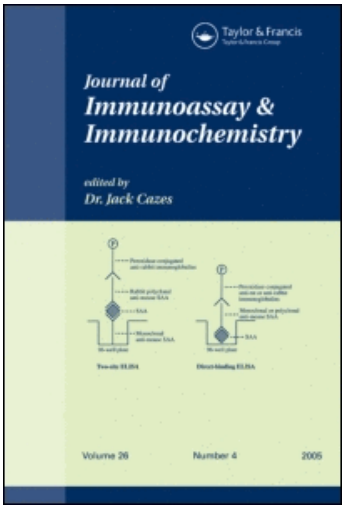

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A CAPTURE ENZYME-LINKED IMMUNOSORBENT ASSAY FOR SPECIES-SPECIFIC DETECTION OF *BOTHROPS* VENOMS

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ABSTRACT

A direct sandwich enzyme-linked immunosorbent assay (ELISA), employing affinity purified antivenom antibodies specifically recognizing the homologous venom, was developed for species-specific detection of bothropic venom. The method is based on a two-step affinity purification of the specific antibodies. A species monovalent antivenom is adsorbed onto a venom adsorbent containing heterologous venoms from the *Bothrops*, *Crotalus* and *Lachesis* genera. The species-specific antibodies obtained, are then adsorbed onto a second venom adsorbent containing only the homologous venom for the removal of non antivenom antibodies. Venom concentrations of 0.1 and 1,000 ng/ml were specifically identified for *Bothrops jararacussu* and *B. alternatus* venom respectively.

(KEY WORDS: snake bite; bothropic venom detection; ELISA)

INTRODUCTION

The high degree of crossreaction between the venoms of the *Bothrops* genera (1, 2) has hampered the development of a species-specific immunoassay for the venom detection. A reported enzyme assay for the specific detection of the *B. jararaca* venom that detected instead *B. atrox* along with other correlated venoms (3). There are reports on the variation of the *Bothrops* snake venoms toxic activities(4) and antivenom efficacy (5) in Brazil. In the state of Minas Gerais, Brazil, there were 1,460 snakebites reported from January to April, 1994, with

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seven deaths (6). This figure rose to 3,220 accidents in September of the same year with 2,759 due to bothropic species. Despite the low mortality, early reactions due to the injection of horse antivenom in victims of *Bothrops* snake bites in Brazil were found to be high, 56%, 87% and 36% respectively for the three different commercial antivenoms tested (7). An assay to specifically detect venoms in snakebite accidents would bring benefits to the study of ophidism in Brazil where, a large diversity of snake species share a same geographical area. We have previously demonstrated the possibility of specifically differentiating venoms from the *Bothrops* genera using an indirect sandwich ELISA and the existence of especies-specific venom components for bothropic and lachetic venoms (8). In this study we have developed a direct sandwich ELISA that detects species-especifically and with improved sensititivity and specificity the venoms of *Bothrops jararacussu* and *B. alternatus*.

MATERIALS AND METHOS

Venoms

Venoms were obtained from the Serpentarium of Fundação Ezequiel Dias (FUNED- Belo Horizonte, Brazil). They are a pool of adult specimens from different geographical localities, kept at the Serpentarium. Venoms were suppied liophylised and a single batch was used throughout the study. Venoms from *Bothrops jararaca*, *B. jararacussu*, *B. alternatus*, *B. moojeni*, *B. neuwiedi* and *Crotalus durissus terrificus* were used.

Antivenoms

Anti-*B. jararacussu* and anti-*B. alternatus* serum was obtained by immunization of sheeps with the specific venom. Venoms were weighed and dissolved in 1.0 ml of 10 mM sodium phosphate buffer, pH 7.2, containing 150 mM NaCl (PBS). After centrifugation, 10,000 g for 30 min, the surpenatant was removed and emulsified with a same volume of Freund's Complete Adjuvant (FCA), for the first dose and with Freund's Incomplete Adjuvant (FIA) for the booster.

All doses were subcutaneous and prepared just prior to use. The schedule was: day 0: 4.0 mg/ml; day 31: 4.0 mg/ml. Bleeding was performed on day 41 and the antivenom titre tested by ELISA. Samples were aliquoted and kept at -20 ^{0}C until use.

Antivenom adsorption

Species-specific antivenom was obtained by removing the crossreacting antibodies on venom affinity adsorbents. The adsorbents were prepared specifically for the adsorption of each antivenom serum, where the heterologous venoms were added according to a crossreactivity index (8). The glutaraldehyde method (9) was used. A total of 100 mg of venom + 400 mg of Bovine Serum Albumin (BSA) was used for each venom adsorbent. Aliquots of 4 ml of antivenom serum diluted in the same volume of PBS, was added to the specific adsorbent and slowly rotated for 1 hour at room temperature. After centrifugation at 3500 g, the supernatant was removed and tested for specificity by ELISA. After two adsorption steps the specific antivenom sera was pooled, dialysed against 10 X diluted PBS and concentrated by lyophilization. Adsorbents were regenerated with 0.1 M Glycine pH 2.5. The pH was restored to neutral with 0.5 M K_2HPO_4 and reequilibrated with 5 washings of PBS (50ml each).

Purification of specific antivenom IgG

Aliquots from absorbed antivenom sera were separately resuspended in 2ml of 0.1 M Tris-HCl pH 8.0. Then each antivenom was applied to an affinity column containing 100 mg of their respective homologous venom attached to 1 g of Superose CL-4B (Pharmacia). Venom was coupled to the support matrix according to the manufacturers instructions. The column was washed with 40 ml of 0.1 M Tris and 40 ml of 0.01 M Tris until absorbance at 280 nm was below 0.05. Then the specific antivenom IgG was eluted with 0.1 M Glycine-HCl pH 2.5. Aliquots of 900 µl were collected in vials containing 100 µl of 1.0 M Tris-HCl pH 8.0. Samples were pooled dialysed against 10 X diluted PBS and liophylised. Purified specific antivenom IgG was conjugated to HRP (10).

ELISA for antivenom titration

Microtiter plates were coated with 1.0μg/ml of the studied venoms in 100 μl of 100 mM sodium carbonate buffer, pH 9.6 (coating buffer) and incubated overnight at 4 °C. Plates were washed 3 times with 10 mM sodium phosphate buffer. PH 7.4, containing 150 mM NaCl (phosphate buffered saline, PBS) and 0.05 % Tween-20 (PBS-Tween). Blocking was achieved by an 1 hour incubation at 37 °C with 100μl/well of PBS containing 3% BSA (PBS-BSA). Absorbed antivenom was added to wells containing the homologous or the heterologous venoms in PBS-Tween, and incubated for 1 hour at 37 °C. The plates were then washed 3 times with PBS-Tween. Conjugate solution (100 μl/well) was added and incubated for 1 hour at 37 °C. After washing, 100 μl/well of substrate medium (10 mM sodium phosphate, pH 7.3, containing 2 mg/ml of OPD and 0.06% H_2O_2) was added for 1 hour at 37 °C in the dark. The reaction was stopped by the addition of 20% sulfuric acid (50 μl/well). Absorbance was measured at 492 nm with a Titerteck ELISA reader.

Direct Sandwich ELISA for Venom Detection

Microtiter plates were coated either with 100 μl/well of the specific anti-*B. jararacussu* IgG or of the specific anti-B. alternatus IgG in coating buffer.The plates were incubated, blocked and washed as above. Then different venom preparations, in concentrations ranging from 1,000-0.1 ng/ml, were added to each plate in PBS-Tween and incubated for 1 hour at 37 °C. After a washing step, the venom specific IgG-HRP conjugate was added to the plate coated with its homologous absorbed antivenom IgG. The anti-*B. jararacussu* conjugate was used at 1:200 dilution and the anti-*B. alternatus* conjugate at 1:50 dilution. The following steps were as above.

Serum Kinectics Studies

Kinectic studies were performed only for the venom of *B. jararacussu*. C57BL/6 mice were divided in 12 groups of five animals each. Four groups were inoculated i.m. in the left hind with 1 LD_{50} (75.0 μg) of *B. jararacussu* venom diluted in 0.5 ml of PBS and four other groups with 4

LD_{50}. A third group was injected with PBS alone (control group). The groups of four animals were sequentially sacrificed by ether inhalation and bled at 15, 30, 45, 60 min. Serum from individual animals of each group was pooled and venom levels assessed by ELISA as described above with the following modifications. Serum samples were used diluted at 1:2 with the incubation buffer. A reference curve was constructed by adding *B. jararacussu* venom (0.47 to 240μg/ml) to normal rabbit serum (NRS) diluted 1:2 as for the sample. To the conjugate buffer we added 5% NRS and 3% BSA. NRS diluted 1:2 was used as background control. A computer program, Standard Curve, Window Chem, Inc., was used to calculate the logarithimic regression for the reference curve and to fit the sample absorbance values to the curve and obtain the concentration of the venom in the mice serum.

RESULTS

The specificity of the purified IgG was first examined by direct ELISA (Figure 1). The response obtained with the two venoms (*B. jararacussu* and *B. alternatus*) were very similar. The cross-reactivity to the venom of related and unrelated species was negligible. In Figure 2, the sensitivity was tested by an antigen capture sandwich ELISA. For *the B. jararacussu* venom a concentration of 0.1 ng/ml could be specifically detected. The difference between the specific and nonspecific reactivity at the lowest concentration detected is six fold. A good linearity is observed for the specific reaction throughout the dose-response curve $r = 0.93$. The standard deviation values indicate a small inter-assay variability. The sensitivity for the *B. alternatus* venom dose-response curve is between 10 and 100 ng/ml. Despite the lower sensitivity there is a good linearity ($r = 0.96$) and small inter-assay variability. Within the detection range no cross-reactivity was observed. Parallelism of serial dilution curves for envenomed mice serum was assessed. Figure 3 shows dilution curves for two separate samples (serum from experimentally envenomed mice with $1LD_{50}$ and $4LD_{50}$ of *B. jararacussu* venom, with highest absorbance values at 492 nm were used) and although results fell in the lower part of the reference curve it clearly

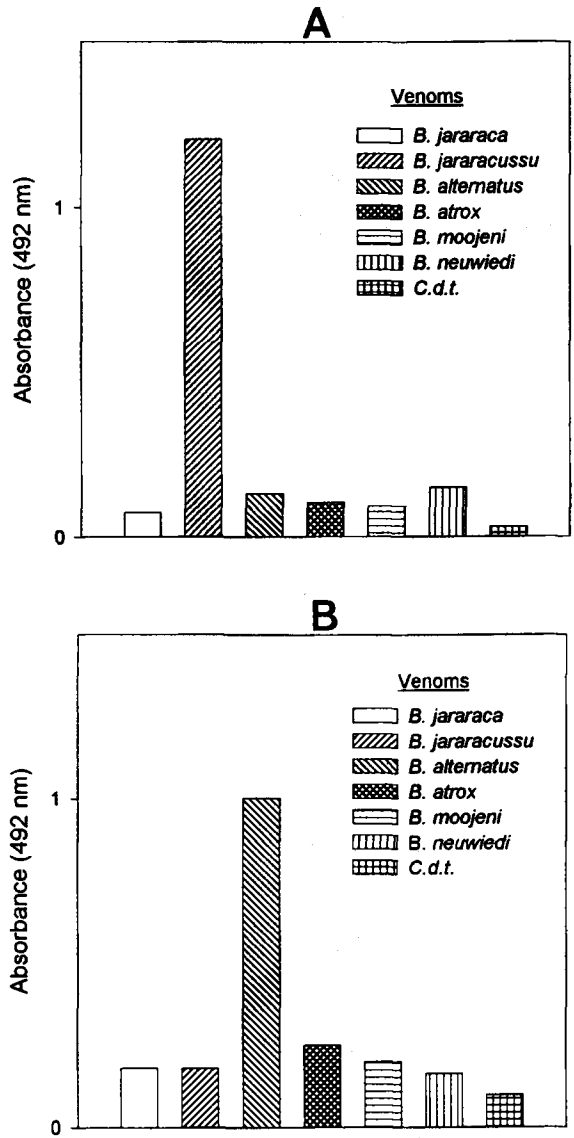

FIGURE 1. Specificity of purified antivenom IgG tested by ELISA. Plates were coated with 1.0μg/ml of snake venoms and reacted against (A) anti-*B. jararacussu* IgG diluted at 1:10; (B) anti-*B. alternatus* diluted at 1:25. Anti-rabbit IgG conjugated t HRP was used at 1:3000 dilution.

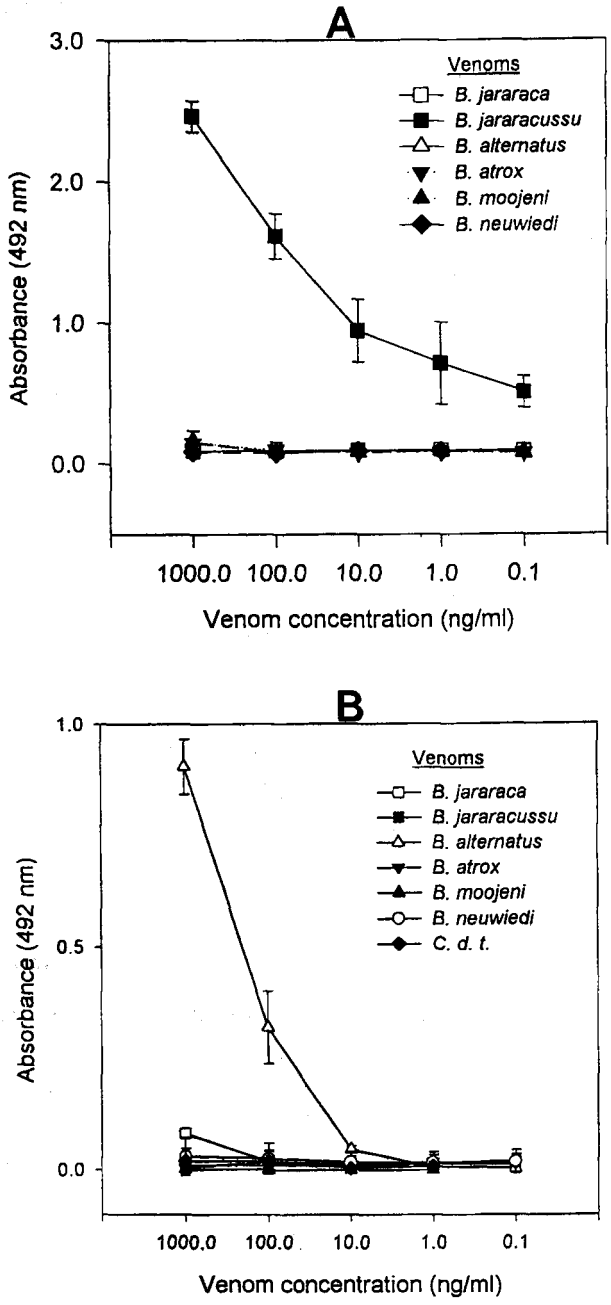

FIGURE 2. Antibody sandwich ELISA for the specific detection of (A) *B. jararacussu* venom and (B) *B. alternatus* venom. Different venom preparations in concentrations ranging from 1,000 to 0.1 ng/ml were assayed. Error bars show standard deviations obtained for the mean values of the measuring points from two separate determinations.

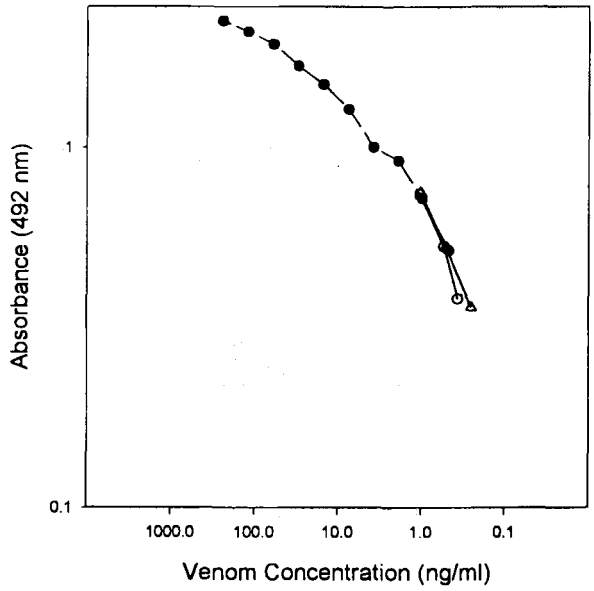

FIGURE 3. Parallelism of serial dilutions of experimentally *B. jararacussu* envenomed mice serum with the venom standard curve in ELISA. Serial dilutions of two serum samples with $1LD_{50}$ (open circle) and $4LD_{50}$ (open triangle) were compared with the *B. jararacussu* venom standard curve (solid circles).

demonstrates that these curves are parallel. The quantitative detection of *B. jararacussu* venom in experimentally envenomed mice was tested. The results were similar for the two different doses used (Figure 4). A rapid transient increase of the venom antigens in the blood which peaked at 30 min after the intramuscular injection is observed for venom dose of 1 LD_{50}. For the dose of 4 LD_{50} maximum venom level in mice blood is observed earlier, at 15 min remaining high until 30 min after the injection. Then like the previous dose venom levels decreases steadily.

DISCUSSION

The present study show the specific detection of *B. jararacussu* and *B. alternatus* snake venoms by ELISA. The assay is sensitive and reproducible, displaying a linear correlation

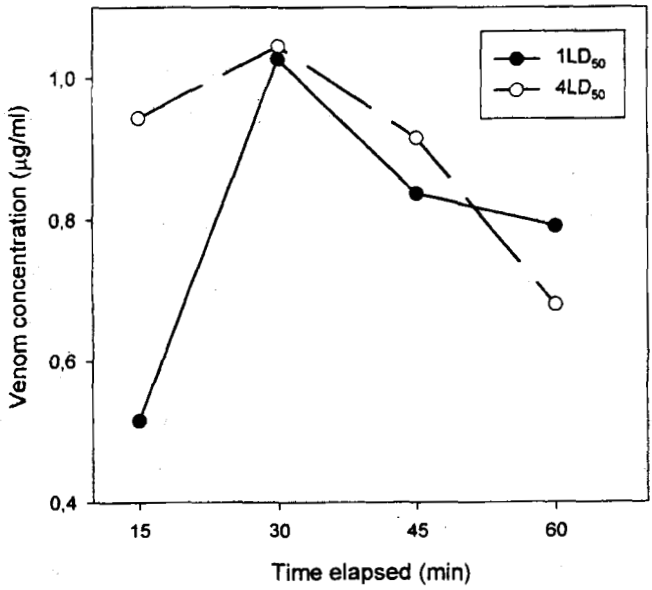

FIGURE 4. Serum kinetics of experimentally evenomed mice. Two groups of mice were injected with different doses of *B. jararacussu* venom in the hind footpad and bled at 15, 30, 40, 45 and 60 min after the inoculation. The serum samples were assayed by the antibody ELISA for the detection of *B. jararacussu* venom. Values represent means of groups.

between the venom concentration and detection levels. Thus, this test is very reliable in the evaluation *B. jararacussu* and *B. alternatus* venom concentrations, in the study of serum kinetics and should be useful in the epidemiological study of the ophidism of both species.

The use of venom affinity columns reduced the cross-reactivity of the monovalent antivenoms to a minimal, even against the venom of close related species of the *bothrops* genus and that of the *Crotalus durissus terrificus*. There is a high antigenic similarity between the *bothrops* venoms mostly due to common components (1, 2). A previous report for the specific detection of *B. jararaca* venom resulted in the specific detection of the heterologous *B. atrox* venom and equally most of the other *Bothrops* venoms used (3). In that particular study, the ELISA reagents used consisted of nonabsorbed horse $F(ab')_2$ and rabbit IgG antibodies obtained from monovalent antivenom raised against the venom of *B. jararaca*. We have shown previously (8) the specific

detection of *B. jararaca*, *B. jararacussu*, *B. alternatus*, *B. moojeni* and *B. neuwiedi*, at concentrations between 0.01-10 mg/ml using indirect antigen-capture ELISA. Then, affinity adsorbents were also used for the removal of crossreacting antibodies. In this study, after the removal of the crossreacting antibodies, venom specific antibodies were purified by affinity chromatography. For the venom of *B. jararacussu* the detection limit was increased 100 fold to 0.01 ng/ml, as for the *B. alternatus* venom the detection limit remained somewhat unchanged. However, specificity was increased in both cases, possibly as a result of the two step antibody purification procedure. For the kinetics of *B. jararacussu* venom in mice sera the venom level detected peaked at 30 min for the dose of $1LD_{50}$ followed by a progressive drop in serum levels. For the dose of 4 LD_{50}, venom level peaked at 15 min, possibly as the result of the larger amount of venom injected, remaining high until 30 min after the injection where a progressive drop is observed. At this point, the clinical use of the kinetics studies is not possible as there is no indication that the specific venom component detected is toxic. We believe that the present method may be applicable as an additional tool in the identification of *bothrops* venoms. The method can be extended to other species.

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