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Anti-botulism single-shot vaccine using chitosan for protein encapsulation by simple coacervation

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ABSTRACT

The aim of the present study was to compare the potency and safety of vaccines against *Clostridium botulinum (C. botulinum)* type C and D formulated with chitosan as controlled release matrix and vaccines formulated in conventional manner using aluminum hydroxide. Parameters were established for the development of chitosan microspheres, using simple coacervation to standardize the use of this polymer in protein encapsulation for vaccine formulation. To formulate a single shot vaccine inactivated antigens of *C. botulinum* type C and D were used with original toxin titles equal to 5.2 and 6.2 log LD50/ml, respectively. For each antigen a chitosan based solution of 50 mL was prepared. Control vaccines were formulated by mixing toxoid type C and D with aluminum hydroxide [25% $Al(OH)_3$, pH 6.3]. The toxoid sterility, innocuity and potency of vaccines were evaluated as stipulated by MAPA-BRASIL according to ministerial directive no. 23. Encapsulation efficiency of BSA in chitosan was 32.5–40.37%, while that the encapsulation efficiency to toxoid type C was 41,03% (1.94 mg/mL) and of the toxoid type D was 32.30% (1.82 mg/mL).

The single shot vaccine formulated using chitosan for protein encapsulation through simple coacervation showed potency and safety similar to conventional vaccine currently used in Brazilian livestock (10 and 2 IU/mL against *C. botulinum* type C and D, respectively). The present work suggests that our single shot vaccine would be a good option as a cattle vaccine against these *C. botulinum* type C and D.

1. Introduction

The *C. botulinum* is an anaerobic Gram-positive bacterium, sporeforming bacillus that can remain in the soil and organic matter for long periods in its resistant form, the spores, without causing disease. However, when they find a favorable environment for anaerobic conditions the spores germinate and produce neurotoxins (BoNT). After intestinal tract absorption, toxins bind to nerve endings receptors, resulting in flaccid paralysis and death of the animal due to respiratory arrest [1]. Neurotoxins of *C. botulinum* type C and D are the largest epidemiological importance, including Brazil [2–4], where it frequently presents fatality rates up to 100% [2,4,5].

The immunization of the entire flock with toxoids type C and D is the main of the disease control. The high production operational costs of these immunogens makes it difficult the deployment of other preventive methods [6]. The immunization is performed with two doses, usually with an interval of four to six weeks between doses, followed by an annual revaccination scheme. The most commonly used adjuvant is aluminum hydroxide [7].

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New approaches, such as the development of new adjuvants which allows the antigens gradual release and in a single shot for cattle immunization against botulism is an excellent alternative to currently available vaccines.

It may be able to minimize the stress effects on the animals as a result of excessive handling during vaccination periods and adverse reactions resulting from the action of adjuvant (aluminum hydroxide or mineral oil) in the animal organism, resulting in a higher productivity and carcass quality.

Chitosan is a hydrophilic, non-toxic, biocompatible and biodegradable amino-polysaccharide obtained through chitin deacetylation in alkaline solution. It is the second most abundant polysaccharide obtained from crustacean exoskeleton and from other marine animals [8]. Studies have focused on the use of chitosan to produce protein-loaded microspheres. Chitosan microspheres with a controlled release mechanism have been used to give antibiotics, antihypertensive, anticancer agents, proteins, peptides and vaccines [9]. In the biomedical field, chitosan is mainly used in biological tissue engineering and vaccinations [10]. It has been demonstrated that a trivalent vaccine against botulism showed high immunization potential by vaccine formulated with chitosan and administered orally [11]. Peptides from the carboxy-terminal portion of native toxins were employed as antigens with good binding capacity and epithelial barrier penetration in vitro. Mice treated with these peptides in adjuvants chitosan based produced high levels of IgA and IgG besides resisting after infection with virulent strains [11].

Chitosan's physical and chemical properties, such as inter- and intra-molecular hydrogen bonding and the cationic charge in acidic medium, makes this polymer more attractive for the development of conventional and novel pharmaceutical products.

Thus, the aim of the present study was to compare the potency and safety of vaccines against *C. botulinum* type C and D formulated with chitosan as controlled release matrix and vaccines formulated in conventional manner using aluminum hydroxide.

2. Material and methods

2.1. Preparation of chitosan-based solutions and precipitation with sodium sulfate

Chitosan powder (COGNIS) was used to prepare a background chitosan solution by diluting 0.125 g of this polymer into 10 mL of 0.5% (v/v) acetic acid (VETEC), adding to this solution 10 mL of 6.25% surfactant and purified water to a final volume of 50 mL. Background chitosan solutions containing the proteins tested (bovine serum albumin – BSA- and toxoid type C and D) for encapsulation the volume of purified water was replaced by the protein solution.

Sodium sulfate was the precipitation agent used to precipitate chitosan in a microparticle system. Nine flasks with 50 mL of chitosan background solution, without antigens, were added with 20% (m/v) sodium sulfate solution at a volume of 0.3, 0.4, 0.5, 0.6, 0.9, 1.2, 1.5, 1.8 and 2.1%. Precipitation of the solutions was evaluated in triplicates using a spectrophotometer with absorbance reading taken at a wavelength of 500 nm.

Chitosan was precipitated in a microsphere system by increasing the pH of the background solution with 20% (m/v) sodium sulfate solution at approximately 1.0 mL min^{-1}, until precipitate was visually perceived.

2.2. Test of surfactant interference

The precipitated background chitosan solution added to the antigen protein solution was used to test the efficiency of antigen encapsulation through simple coacervation, which was determined using the method described by Bradford [12]. However, the Bradford assay is affected by detergents found in the analyzed samples, such as sodium dodecyl sulfate (SDS) and octyl phenol ethoxylate. To avoid result distortions, this study evaluated the interference of the surfactants used (Polysorbate 80 and poloxamer, BASF) on total protein quantification through the Bradford assay.

After building a standard curve for different BSA levels measured by the adapted Bradford assay, BSA determination was repeated in the presence of the surfactants tested. The standard curve for BSA concentration as a function of absorbance reading was obtained through linear regression. To Polysorbate 80 and Poloxamer quantification, seven solutions of Polysorbate 80 and seven solutions of Poloxamer were prepared at 0.3, 0.5, 0.7, 0.9, 1.1, 1.25 and 1.5%. A volume of 20 μL of each solution was mixed with 1 mL of Bradford reagent.

The protein quantification in the presence of surfactants was realized by analysis of seven BSA samples prepared at 0.2, 0.5, 0.8, 1.1, 1.4, 1.7 and 2.0% added to 1.25% of Polysorbate 80 or Poloxamer. A volume of 20 μL of each solution was mixed with 1 mL of Bradford reagent. Solutions were homogenized, incubated for 20 min and its absorbance was read in a spectrophotometer at a wavelength of 595 nm (Pharmacia Inc).

2.3. Evaluation of protein encapsulation efficiency

Six different chitosan background solutions were added to crescent BSA concentrations (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/mL) and precipitation with 20% (m/v) sodium sulfate was used to the encapsulation test. To evaluate BSA encapsulation, the samples were suspended through centrifugation for 30 min at 8000 rpm at 4 °C [13]. Protein concentration was subsequently determined in the supernatant using the adapted Bradford technique and the standard BSA curve. The content of encapsulated microspheres was determined by subtracting the protein content determined in the supernatant from initial BSA concentration to obtain encapsulation efficiency.

After testing BSA encapsulation, 1.94 mg/mL solution of toxoid type C and a 1.82 mg/mL solution of toxoid type D were prepared. These solutions were subsequently added to chitosan background solutions for a final antigen concentration of 0.50 mg/mL for toxoid type C and 0.46 mg/mL of toxoid type D. Similarly, both solutions were precipitated with 20% (m/v) sodium sulfate solution and the efficiency of antigen encapsulation was calculated.

2.4. Vaccine production, formulation and safety

The amount of antigen used in each formulation was determined with the title of toxins through measure of Lethal Dose (LD50/ml) after the fermentation. It was considered toxin titles of *C. botulinum* type C of $10^{5.2}$LD50/mL and D of $10^{6.2}$ LD50/ml. After that, toxins of *C. botulinum* type C and D were inactivated with formaldehyde 37% for 7 days. In each antigen a chitosan based solution of 50 mL was used. Each antigen was added to the base solution slowly, stirred and maintained under those conditions for 1 h at room temperature. Subsequently, the precipitation of the base solutions containing antigens took place through the addition of 1.5 mL of sodium sulfate 20% (w/v) and gentle stirring for a short period of time.

After joining the two fractions containing *C. botulinum* type C and D, 25% chitosan gel was added, and the stirring maintained for more 30 min. At the end, the pH of the vaccine was adjusted to 6.3, with the support of sodium hydroxide solution 2 N. Control vaccine was formulated by mixing *C. botulinum* toxoid type C and D with aluminum hydroxide [25% $Al(OH)_3$, pH 6.3]. The sterility and innocuity were evaluated as stipulated by the Brazilian Ministry of

Agriculture, Livestock and Food Supply according to ministerial directive no. 23 (MAPA – BRASIL) [14].

2.5. Guinea pig vaccination and serum neutralization assay

The potency of the vaccines was evaluated as stipulated the MAPA - BRASIL according to ministerial directive no. 23 [14]. Immunization was performed subcutaneously in 12 guinea pigs with 60 days age and 350–450 g immunized with 5 mL doses for each formulation. Booster was performed after 21 days of primary immunization with antigen adsorbed in aluminum hydroxide adjuvant (control group).

The method was adapted for testing the controlled-release vaccine (single shot), because there is no booster dose in the vaccination procedure. The remaining steps of the method remained unchanged. Forty-two days after the first vaccination, blood samples were collected to obtain serum samples to perform a neutralization bioassay in mice. Samples collected were stocked at −20 °C until further use [14]. Toxin and Serum of *C. botulinum* type C and D, were provided by MAPA - BRASIL and standardized at 1 l+/mL and 5 UI/mL, respectively [13]. The exsanguination was performed through cardiac puncture with animals anaesthetized. After, the animals were euthanized with anesthetic overdose. All sera were titrated through serum neutralization bioassay in Swiss mice as described by MAPA - BRASIL according to ministerial directive no. 23 [13].

The neutralization bioassay consists of sera dilutions combined with standard toxins at 37 °C for 30 min with 0.2 mL of each dilution injected via intravenous in two Swiss Webster mice weighing between 18 and 22 g with 4 weeks. The animals were observed for death or survival for a period of three days. Serum neutralization with standard antitoxins of *C. botulinum* type C (5 IU/mL) and D (2 IU/mL) was performed to check the toxins standardization [4,15]. Vaccine formulation is considered approved if it achieves antibodies levels to toxoid type C (5 IU/mL) and D (2 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 (Permit No. 233/2010).

2.6. Statistical analysis

The analyses were carried out using SPSS 17.0 software. Statistical tests were selected according to the characteristics of the samples and the distribution of variables and probability values < 0.05 were considered statistically significant. Assuming a linear correlation between the values obtained in spectrophotometer analysis, the method of linear calibration regression was applied to predict the protein concentration. ANOVA and Tukey's test were implemented to identify significant differences in antibody titers among the groups.

3. Results

3.1. Surfactant interference and protein quantification in the presence of surfactant

Samples with Polysorbate 80 were strongly detected in absorbance readings, describing a strong linear correlation with optical density at 595 nm ($R^2 = 0.984$) (Fig. 1A). The increase in Polysorbate 80 concentration showed increase in absorbance readings. This result corroborates that described by Bradford [12], who asserts that the method could be jeopardized due to detergents in the samples. On the other hand, Poloxamer samples were weakly detected and non-correlated to sample concentration (Fig. 1B). The variation on Poloxamer did not affect the readings.

The presence of 1.25% Poloxamer demonstrates that BSA quantification was similar to the concentration of BSA control for the different concentrations tested. The quantification of the protein level did not show difference between samples with surfactant Poloxamer and samples with BSA (control) in each BSA concentration tested (Fig. 1C). However, the use of Polysorbate 80 clearly affected protein sample quantification. The protein concentration in samples with Polysorbate 80 was smaller than control samples (Fig. 1D).

The different influence of Polysorbate 80 and Poloxamer on the performance of the Bradford assay can be explained through the structural specificities of these compounds. The molecular structure of Polysorbate 80 has oxyethylene radicals disposed in a, b, c and d chains (Fig. 1E).

They are bonded to a sorbitol molecule and they have hydroxyl end groups, except for the d chain, which carries a monounsaturated fatty acid at the end of the chain. Poloxamer has oxyethylene radicals interposed on the oxypropylene radical b (Fig. 1F).

3.2. Determination of protein encapsulation efficiency

The chitosan was precipitated using a 0.8% sodium sulfate solution, which corresponds to 2.0 mL of 20% sodium sulfate solution in 50 mL of chitosan background solution. However, a high margin of safety was established since chitosan started precipitating under 0.6% sodium sulfate, which corresponds to 1.5 mL of the solution. In the present study, absorbance values from the nine background chitosan solutions precipitated with different volumes of 20% sodium sulfate solution are shown in Fig. 2. Solution stabilization started with the addition of 1.2 mL sodium sulfate and was practically achieved with 1.5 mL, which was defined as the standard volume to use to precipitate chitosan in 50 mL of background solution.

To determine the efficiency of BSA encapsulation it was used BSA protein through simple coacervation with chitosan solution from 0.5 to 3.0 mg/mL. The proportion of protein encapsulated or adsorbed to chitosan microspheres after coacervation ranged from 32.55% to 40.37% (Table 1).

The percentage of adsorbed protein was obtained by equation $A/I \times 100$ (Table 1). Similar values were obtained for encapsulation of the *C. botulinum* toxoid type C and D. The encapsulation efficiency was 41.03% for toxoid type C and 32.30% for toxoid type D. The percentage of adsorbed protein was obtained through equation $CSB - C/C \times 100$ (Table 2). The data were compared with standard BSA curve of equation $DO_{595} = 0.8867\ x$ (protein concentration in mg/mL) + 0.0094, with $R^2 = 0.9938$.

The encapsulation efficiency obtained in the present study for toxoid type D (32.30%), toxoid type C (41.03%) and BSA (32.55–40.37%) was obtained from adaptations in the method proposed by Lourenço [16]. However, compared to the studies described above, the technique used herein can be improved to the objectives proposed. Nevertheless, the results obtained corroborate with the results described in the literature, which support the efficiency of the method used.

Animals vaccinated with the single shot vaccine were able to develop titers of 10 and 2 IU/mL against *C. botulinum* type C and D respectively (Fig. 3). These titers are compatible with established by the MAPA - BRASIL according to ministerial directive no. 23 [14]. The group vaccinated with toxoid type C and D adsorbed with aluminum hydroxide adjuvant presented the same results (Fig. 3). ANOVA and Tukey's test did not indicate significant difference ($P < 0.001$). Although both strategies met the requirements established by Brazilian legislation [14], only levels of neutralizing antibodies against toxoid of *C. botulinum* type C exceeded the minimum, while levels of neutralizing antibodies against toxoid of

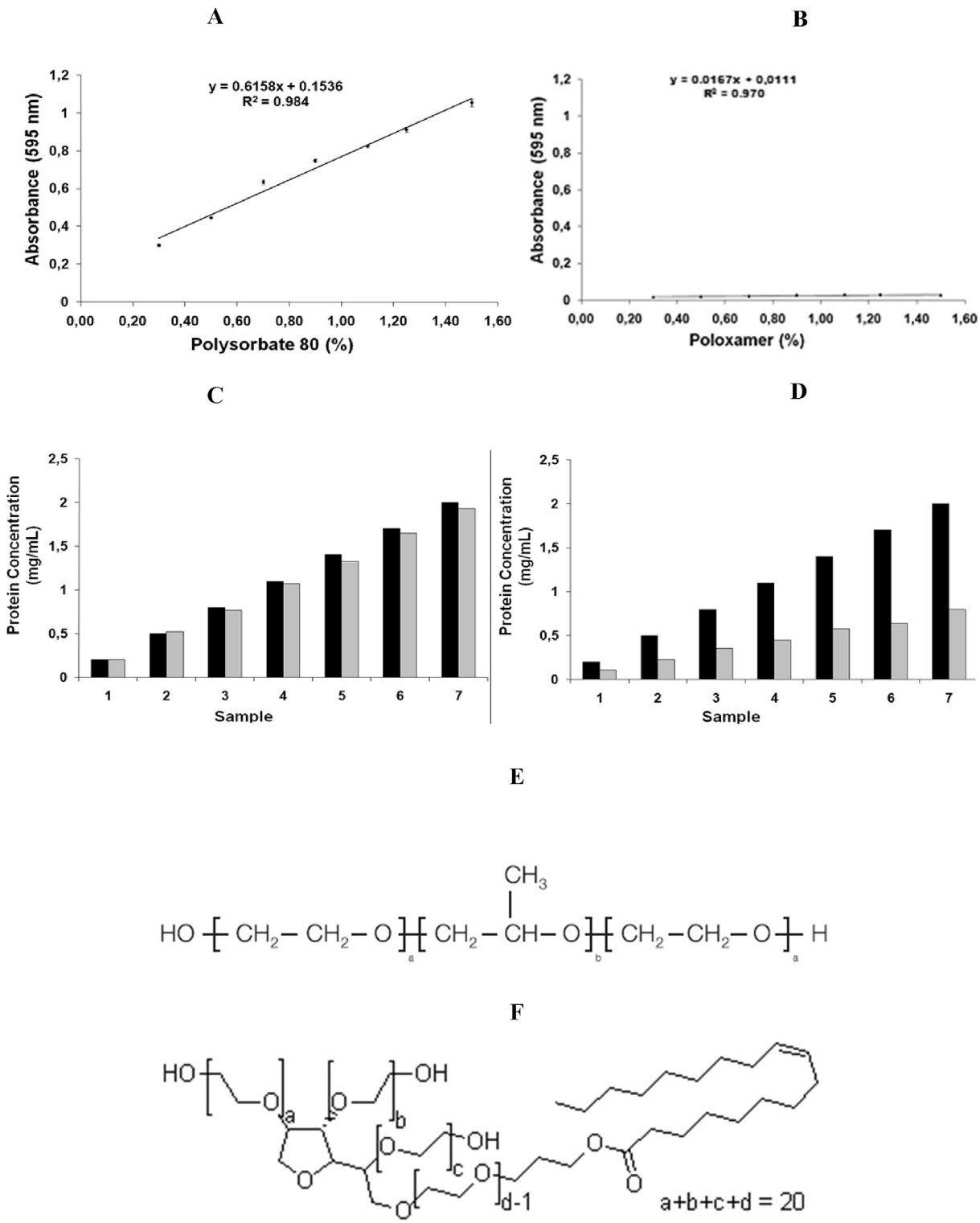

Fig. 1. Interference analysis of surfactant Polysorbate 80 and Poloxamer evaluated by spectrophotometer methods. A - Interference of Polysorbate 80 in absorbance readings. Different concentrations of Polysorbate 80 were tested at absorbance of 595 nm. B - Interference of Poloxamer 80 in absorbance readings. Different concentrations of Poloxamer were tested at absorbance of 595 nm. C - BSA concentration after adding Poloxamer measured by Bradford methods. (dark bars - BSA control); (gray bars - with 1.25% Poloxamer concentration). D - BSA concentration after adding Polysorbate 80 measured by Bradford methods. (dark bars - BSA control); (gray bars - with 1.25% Polysorbate 80 concentration). E - Molecular structure of Poloxamer. Available: http://www.pharma-ingredients.basf.com/product.aspx?PRD=30035120). F - Molecular structure of Polysorbate 80. The structure describes that sum of chains CH_2CH_2O identified in (a) (b) (c) and (d) is equivalent to 20. Available: http://www.chemblink.com/products/9005-65-6.htm).

C. botulinum type D presented the minimum accepted. The standardization of the toxin of *C. botulinum* type C and D was verified through serum neutralization using standard antisera. As expected, 5 and 2 IU/mL of neutralizing antitoxins were detected against toxin of *C. botulinum* type C and D, respectively, indicating that the material used and the assay are reliable.

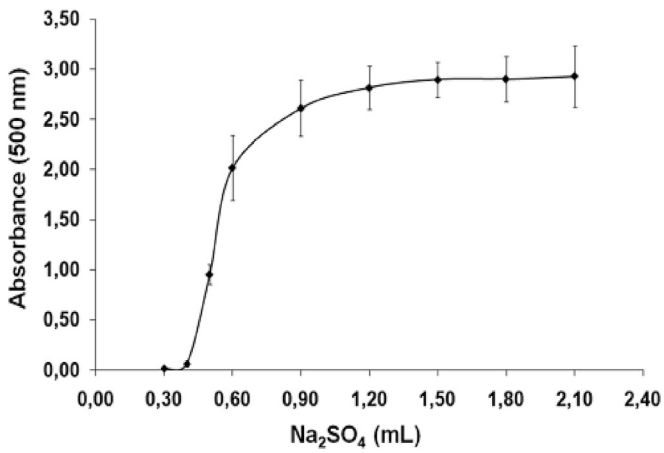

Fig. 2. Chitosan precipitation curve obtained by nine chitosan solutions precipitated gradually with 20% sodium sulfate. (1.2–1.5 mL) sodium sulfate was defined as the standard volume to use to precipitate chitosan in 50 mL of background solution. ($p > 0.05$).

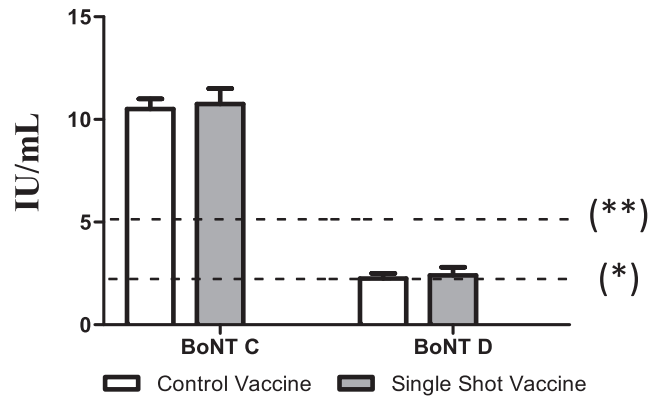

Fig. 3. Neutralizing antibodies titers against toxoid *C. botulinum* type C and D after immunization with a single shot vaccine and control vaccine with aluminum hydroxide adjuvant measured through serum neutralization methods. ANOVA and Tukey's test ($P < 0.001$). (*) - antibodies required for MAPA- BRASIL approval (*C. botulinum* type D); (**) - antibodies required for MAPA- BRASIL approval (*C. botulinum* type C).

Table 1
Evaluation of method efficiency through determination of BSA encapsulation (%) after chitosan precipitation.

#	Mean $_{(OD)}$	Dil.	ROD	C (mg/mL)	I (mg/mL)	A (mg/mL)	A (%)
1	0,533	1,5	0,7	0,327	0,5	0,173	34,66
2	0,870	3,0	2,3	0,674	1,0	0,326	32,55
3	1175	5,0	5,4	0,975	1,5	0,525	34,98
4	1512	6,0	8,6	1193	2,0	0,807	40,37
5	1846	8,0	14,1	1602	2,5	0,898	35,90
6	2171	10,0	1,7	1912	3,0	1088	36,27

- represent the sample number. Mean$_{(OD)}$ - optical density measured at 595 nm. Dil - sample dilution factor. ROD - real optical density. C - Protein concentration in the supernatant after the centrifugation of the precipitated samples. I (mg/mL) - initial protein concentration in the background solution. A (mg/mL) - adsorbed protein obtained by the difference of the initial concentration (I) and the protein concentration in the supernatant (C). A (%) - is percentage of adsorbed protein obtained from $A/I \times 100$.

Table 2
Efficiency of encapsulation of toxoid type C and D using the proposed method.

#	Mean $_{(OD)}$	C (mg/mL)	CSB (mg/mL)	Ads. (%)
SPN *C. bot.* C	0,330	0,30	0,50	41,03
SPN *C. bot.* D	0,343	0,31	0,46	32,30

- represent the supernatant of samples with botulinum toxoid type C and D encapsulated.
Mean $_{(OD)}$ - optical density median at 595 nm. C - Protein concentration in the sample. CSB - concentration in the background chitosan solution. Ads (%) – is percentage of adsorbed protein obtained from $CSB - C/C \times 100$.

4. Discussion

Ovalbumin encapsulation efficiency of simple coacervation also was evaluated [17] through the determination of the remaining protein in the supernatant after adsorption, according to Lowry's method for protein quantification. Encapsulation efficiency ranged from 32 to 85%. This result corroborates with data obtained in the present study. Other data also suggest that the encapsulation procedures used could be improved by modulating parameters such as protein and chitosan concentration [17]. However, for the tetanus toxoid encapsulation within chitosan microspheres cross-linking associated to emulsion techniques was used. The result achieved was 70–84% of efficiency, which was determined through the time flocculation test procedure [18]. However, high efficiency was obtained through encapsulation techniques and efficiency evaluation very different from those applied on the present study.

Botulism in cattle livestock has attracted close producers and researchers' attention due to its extremely high fatality rate [5]. Botulinum toxin is one of the most lethal substances known throughout the world as doses of 1 ng kg^{-1} can be fatal to man and certain animals [19]. Toxin of *C. botulinum* type D seems to have a very large toxigenic capacity in mice. However, when *C. botulinum* neurotoxin undergoes an inactivation with formalin process depending on the conditions under which the reaction occurs, its antigenicity can be greatly decreased, which may cause low immunogenicity in immunization procedures, as is it commonly observed in some botulinum toxoids commercially available [20]. In our work it was performed the evaluation of chitosan and aluminum hydroxide adjuvants of the absorbed antigen after the inactivation process. This is an industrial routine of the toxoid production of *C. botulinum* types C and D. However, a lower immunogenicity of an antigen is normally corrected by the presence of adjuvants or administration of booster doses as it is done for most vaccines, both human and animal [21].

Chitosan, a natural, non-toxic, biodegradable and biocompatible polymer is quite applicable in tissue engineering and controlled drug release [22]. Chitosan is capable of enabling humoral and cellular immune responses, and in some studies it is more efficient and safe compared to the incomplete Freund's adjuvant and aluminum hydroxide [23]. In our work, it was observed that animals vaccinated with the single shot vaccine were able to develop the same titers of 10 IU/mL (toxoid type C) and 2 IU/mL (toxoid type D) obtained with the antigen adsorbed with aluminum hydroxide adjuvant (Fig. 3).

Several studies have reported the safety of the polymer for use in vaccines. However, this study related viral vaccines. Chang et al. [24] showed that the chitosan used in viral vaccines induced humoral immune responses besides protecting mice against lethal challenge. Chitosan was comparable to the alum adjuvant in efficacy and might be a candidate adjuvant for parenteral administration of inactivated influenza vaccines [24]. Günbeyaz et al. [25] evaluated effect chitosan in bovine herpesvirus 1 (BHV-1) and infectious bovine rhinotracheitis (IBR) and considered that chitosan based formulations as promising adjuvant/delivery systems for mucosal immunization against BHV-1 of bovines.

There are few reports on the chitosan as a vaccine adjuvant for clostridiosis disease control. Ravichandran et al. [11] evaluated the

immunoglobulin responses with use chitosan adjuvant as mucosal vaccine. It is shown that levels of resistance to challenge were increased by coadministration of chitosan adjuvants [11]. Ravichandran et al. [11] report that efforts were made to generate a mucosal vaccine that provides protection against the *C. botulinum* type A, B, and E, however, most reports dealing with vaccines against *C. botulinum* neurotoxin have focused in different routes of administration, fact which makes it difficult the comparison of the research. Besides, it uses in most of the vaccines the aluminum hydroxide adjuvant. There are a few uses of chitosan as an adjuvant for these antigens.

In the sterility test it was not observed microbial growth and neither adverse nor unexpected reactions occurred in guinea pig subcutaneously vaccinated, thus indicating the innocuity of our vaccine formulation. The results did not surprise us, as aluminum hydroxide is widely used as an adjuvant in cattle vaccines and, as demonstrated elsewhere, the HC domains of *C. botulinum* toxins, alone or as a fusion protein, are non-toxic [7,26]. Absence of strong local reactions were expected for the vaccines formulated with chitosan polymers, which is a biodegradable polysaccharide and it is less aggressive to the host than the aluminum hydroxide or oils adjuvants.

Therefore the single shot vaccine formulated using chitosan for protein encapsulation through simple coacervation showed potency and safety similar to conventional vaccine currently used in Brazilian livestock. This work will help understanding the chitosan adjuvant efficacy of commercial vaccines to clostridiosis of the Brazilian and world veterinary market. Thus this work suggests that our single shot vaccine would be a good option as a vaccine for cattle against *C. botulinum* type C and D.

5. Conclusion

Single-shot vaccine formulated with chitosan microparticles were able to meet the minimum specifications required by MAPA-BRASIL. The chitosan adjuvant was considered efficient and innocuous. This indicates the technical feasibility of a single shot vaccine against the animal botulism, formulated using chitosan microspheres by the simple coacervation technique. Further work will be needed to optimize a new formulation with antigen adsorbed in chitosan to stimulate protection above the accepted limit.

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