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Comparison of humoral neutralizing antibody response in rabbits, guinea pigs, and cattle vaccinated with epsilon and beta toxoids from *Clostridium perfringens* and *C. botulinum* types C and D toxoids

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ABSTRACT

Rabbits and guinea pigs are used in the official control and validation of clostridial vaccines, but it is unknown whether the antitoxin titers obtained in these animals corroborate with the humoral response in bovine. The objective of the study was to compare the humoral antibody response of guinea pig and rabbits to those obtained in cattle vaccinated with a commercial vaccine containing *Clostridium perfringens* epsilon and beta, and *Clostridium botulinum* types C and D toxoids. This study revealed the same level of humoral response in rabbits and cattle for all four toxoids tested, including *C. botulinum* types C and D toxoids. In contrast, the titers of neutralizing antibodies against *C. botulinum* type C toxin in guinea pigs differed from those obtained in cattle. Thus, the present work suggests that the potency test for *C. botulinum* types C in rabbits agrees more with the humoral response in cattle than the potency test in guinea pigs, thereby making it possible to use only rabbits as models in the official control and validations of clostridial vaccines.

1. Introduction

Brazil has more than 200 million bovines, with the second largest cattle herd in the world, and it is the world's largest cattle meat exporter, selling to more than 180 countries [1]. Among several diseases that affect the cattle production, botulism, necrotic enteritis, and enterotoxaemia, caused by *Clostridium botulinum* and *C. perfringens* types C and D, respectively, stand out due to their high prevalence and high mortality [1–4]. In Brazil, these diseases are responsible for the deaths of approximately 500,000 cattle per year, resulting in an estimated loss of about US$350 million [5]. Botulism is also an emerging disease in cattle, laying hens, and broiler chicken in some European countries [6–8], but it is still under-diagnosed and under-reported [9].

While the total eradication of botulism, necrotic enteritis, and enterotoxemia is considered impossible, regular vaccination of the herd with toxoids remains the best approach to control these diseases [2,4,6]. Annually, more than 200 million doses of vaccines against clostridia are sold in Brazil. These vaccines are commonly produced by fermentation processes, which are known to yield variable amounts of toxoids between batches [10,11]. Thus, to ensure quality control, the Brazilian Ministry of Agriculture, Livestock and Food Supply (MAPA) regularly submits samples of commercial vaccines containing epsilon, beta, *C. botulinum*type C, and *C. botulinum* type D toxoids to potency tests [12]. In addition, several studies aiming to identify new vaccines against botulism, enterotoxemia and necrotic enteritis are published every year [10,11,13,14]. Because potency tests in cattle are expensive, these studies and other official tests commonly use laboratory animals, mostly rabbits and guinea pigs in the evaluation of the humoral response to these toxoids.

The protocol used for potency test of epsilon and beta toxoids is commonly based on the Code of Federal Regulations of United States Department of Agriculture (CFR) [15,16] or European Pharmacopoeia [16], which recommends the vaccination of rabbits followed by quantitative determination of the induced antibodies in the rabbit sera using the toxin neutralization test in mice. In contrast, for *C. botulinum* type C and D toxoids, the European

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Pharmacopeia and CFR recommend a vaccination-challenge protocol in mice and mink, respectively, which is impractical and may lead to bioethical issues [17,18]. Thus, several studies on the potency tests *C. botulinum* toxoids and even the official potency test in Brazil and South Africa are based on the vaccination of guinea pigs [11–14,19,20].

There is no study comparing the humoral antibody responses inrabbits and guinea pigs with the response of cattle vaccinated with these toxoids. Consequently, it is unknown whether the antitoxin titers obtained in these animals corroborate with the humoral antibody response in cattle. Thus, the objective of this study was to compare the humoral response of guinea pig and rabbits to those obtained in cattle vaccinated with commercial vaccines.

2. Material and methods

2.1. Vaccine used

In this study, the most-commonly sold clostridial vaccine (Poli-Star, Vallée, Brazil) in Brazil was provided by MAPA after passing the recommended tests for *C. perfringens* epsilon, beta, and *C. botulinum* types C and D toxoids. The vaccine has aluminum hydroxide as an adjuvant and, in addition to these four toxoids, it contains *C. chauvoei* bacterin and *C. septicum*, *C. novyi* and *C. sordellii* toxoids. The vaccine was kept under refrigeration (4 °C) as recommended by the manufacturer.

2.2. Cattle

This study was conducted according to the Brazilian National Council for Animal Experimentations (CONCEA) and was approved by the Federal University of Minas Gerais Ethics Committee on the Use of Animals (CEUA/UFMG) permit number 373/2014. Eight cattle (*Bos Taurus*), aged 4–6 months, received two doses of the selected vaccine on days 0 and 30, according to the manufacture's recommendation. Approximately 15 mL of blood samples were collected from the jugular vein at day 0 and15 days after the booster (day 45).

2.3. Rabbits

Sixteen New Zealand rabbits (*Oryctolagus cuniculus*), weighing between 1.8 and 3.6 kg, were used. The first group, which consisted of eight animals, was vaccinated as recommended by The Code of Federal Regulations (CFR) [15,16]. The vaccine was administrated two times, with a 21-day interval, and half of the dose (2.5 mL) recommended for cattle according to the manufacture's instructionwas used. In addition, the second group, which consisted of eight animals, was vaccinated two times with a 21-day interval between the vaccinations. This group received the same dose used for cattle (5 mL), similar to the protocol recommended by MAPA for the potency test of *C. botulinum* C and D toxoids in guinea pigs [12]. Blood samples were collected from all the rabbits 14 days after the booster vaccination.

2.4. Guinea pigs

Two groups of 12 guinea pigs, weighing between 350 and 450 g, were used. The first group received the same dose used for cattle (5 mL), as recommended by MAPA, while the second group received half of the dose(2.5 mL) recommended for cattle similarly to the protocol recommended by CFR for the potency test of *C. perfringens* epsilon and beta toxoids in rabbits [15.16]. Both groups were vaccinated two times, with a 21-day interval in-between the doses. The blood samples were collected 21 days after the booster, as recommended by MAPA, and similar to the protocol applied in several studies [11–14,21].

2.5. Serum neutralizing antibodiestiter determination and statistical analysis

Blood samples were centrifuged at 1000 x g for 15 min at 4 °C and kept at −20 °C until used. Serum neutralization test in mice were used to determine antitoxin titers in the collected samples, as recommended by CFR. Serum dilutions were briefly mixed with standard toxins (Table 1) at 37 °C for 30 min, before 0.2 mL of each dilution was inoculated intravenously in two Swiss Webster mice weighing between 18 and 22 g. The animals were observed every 24 h to determine if they were alive or dead for a total period of 72 h. Retro-titration [Not sure what you mean by this] with standard antitoxins (Table 1)was performed to check the standardization of the toxins.

Although CFR, European Pharmacopeia, and MAPA recommend the determination of titers in pool of vaccinated animals, individual titers were estimated to allow for statistical analysis. The following titers were tested: 1IU/mL, 2 IU/mL, 5 IU/mL, and 10 IU/mL for *C. botulinum* types C and D [12] and *C. perfringens* epsilon toxin (CFR), and 10 IU/mL, 15 IU/mL, 20 IU/mL, and 25 IU/mL for beta toxin (CFR). Serum titers were analyzed by Kruskal-Wallis test using SPSS statistical software (SPSS, USA) and a 95% confidence interval was used to determine the agreement between the titers obtained in guinea pigs, rabbits and cattle.

3. Results

The titers of the pooled sera from vaccinated rabbits and cattle were higher than those recommended by CFR and European Pharmacopeia for beta toxoid (10 IU/mL) and epsilon toxoid (2 and 5 IU/mL, respectively). Similarly, the pooled sera from guinea pigs, rabbits and cattle were also higher than the titers recommended by MAPA for *C. botulinum* types C and D toxoids (5 and 2 IU/mL, respectively) (Table 2). The results of statistical analysis tests of the individual titers revealed the same level of humoral antibody response in rabbits and cattle for all four toxoids tested. In contrast, the titers of neutralizing antibodies against *C. botulinum* type C toxin in guinea pigs vaccinated with half or the full dose were different ($p > 0.05$) from those detected in cattle.

4. Discussion

Although the magnitude of the titers in the pooled sera seems to be similar among the three animal species (Table 2), the results of statistical analysis of the individual titers revealed some discrepancies. The present study showed the same level of humoral antibody response in rabbits and cattle for all four toxoids tested, including *C. botulinum* types C and D toxoids, which are commonly tested in guinea pigs. First, these results suggest that the protocol of potency test for epsilon and beta toxoids in rabbits, as recommended by CFR and European Pharmacopeia, are adequate to predict the titers in cattle. Secondly, the protocol described for these two *C. perfringens* toxoids could also be useful in the evaluation of vaccines containing *C. botulinum* types C and D toxoids. This would allow the use of only one animal species in the official potency tests, and given that most clostridial vaccines are multivalent, the group of rabbits could be submitted to the same vaccination protocols for all these four toxoids, thereby reducing the number of animals used.

In contrast, the titers of neutralizing antibodies against *C. botulinum* type C toxin in guinea pigs vaccinated with half or full dose were different from those detected in cattle. These results