ORIGINAL ARTICLE

NEUTRALIZATION OF THE LETHAL ACTIVITY FROM *Bothrops atrox* VENOM BY HYPERIMUNE LLAMA SERUM *(Lama glama)*

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ABSTRACT

Objectives: To evaluate the capacity of the hyperimmune llama serum (*Lama glama*) to neutralize the lethal activity of *Bothrops atrox* venom in laboratory mice. **Materials and methods:** Mean lethal dose (LD_{50}) was calculated from a *Bothrops atrox* venom sample pool from Peru. The antibody titers were measured by ELISA assay; and the immune serum neutralization potency was measured by calculating the mean effective dose (ED_{50}) during the immunization period. **Results:** The venom's LD_{50} was 3.96 μg/g; similar to what was found in other studies about *Bothrops atrox* carried out in Peru. The titers of antibodies against the venom increased rapidly in the llama, demonstrating a fast immune response; however, the neutralization capacity increased slowly and required several doses and immunization reinforcements, obtaining a ED_{50} of 3.30 μL/g mouse and a neutralization potency of 3.6 mg/mL after 15 immunizations. **Conclusions:** The hyperimmune llama serum is able to neutralize the lethality of the *Bothrops atrox* venom from Peru in laboratory mice.

Keywords: *Bothrops atrox*; *Lama glama*; Neutralization; Immune Sera; Lethal Dose 50; Mortality (Source: MeSH NLM).

INTRODUCTION

Ophidism is a syndrome caused by snake venom poisoning, usually species from *Viperidae* and *Elapidae* families [1,2]. In Peru, the *Bothrops atrox* snake is known as the species of greatest medical relevance, responsible for 88%-92% of the ophidism cases in the country [1,3], causing morbimortality and leaving sequelae, such as amputations or lacerations in the affected parts. Tissue damage is caused by several biological activities from venom's enzymes, such as the proteolytic (destruction of structural proteins), coagulating, vascular toxic, and nephrotoxic effects [4].

The clinical manifestations are characterized by pain, edema, ecchymosis, erythema, and necrosis. In severe cases, vesicles or blisters with serous and hemorrhagic contents may appear, as well as hematemesis and hemorrhagic shock. The only pharmacologically valid treatment for cases of ophidism is the application of passive artificial immunotherapy, by means of the transfer of antivenom IgG antibodies, generally heterologous, of equine origin [5].

The Instituto Nacional de Salud (INS) produced the polyvalent anti-bothropic serum, a purified solution of specific IgG immunoglobulins obtained from the plasma of hyperimmunized equines with a pool of *Bothrops* snake venom, specifically *Bothrops atrox, Bothrops pictus, Bothrops barnetti, Bothrops braziili* and *Bothrocophias hyoprora* [2,3,6].

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Some of the problems associated to the production of the venom antidote are the need to use snakes to obtain the venom, the implicit risk in obtaining the venom, the use of horses to obtain the hyperimmune serum, and the subsequent extraction of the total antibodies which contain the venom neutralizing antibodies and other antibodies from the horse [1,2]. The equines used often suffer from hepatomegaly, because of the venom immunization. There is some research aimed at improving the physiological and biochemical processes, including testing alternative adjuvants that improve the release and assimilation of the venom within the horse, in order to reduce the negative effects of the inoculations [5].

In addition, complete equine antibodies contain the constant fraction in the heavy chain, which are recognized by the receptors in various types of cells, such as NK lymphocytes, neutrophils, macrophages, B cells, and mast cells, and by complement factors. Complete equine antibodies can also cause other adverse reactions known as serum sickness, which is mainly characterized by urticaria, cough, nausea and vomiting, tachycardia, and headaches. Additionally, in many cases the patient may develop systemic anaphylaxis with symptoms, such as hypotension, bronchospasm, and angioedema [7].

The main objective of this study was to determine the effectiveness of the hyperimmune llama serum *(Lama glama)* as a neutralizer of the lethal activity of the Peruvian snake venom *Bothrops atrox*; and to provide knowledge regarding new alternatives to biological products, which will contribute to the INS production capacity of antiophidic serums.

KEY MESSAGES

Motivation for the study: In Peru there are few studies on the use of animals other than horses for the production of antidotes to venomous snake bites. It is necessary to design and evaluate new alternatives to address this problem.

Main findings: The hyperimmune serum of the llama neutralizes the venom of the Bothrops atrox snake in a similar way to the antidotes currently used for this type of snake.

Implications: If further studies are conducted and the efficacy of llama serum for antidote production is demonstrated, a new alternative for the production of anti-bothropic antidote could be available.

MATERIALS AND METHODS

This is an analytical experimental study.

Experimental animals

A male llama *(Lama glama)* was used to obtain the pre-immune and post-immune serum. The animal was obtained from an agricultural center (SAIS Pachacutec), it was healthy, and passed a period of veterinary evaluation and quarantine of 40 days prior to experimentation. BALB/c mice *(Mus musculus)* of 17 g to 19 g were used from the biotherium of the National Center of Biological Production of the INS, which were kept in a controlled environment in the laboratory. For mice handling, the researchers followed the Guide for Handling and Care of Laboratory Animals: Mice, from the INS [8].

Procedures

Obtaining and preserving the venom

Venom samples were obtained by manual extraction, collected in a beaker, and transported to the laboratory via cold chain. They were centrifuged at 5,000 rpm for 20 minutes to remove foreign components, and 2 mL aliquots were taken in glass vials. The vials were frozen at –80 °C overnight, and placed in a freeze-dryer until a homogeneous tablet was formed; the net weight of the freeze-dried venom was registered and stored at –80 °C until use.

Protein electrophoresis by SDS-PAGE

The venom was treated with a protein buffer under reducing conditions for 5 minutes at 100 °C and then underwent electrophoresis by the SDS-PAGE method at 12% for 1 hour at 100 volts. A protein molecular-weight marker was used (Thermo Scientific #26623). The gel was stained with Coomassie blue for 1 hour and then washed with a bleaching solution. For the serum, the conditions of electrophoresis, staining and gel processing were the same as for the venom.

Determination of the average lethal effect

The stock venom solution was prepared at a 1 mg/mL concentration using sterile saline solution as solvent. Mean lethal dose (LD50) values reported in previous studies [12-15] were taken as a reference; for *Bothrops atrox* venom, a range from 3 μg/g to 6 μg/g mouse has been reported. Solutions with higher and lower venom concentration were prepared, maintaining a constant dilution factor of 1.22 μg/g mouse. A volume of 0.5 mL of venom solution was inoculated intrape-

ritoneally into each mouse. The number of live/dead (L/D) mice in each group was registered at 24 and 48 hours. The number of dead mice in each box at 48 hours was considered for probit analysis.

Lama glama immunization with Bothrops atrox venom

Immunizations were performed in a stock or with an inoculation sleeve for older animals. Before venom inoculation, the injection area was shaved on the llama's back side. The injected substance had a 1:1 ratio of venom and the GERBU adjuvant [9,10], its total volume was 4 mL (2 mL of venom and 2 mL of the GERBU adjuvant). The venom was inoculated subcutaneously (distributed proportionally in four places on the back and alternating between both lateral parts of the back between each inoculation).

Llama immunization scheme with Bothrops atrox venom

The primary immunization scheme comprised 8 immunizations with total venom from *Bothrops atrox* plus the GERBU adjuvant in a 1:1 ratio (V/V). The amount of venom inoculated in each immunization dose, as well as the time intervals between doses, are indicated in the supplementary material. After the primary scheme, 7 booster doses were applied, as indicated in Table 1.

Obtaining serum from Lama glama

A blood sample (approx. 20 mL) was taken from the right jugular vein with a 20 mL syringe and an 18-gauge needle. The blood was collected in vacutainer tubes containing a serum separator gel and centrifuged at 3,000 rpm for 5 minutes. The serum was collected in 1.5 mL microcentrifuge tubes, which were then stored at –80°C. Pre-immune serum was obtained before the first immunization; post-immunization serums were collected the same day before each immunization (Table 1).

ELISA for the measurement of Lama glama antibodies against Bothrops atrox venom

The poison was diluted in carbonate buffer (Na_2CO_3 0.015 M, $NaHCO_3$ 0.035 M) to a final concentration of 0.5 µg/mL. Then, 100 µL/well of the diluted poison was applied, the plate covered and incubated overnight at 4 °C. The poison solution was removed by inversion. Then, 250 µL of the blocking buffer (AFB or skimmed milk) was added to each well and incubated for 1 hour at room temperature. Titrated sera were diluted from 1/200 to 1/800 in carbonate buffer; as well as the negative control (pre-immune serum) and the blank (antigen-free carbonate buffer). The blocking buffer was removed and washed 5 times, then 100 µL of the titrated serum was added to each well and incubated for 1 hour at room temperature, in duplicate. The serum was removed and washed 5 times; 100 µL of conjugate (anti-*Lama*-IGG (H+L)-Peroxidase) which was diluted to 1:10,000 in conjugate dilution buffer, was added to each well; it was then incubated for 1 hour at room temperature. Then, the conjugate was removed and washed 5 times; 100 µL of TMB (tetramethyl-benzidine) substrate was added to each well and incubated for 5-15 minutes at room temperature and in the dark. 50 µL of 0.5 M stopping solution (H_2SO_4) was added to each well. The plate was read at a wavelength of 450 nm.

Determination of the lethal effect neutralization

The recommendations from the manual of procedures of the Clodomiro Picado Institute were followed to determine the toxic activities of the venom and its neutralization [11]. Male mice of 17 g to 18 g were used, 5 groups of 6 mice per box and an additional box for controls.

The venom dose was determined by considering 4 times the LD_{50} value for each gram of mouse to be inoculated (µg venom/g mouse). The venom and antivenom (llama serum) were preincubated at 37 °C for 30 minutes with fixed doses of poison ($4DL_{50}$) and 5 venom dilutions. The serum volume of each inoculum was determined, assuming that 1 mL of serum should neutralize at least 2.5 mg of venom (anti-bothropic serum-INS). Venom solution (without serum) containing 4DL50 was prepared for control mice. A volume of 0.5 mL of the prepared solution was inoculated intraperitoneally into each mouse. The mice were observed during the following hours. The quantity of L/D animals was registered for each group at 24 and 48 hours after inoculation, the number of dead mice in each box was noted at 48 hours for statistical calculation.

Statistical analysis

Statistical analysis was performed using Stata version 11 and the probit function to determine the LD_{50}; lethality neutralization data was also analyzed to calculate the serum ED_{50}. The differences between the groups were determined with the Chi-square test, considering p values under 0.05 as statistically significant.

Ethical aspects

The Institutional Ethics Committee on Human Beings and the Ethics Committee for the Use of Experimental Animals of the INS approved this study.

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Table 1. Schedule of primary immunization and boosters of *Lama glama* with *Bothrops atrox* venom.

Day	Bothrops atrox venom	Inoculation volume (mL)	Adjuvant	Procedure [a]
0	-	-	-	Pre-immune serum collection
Primary immunization schedule				
7	0,5 mg	4	GERBU	Serum collection / immunization
14	1 mg	4	GERBU	Serum collection / immunization
21	2 mg	4	GERBU	Serum collection / immunization
28	3 mg	4	GERBU	Serum collection / immunization
35	4 mg	4	GERBU	Serum collection / immunization
42	4 mg	4	GERBU	Serum collection / immunization
51	4 mg	4	GERBU	Serum collection / immunization
65	4 mg	4	GERBU	Serum collection / immunization
Immunization booster scheme				
126	4 mg	4	GERBU	Serum collection / immunization
201	4 mg	4	GERBU	Serum collection / immunization
229	4 mg	4	GERBU	Serum collection / immunization
236	4 mg	4	GERBU	Serum collection / immunization
245	4 mg	4	GERBU	Serum collection / immunization
253	4 mg	4	GERBU	Serum collection / immunization
268	4 mg	4	GERBU	Serum collection / immunization

[a] Except for day 0, serum was obtained the same day before each immunization.

RESULTS

The LD_{50} of the Peruvian Bothrops atrox snake venom pool was of 3.96 µg venom/g mouse at 48 hours, with a lower limit of 3.57 µg/g and an upper limit of 4.38 µg/g, according to its 95% confidence interval. In addition, an R^2 of 0.99 was found, which indicates a high correlation between the applied dose and the lethal effect of the venom (Figure 1).

Bothrops atrox venom analysis by SDS-PAGE electrophoresis shows a characteristic protein profile that matches the protein profile reported for this species [6]. Four strong bands were observed (more proteins) and at least 6 weak bands; from those 4 with more proteins, 2 bands had a high molecular weight (45- 60 kDa) and 2 bands had a lower molecular weight (10-20 kDa) (Figure 2A). In the serum electrophoresis analysis, we found a band protein profile, which represented heavy and light chains of IgG antibodies (Figure 2B).

Standardization of the ELISA assay for detecting *Bothrops atrox* venom antibodies against the llama serum showed that the optimal amount of antigen to use is 50 ng, the optimal serum dilution is 1:800 and the ideal dilution of peroxidase conjugated antibody is 1:10,000. Bovine serum albumin (BSA) proved to be a better blocking agent than skimmed milk for the ELISA. The difference in absorbance between pre-immune control serum and immune serum was considered when choosing the optimal assay parameters. The specific antibody titer for *Bothrops atrox* venom increased rapidly in the serum from the second week after the beginning of the immunizations, reaching a maximum concentration in the fourth week and then remaining constant.

After primary immunization, the expected potency was not achieved, which was similar to the reference serum (equine serum) (2.5 mg/mL); therefore, after this first scheme, 7 immunization boosters of 4 mg of venom in a volume of 2 mL were carried out, until achieving a neutralizing potency equal or higher than 2.5 mg/mL. After the boosters, the ED_{50} was 3.30 µL/g mouse and the upper and lower limits were 3.86 µL/g mouse and 2.90 µL/g mouse, respectively. This ED_{50} is equivalent to a venom neutralization potency of 3.6 mg/mL; 1 mL of llama serum is capable of neutralizing 3.6 mg of *Bothrops atrox* venom. A comparison was made between antibody titers and potencies according to the days elapsed (Figure 3).

DISCUSSION

Although antivenoms produced in horses are the main treatment used in cases of ophidism, there are still some problems

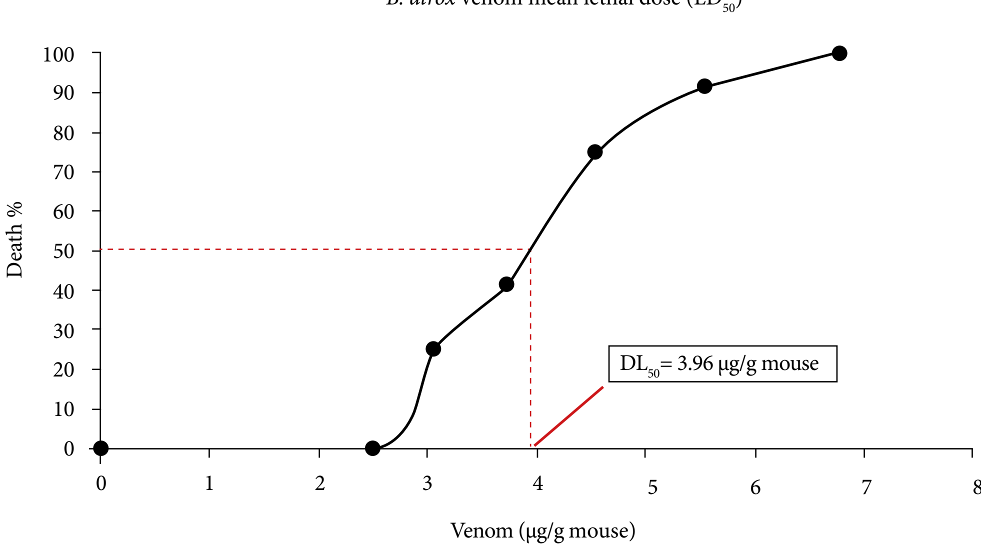

Figure 1. Determination of *Bothrops atrox* venom mean lethal dose (LD_{50}). Different amounts of venom were evaluated, from 2.5 to 6.75 µg of venom per gram of mouse in groups of 12 mice. The venom solutions were inoculated intraperitoneal and the mice were counted alive and dead after 48 hours.

associated with the use of these medications, such as the occurrence of adverse reactions in patients [3]. The present study analyzes the llama's immune response to the venom of the *Bothrops atrox* snake and the capacity of this hyperimmune serum to neutralize the lethality of the venom, as an approach to evaluate other serum-producing species.

Ruiz *et al.* [12] determined an intraperitoneal *Bothrops atrox* LD^{50} at 48 hours of 2.3 mg/kg. Meanwhile, Ohsaka [13] determined an intraperitoneal LD_{50} at 48 hours of 3.8 µg/g.

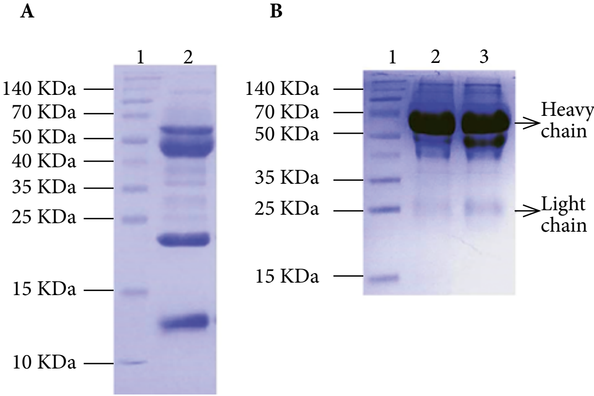

Figure 2. SDS-PAGE electrophoresis of *Bothrops atrox* venom and llama serum. A) Snake venom protein profile of *Bothrops atrox*. Lane 1: protein molecular weight marker (Termo Scientific #26623). Lane 2: *Bothrops atrox* total venom. B) Llama serum electrophoresis (*Lama glama*). Lane 1: protein molecular weight marker (Thermo Scientific #26623). Lane 2: llama pre-immune serum. Track 3: llama immune serum.

Barros *et al.* [14] determined a LD_{50} of 6.85 µg/g, and Meier and Theakston [15] determined a LD_{50} of 3.95 µg/g under the same conditions. In this study we obtained a LD_{50} of 3.96 µg/g, a figure similar to those found in the mentioned previous studies; this could be due to the fact that we used the same assay and the biological samples are from the same species. However, some differences could be found due to variations between the populations of snakes studied, since the characteristics of venom from snakes of the same species can vary depending on the age of the specimen, their origin or whether they are found in the wild or in captivity [4,16].

Previous studies [17] have shown that bothropic venoms are 1.2 to 3.6 times more toxic when inoculated intravenously than intraperitoneally. Previous data also suggest that the subcutaneous route is not recommended for the evaluation of toxic potency [17]. For these reasons, in this study, inoculation by the intraperitoneal route was used, showing good results in the lethality neutralization test.

The antivenoms produced in horses are evaluated by the same institutions that manufacture them, in our case the INS, but studies are also carried out to verify their effectiveness, their range of action in terms of snake species, and their neutralizing power [18]. In our study, the llama hyperimmune serum presented a neutralizing power of 3.6 mg/mL, which means that 1 mL of the llama hyperimmune serum can neutralize 3.6 mg of *Bothrops atrox* venom.

In this study, the dose-response data were analyzed with

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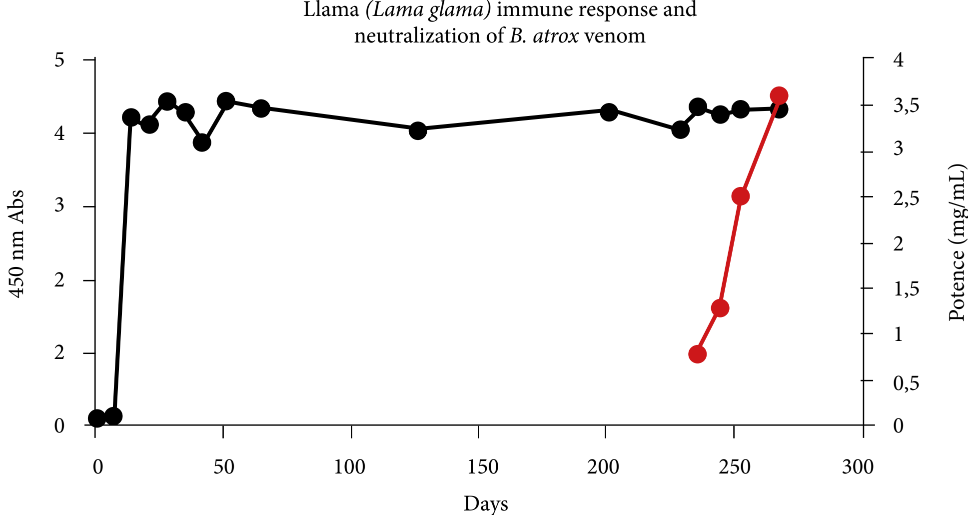

Figure 3. Measurement of antibodies against *Bothrops atrox* venom by ELISA and neutralization of the lethal effect in mice. The antibodies were measured in the llama serum after the immunizations by ELISA assay (values in left Y axis, black color line), and the potency of lethality neutralization of the llama hyperimmune serum was analyzed, in laboratory mice (values in right Y axis, red color line).

the probit model, although other types of possible analyses were found in references, probit was chosen because it was the most suitable for this analysis. Due to the need to obtain objective data, statistics has been used for decades and as it has been advancing, some ways of calculating the LD_{50} have been devised. For example, in 1985 Meier and Theaicston [15] proposed a statistical analysis method to reduce the number of experimental mice from 30 to 10 that did not differ significantly in the results and was more desirable for economic and ethical reasons. But this has been later overcome by the probit model, which is particularly useful against dose-response data of this type.

Previous studies have already demonstrated the great importance of the ELISA technique in *in vitro* assays for analyzing antibodies generated in response to venom immunization, compared to other methods used, as immunodiffusion or hemagglutination [19].

Simultaneous concordance between the antibody titer and the neutralization capacity during the immunization process was not observed, since the antibodies against *Bothrops atrox* venom increase rapidly; but the neutralization potency increases slowly, requires more time to reach expected values and also requires the administration of immunization reinforce doses.

Other studies involving llama serum also found no correlation between titer and effectiveness of venom neutralization in preclinical trials [20]. This observation is probably due to the fact that the ELISA assay detects early all the antibodies produced against all the venom antigens, while the neutralization effect depends only on a small group of specific antibodies that neutralize or block the toxicity produced by the toxins or proteins of the venom.

The slow development of the neutralization potency in the llama is understandable, considering that 12 or more immunization doses are required in horses to reach the expected values of venom neutralization potency [21].

Harrison *et al.* [22] measured the neutralizing capacity of *Lama glama* hyperimmune serum against *Echis ocellatus* venom, and found that an elevated llama antibody titer is produced at 2 weeks; similar to our results, which indicate that it is feasible to prepare bothropic antiserum from llama serum. In addition, they found a yet undetermined component within the llama serum that is not an IgG and that exhibits antihemorrhagic activity. Fernandez *et al.* [23] found that llama serum is effective against *Bothrops mattogrossensis* snake venom, which also represents a valuable alternative for antidote manufacture in South America.

Among the limitations of the study, it should be considered that only the immune response to *Bothrops atrox* snake venom has been evaluated in a single llama and it is recommended to do the analysis in more than one animal; the neutralizing capacity of the llama serum has not been compared to the anti-bothropic serum produced in horses, because the latter is a polyvalent serum and the llama serum is only against *Bothrops atrox* snake venom. It should be noted that this is one of the few studies that has evaluated the capacity of the llama

serum to neutralize the snake venom, and although there are other similar studies with snake venom from the genus *Bothrops*, such as *Bothrops matogrosensis,* this is the only known study with the species *Bothrops atrox*.

In conclusion, the results show that the llama hyperimmune serum can neutralize the venom of Peruvian *Bothrops atrox*, with a ED_{50} of 3.30 µL of serum/g mouse and a venom neutralization potency of 3.6 mg/mL, which means that 1 mL of hyperimmune serum can neutralize 3.6 mg of venom. The LD50 of the venom from Peruvian *Bothrops atrox* was 3.96 µg venom/g mouse and 4 times this amount was used as a challenge dose to test for venom neutralization. The immune response of the llama to the venom is rapid, with high antibody titers from the second immunization week, but several more weeks and additional booster doses are required to achieve the expected venom neutralization potency of this hyperimmune serum. The ELISA test is a good *in vitro* method for analyzing antibodies generated by immunization with *Bothrops atrox* venom and allows monitoring the immune response during immunization.

Authors' contributions: HBC, EGC, VOYC, CPR, OCR, CBF, and BTF designed the article, gathered the results, analyzed the data, wrote and reviewed the article, and approved the final version. MGP, DGN, RIA, SSO, HMA gathered the results, analyzed the data, reviewed the article, and approved the final version.

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Supplementary material: Available in the electronic version of the RPMESP.

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