2016) immunised with recombinant α and β vaccines, at a concentration of 200μg of each antigen, were capable of stimulating the immune response with levels above internationally required levels (USDA 2002, Otaka et al. 2017). Aside from verifying the results of studies conducted in these species, the structural characteristics of the α and β epitopes were maintained because antibodies generated in horses inoculated with the recombinant vaccine were able to recognise the standard *C. perfringens* toxins used in the seroneutralization tests.

The concentration of 200μg of recombinant toxoids was the lowest antigen concentration capable of inducing seroconversion in 100% of the inoculated horses. This concentration of α and β toxins was the same as used by Moreira et al. (2016) in ruminants and Salvarani et al. (2013) in swines, which was also effective in inducing neutralizing antibody levels higher than those required by international standards.

In neonatal foals there are an increased susceptibility of β toxin action in the first days of life due to the low level of intestinal trypsin present in colostrum (Lawrence 1997). Additional studies should be performed to check the level of circulating antibodies in newborns, which were acquired through passive immunity from mares previously immunised with the recombinant vaccine, as has been performed in pigs (Salvarani et al. 2013).

CONCLUSIONS

The present study demonstrated that the recombinant vaccine was able to stimulate satisfactory humoral immune responses in vaccinated horses, and may prove to be a prophylactic alternative in *Clostridium perfringens* type A and C infections in horses, which generally present as superacute diseases with a high mortality rate.

Future studies into the dynamic of the immune response in horses immunised with recombinant vaccines will further add to the currently scant data on this topic.

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Conflict of interest statement.- The authors have no competing interests.

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