

**DETECTION AND NEUTRALIZATION OF VENOM BY OVINE ANTISERUM IN
EXPERIMENTAL ENVENOMING BY *Bothrops jararaca***

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ABSTRACT: In this study we optimized an enzyme-linked immunosorbent assay (ELISA) to evaluate bothropic venom levels in biological samples. These samples were obtained by two distinct protocols. In the first one, Swiss mice were injected with 1 LD₅₀ of *Bothrops jararaca* (*B. jararaca*) venom and 15 minutes later, animals were treated with ovine antiobothropic serum. Blood and spleen homogenate samples were obtained 6 hours after antiserum therapy. Ovine antiobothropic serum significantly neutralized venom levels in serum and spleen. In the second protocol, BALB/c mice were injected with 1 LD₅₀ of bothropic venom by either intraperitoneal (IP) or intradermal (ID) route and venom levels were evaluated 1, 3 and 6 hours after, in blood, spleen homogenates and urine. Serum and splenic venom levels were significantly higher in animals envenomed by IP route comparing with animals envenomed by ID route. Higher venom levels were also detected in urine samples from animals envenomed by IP route. However, these differences were not statistically significant. These results demonstrated that the optimized ELISA was adequate to quantify venom levels in different biological samples. This assay could, therefore, substitute the *in vivo* neutralizing assay and also be useful to evaluate the severity of human and experimental envenomations.

KEY WORDS: *Bothrops jararaca*, enzyme-linked immunosorbent assay, ovine antiobothropic serum, venom neutralization.

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INTRODUCTION

About 20,000 cases of snakebites are reported annually in Brazil, with a mortality rate of 0.5% (8). *Bothrops jararaca* is the species responsible for the majority of snakebites in southeast Brazil (20).

The most effective therapy to counteract toxic effects of ophidic venoms is treatment with specific antiserum produced in horses. However, this kind of therapy can be associated with side effects as type I and III hypersensitivities (15). Alternative antibody sources, devoid of deleterious effects, are highly desirable. Sjostram *et al.* (21) compared monospecific antivenoms raised in sheep against a variety of snake venoms with commercially available antisera. They observed that ovine antivenoms generally contained higher concentration of specific antibodies than equine derived material. In addition, the ovine product usually provided better *in vivo* protection to mice than the equine counterpart (21). Easier ovine management, comparing to equines, has also stimulated sheep usage to generate antivenoms. Antisera to different snake venoms have been raised in sheep by Instituto Clodomiro Picado, including antiserum against *Bothrops asper* (13, 14, 19, 24).

Efficiency and safety in venom immunotherapy have also been improved by application of enzyme-linked immunosorbent assay (ELISA) to snake venom research. Theakston *et al.* (22) first described the use of this assay in detection of antigen and antibody in snake bitten patients. Subsequently, ELISAs have been described for detection of venom antigens from different species of venomous animals (3, 10, 11, 12, 23).

In this study, we reported the development of a sandwich-type ELISA for detection of *B. jararaca* venom antigens in blood, spleen homogenate and urine of mice experimentally envenomed by two different routes. We also used this assay to evaluate the *in vivo* ability of an ovine antiothropic serum to neutralize serum venom levels.

MATERIALS AND METHODS

Venom

A pool of lyophilized venoms was obtained from various adult specimens of *Bothrops jararaca* snakes kept at the Center for the Study of Venoms and Venomous Animals

(CEVAP, UNESP, Botucatu, São Paulo). After lyophilization, the material was stocked at -40°C. For usage, the venom was diluted in endotoxin free salt solution.

Animals

Male Swiss mice, female BALB/c mice, aged 4-6 weeks, and two female Norfolk rabbits (Botucatu Strain, 2-3kg) were supplied by the Central Animal Facility of São Paulo State University, UNESP, Botucatu, São Paulo. The animals were kept at the Animal Facility of the Department of Microbiology and Immunology throughout the experiments with free access to food and water. Four ovines of ideal breed (65-70kg), aged 3-11 years old, were kept at the Veterinarian Hospital at UNESP, Botucatu.

Antibodies

Peroxidase-conjugated goat anti-rabbit immunoglobulin antibody was purchased from Sigmal Chemical Co. Antithropic serum was obtained by rabbit immunization. Animals were initially injected with 250ng of *B. jararaca* venom emulsified with 0.5ml of Complete Freund's Adjuvant (CFA), by subcutaneous (SC) route. Three other doses of 100ng emulsified with 0.5ml of Incomplete Freund's adjuvant (IFA) were delivered by SC route. Time interval among inoculations was 10 days. Two weeks after the last dose, animals were bled and serum kept at -20°C until use.

The ovine antithropic serum was obtained by following the procedures developed at Instituto Clodomiro Picado (7). Briefly, 4 animals were immunized with seven doses of bothropic venom. The following doses: 0.5; 1; 3; 9; 18; 30; and 45mg were injected by SC route on days 0; 15; 30; 40; 50; 60; and 70, respectively. The first dose was associated with CFA, the second one with IFA, and the remaining doses with Al(OH)₃. Booster effect on antiserum levels was measured by an indirect ELISA. The antiserum used in this investigation was obtained from one animal (N.1572), whose maximum titre was observed 30-40 days after the first immunization. Further boosters did not significantly affect the specific antibody level. The last antibody sample, obtained 10 days after the last venom dose, was used due to its expected higher affinity.

Blood, urine and spleen homogenates

Initially, Swiss mice were used to evaluate the neutralizing ability of the ovine antiserum. Animals were allocated in 4 groups (5 animals each). Each group received 2.5µg/g of bothropic venom by IP route. After 15 minutes, three of these groups were treated with ovine antiobothropic serum (diluted 1:10, 1:40, or 1:200). The fourth group was left untreated to be used as a control. Treatment was delivered in a 200µl final volume. After six hours animals were killed and blood and spleen were collected. Blood samples were obtained by cardiac puncture and spleen homogenates were prepared by disrupting the splenic tissue on a sterile fine nylon screen with 500µl of RPMI 1640 medium. Blood samples and splenic homogenates were centrifuged and supernatants stored at -20°C for further venom quantification by ELISA.

To assess the relationship between envenomation route and venom levels, BALB/c mice were injected with 2.5µg/g of bothropic venom by either IP or ID (footpad) route. After 1, 3 and 6 hours, groups of animals were killed and blood, urine and splenic homogenates were also obtained, centrifuged and stocked at -20°C for further evaluation. Samples obtained in a similar way from non-envenomed animals were used as controls.

ELISA for venom quantification

This assay was standardized according to Barral-Netto *et al.* (3) and also to our previous experience in this field (4). Polypropylene microtiter plates were coated with F(ab')₂ fraction from horse anti-*B. jararaca* venom (batch n. 970802, produced by Instituto Vital Brazil, Niterói, Rio de Janeiro) (100 µl diluted 1:200 in carbonate-bicarbonate buffer, pH 9.6, for 1 h at 37°C). The wells were then washed five times with 200µl phosphate buffered saline (PBS) containing 0.05% Tween 20 (PBS-T) and incubated overnight at 4°C with 3% low-fat milk in carbonate-bicarbonate buffer as a blocking step. After another washing cycle, a 2-h incubation was performed with either various concentrations of *B. jararaca* venom (ranging from 65ng/ml to 500pg/ml) or samples from serum, splenic homogenates or urine. Each venom concentration was tested in duplicate. After another washing step, 100µl of rabbit antiobothropic serum (1:200) was added to the plate and incubated 1h at 37°C. The wells were subsequently washed and 100µl of goat anti-rabbit IgG conjugated to peroxidase (1:8000) were added. The plates

were incubated for 1h at 37°C. The wells were then washed and incubated with 100µl of an enzymatic substrate containing 0.04% ortho-phenylenediamine plus 0.012% hydrogen peroxide in citrate-phosphate buffer (pH 5.0) for about 15 min at room temperature in the dark. The reaction was stopped by addition of 50µl of 2M sulfuric acid and the absorbance values were measured at 492nm using an ELISA reader (Multiskan Spectrophotometer, manufactured in Finland by Labsystems). Venom concentrations were interpolated from standard curves of *B. jararaca* venom.

RESULTS

ELISA optimization for Bothrops jararaca venom detection

The best conditions for *B. jararaca* venom detection included a blocking step (12h at 4°C) with a carbonate/bicarbonate buffer (pH 9.6) containing 3% non-fat dry milk. Specificity was increased by this procedure. Samples incubation during 2h at 37°C also increased sensitivity (500pg/ml). The best dilution for horse F(ab')₂ used as capture antibody and for detection antibody (whole rabbit antiothropic serum) was 1:200. The best dilution for the conjugate (goat anti-rabbit serum) was 1:8000. The best incubation condition for these three antibodies was 1h at 37°C. The correlation coefficient (*r*) of this assay varied from 0.96 to 0.98. By using these conditions we were able to detect very low levels of bothropic venom in biological samples. A titration curve done with a crude preparation of venom from *B. jararaca* is shown in Figure 1.

Neutralizing activity of ovine antiothropic serum

As can be observed in Figure 2a, ovine antiserum administration determined a significant drop on serum venom levels. The neutralization effect was, as expected, more pronounced with less diluted serum. On the other hand, when spleen homogenates were used to evaluate venom concentration (Figure 2b), only animals that received the less diluted ovine antiserum (1:10) presented a significant decrease in venom concentration.

Comparison of venom levels in mice envenomed by IP or ID route

As shown in Figure 3a, serum venom levels were significantly higher in animals that were envenomed by IP route in comparison with animals envenomed by ID route. This difference was observed at the three evaluation periods.

A similar pattern of venom concentrations was observed in urine (Figure 3b) and spleen homogenate (Figure 3c) samples. In spite of this similarity, differences observed in urine samples were not statistically significant.

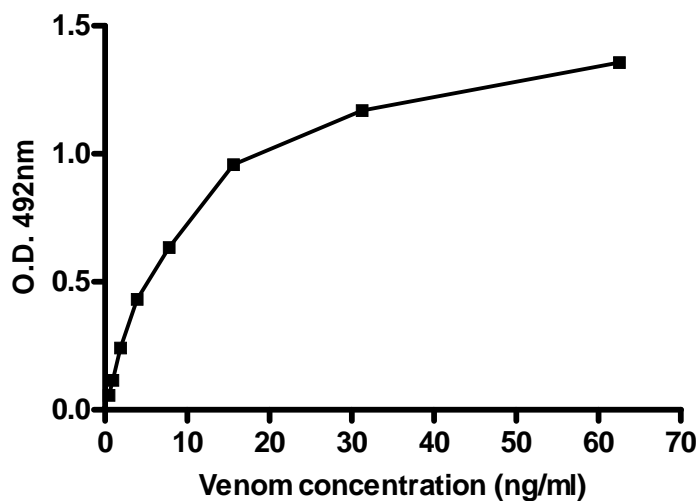


Figure 1: ELISA representative calibration curve for *B. jararaca* crude venom detection. Venom was diluted with PBS/milk 3% and ELISA developed with the reagents described before. Resulting optical densities were correlated to venom concentrations. Optical densities from three separate assays were combined (n=6) and showed in mean \pm SD. The *r* value is 0.98.

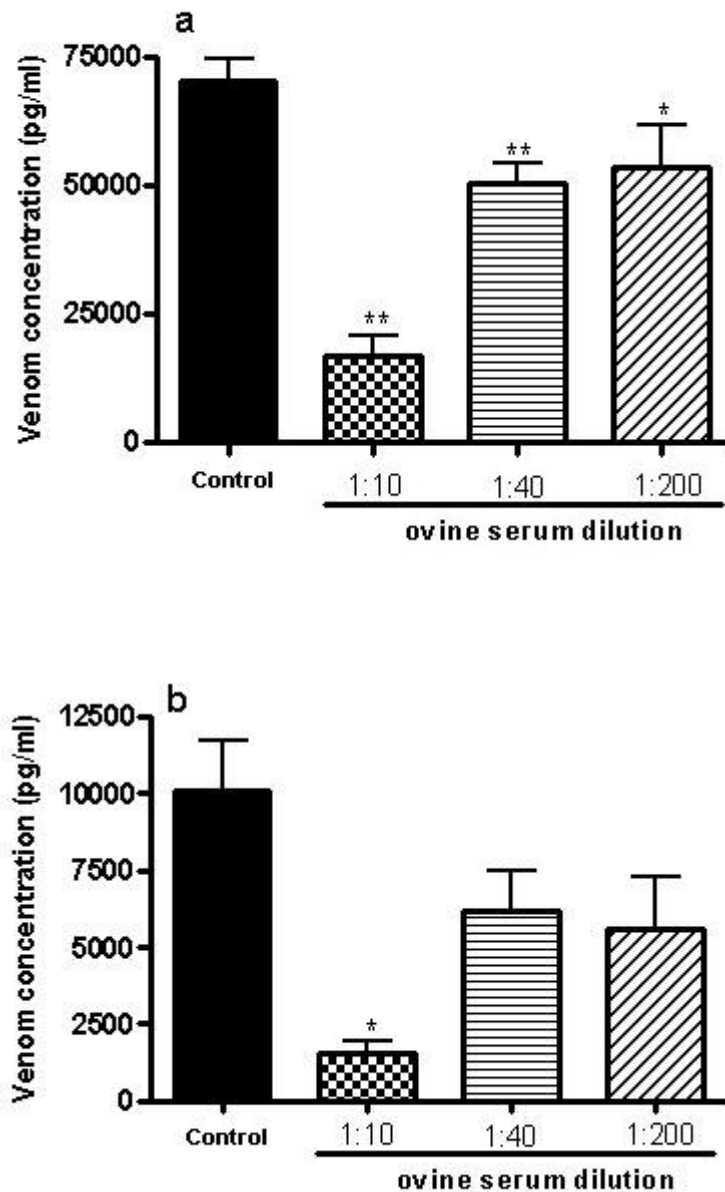


Figure 2: *In vivo* neutralizing activity of ovine antiothropic serum. Swiss mice were injected with 1 LD₅₀ of *B. jararaca* venom by IP route. After 15 min, animals were treated with ovine antiothropic serum (diluted 1:10, 1:40, or 1:200). A group was left untreated and used as a control. Biological samples were obtained 6 hours after venom inoculation and analyzed for their venom concentration by ELISA. Venom concentrations in blood (a) and spleen homogenate (b) samples. Values are means \pm SD. * $p < 0.05$ and ** $p < 0.01$ regarding the untreated group.

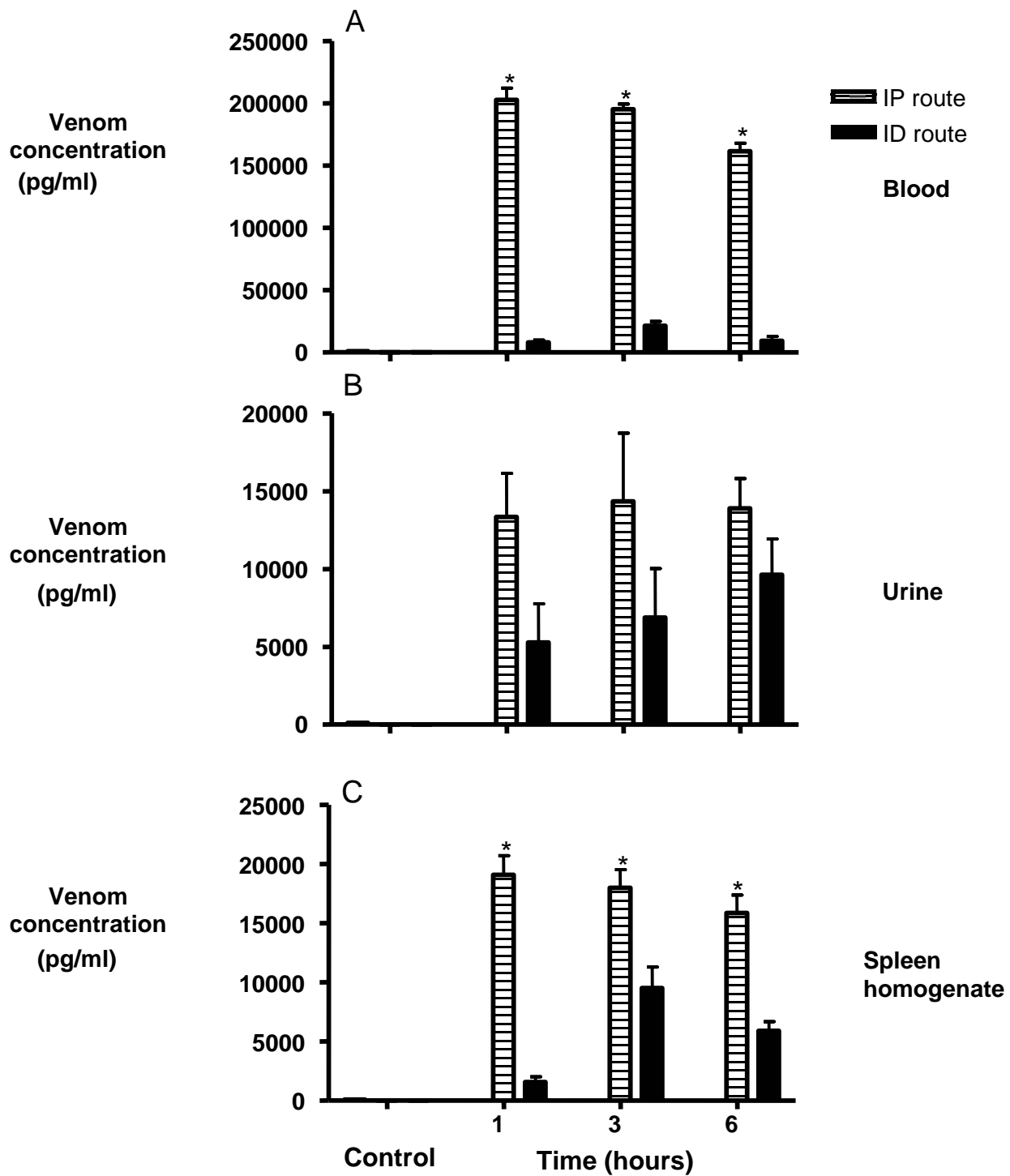


Figure 3: Venom concentrations in biological samples. BALB/c mice were injected with 1 LD₅₀ of *B. jararaca* venom by either IP or ID route and biological samples were obtained 1, 3, and 6 hours after inoculation. Samples were analyzed for their venom concentrations by ELISA. Venom concentrations in blood (a), urine (b) and spleen homogenate (c) samples. Values are means \pm SD. * $p < 0.05$ regarding ID route at the same period.

DISCUSSION

Among the ophidian accidents occurring in Botucatu area, located at São Paulo State, about 80% are caused by *Bothrops* species (6). As in many other parts of the world, treatment is still based on inoculation of horse antiothropic serum (12). There is an increasing interest for development of other sources of antivenom and also of assays to identify the venom and its concentration in body fluids. This investigation was done to develop a sandwich-type ELISA for detection and comparison of venom levels in blood, urine and spleen homogenate samples obtained from mice experimentally injected with bothropic venom. This methodology was also employed to evaluate the neutralizing ability of an ovine anti-*Bothropic* antivenom.

The ELISA test adapted by us presented elevated sensitivity (500pg/ml), similar to values described in other reports (1, 3, 18). The blocking step done by using non-fat milk during 12h at 4°C comparing with 1h at room temperature was very effective, decreasing substantially the background. The usefulness of this step to improve the specificity was not evaluated. We believe that sensitivity and specificity could be improved by substituting the whole rabbit anti-bothropic serum for its IgG fraction. Once optimized, this assay was used to quantify venom in biological samples from experimentally envenomed mice.

Initially, it was observed that non-diluted sheep antiothropic venom was able to neutralize lethal venom activity associated with 2 LD₅₀ (not shown). The potency of antivenom has been traditionally evaluated by testing lethality neutralization (25). As this procedure requires sacrifice of a large number of mice, other alternatives are being sought to replace the *in vivo* neutralization assay. For this reason, this neutralizing ability was then checked by quantification of serum and splenic venom levels by ELISA.

According to the neutralizing effect observed *in vivo*, serum and splenic venom levels were significantly lower in animals subjected to ovine antiserum treatment compared to non-treated animals. This effect depended upon antiserum dilution. Interestingly, higher antiserum dilutions (1:40 and 1:200) significantly decrease serum venom levels but not splenic venom levels. These findings could be related to a differential distribution of ovine antivenom among serum and the other organs, as has been described for horse antiserum (9, 17).

This assay was then used to evaluate venom concentration in samples from BALB/c female mice injected with bothropic venom by two distinct routes (IP or ID). LD₅₀ was established previously by IP route (not shown). The decision to compare venom levels associated with both envenomation routes was based on the fact that IP route is classically employed in envenoming studies whereas ID route best resembles the natural ophidian accident. As expected, serum venom levels were always higher in animals envenomed by IP route. These results are in accordance with the differential severity of the two envenoming procedures. Animals injected by IP route presented more severe envenomation signs as prostration, hemorrhage at the peritoneal cavity and elevated mortality at 6 hours (results not shown). These findings resemble and reinforce clinical data showing a direct correlation between severity of clinical signs and the higher serum venom levels found in human patients (2, 5).

The profile of venom concentrations in spleen homogenates was very similar to blood venom distribution, i.e., significantly higher levels associated with IP route. However, these values did not consider the dilution of spleen homogenates determined by addition of 500µl of RPMI 1640 medium to the spleen to prepare the homogenates and use of 100µl to evaluate venom concentration. If these values were roughly corrected by this factor, they would be similar to blood levels. Certainly, this kind of determination could be better appraised by using homogenate samples from animals previously subjected to perfusion as described by Domingos *et al.* (16). Urine venom levels also followed the serum levels profile, i.e., higher levels in animals injected by IP route. However, these levels were not statistically different from levels determined by ID envenomation.

Taken together, the results demonstrate that the optimized ELISA was adequate to evaluate venom levels in different samples and that this assay could also be useful to evaluate the neutralizing effect of antiothropic serum raised in ovines.

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