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Original paper

USE OF AN ELISA ASSAY TO EVALUATE VENOM, ANTIVENOM, IgG AND IgM HUMAN ANTIBODY LEVELS IN SERUM AND CEREBROSPINAL FLUID FROM PATIENTS BITTEN BY *Crotalus durissus terrificus* IN BRAZIL.

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ABSTRACT: A sandwich-type ELISA technique for specific and sensitive detection of *Crotalus durissus terrificus* venom antigens, horse-antivenom, human IgG and IgM antibodies was set up. Sixteen patients, 13 males and 3 females aged between 13 to 63 years (mean 33 ± 15) bitten by *Crotalus durissus terrificus* snakes were studied. Of the 15 patients, 6 had previously received anti-*Crotalus* venom and no seric venom was detected. For the other 9 patients studied, the venom levels ranged from 2 to 108 ng/ml according to the severity of each case. Seric antivenom was detected up to 44 days after the bite. IgM human antibody levels against *Crotalus* venom were higher between 3 and 18 days after specific treatment. IgG human antibody levels against *Crotalus* venom were detected between 30 and 90 days after envenoming. Venom and antivenom levels in cerebrospinal fluid were not observed 24 h after the bite. This suggests that neither the venom nor the antivenom is capable of crossing the blood-brain barrier. In addition, when either the venom or the antivenom is presented to the immune system cells an immune response is prepared.

☑ **KEY WORDS:** Enzyme-linked immunosorbent assay, *Crotalus durissus terrificus*, venom, antivenom, antibodies, cerebrospinal fluid.

INTRODUCTION

Snakebites are a serious medical, social and economic problem in many parts of the world, especially in tropical and subtropical countries (**4**,**7**,**20**,**21**). In Brazil, envenomations caused by *Crotalus* genus account for about 7 to 40%, depending on the area (**4,20,21**). In Botucatu, State of São Paulo, 20% of the snakebites are caused by *Crotalus durissus terrificus* (**5**,**7**). Among the clinical features, crotalic venom can damage the squeletal

muscle (**2**,**13**), blood coagulation (**18**,**27**), kidneys (**1**), liver (**6**) and the central nervous system (**29**). Regarding to the latter, there are doubts whether the venom or the antivenom is capable of crossing the blood-brain barrier (29).

Venom detection has been attempted using immunodiffusion (**19**,**28**), passive hemagglutination (**9**), counter-current immunoelectrophoresis (**15**) and radioimmunoassay (**11**,**12**). None of these methods was totally satisfactory for use in tropical countries where snakebites are a major public health problem due to their expense, insensitivity or instability of the reagents (**16**).

The enzyme-linked immunosorbent assay (ELISA) replaced radioimmunoassay with advantages because of its simplicity, rapidity, sensitivity and specificity in the detection of low concentration of venom in serum and urine (**17**). In 1977, Theakston *et al.* (**25**) described the use of the enzyme-linked immunosorbent assay (ELISA) for the detection of venom and specific antibodies in snake-bitten patients. They suggested that this method might be useful to the immunodiagnosis of snakebites, study of pathophysiology of envenoming, therapeutic follow up with antivenoms and to epidemiological surveys.

According to Theakston (**23**), ELISA enables to make a differentiated diagnosis of patients bitten by snakes of different species which can produce similar clinical features. Besides, detection of specific venom antibodies in sera of patients constitutes a significant alternative to epidemiological studies (**26**).

On the other hand, the enzyme-linked immunosorbent assay (ELISA) has been continually refined since its introduction and has also allowed the evaluation of venom, antivenom and specific human antibody levels in envenoming (**3**,**23**,**24**,**25**).

In this study, we set up a sandwich-type ELISA technique for specific and sensitive detection of *C. d. terrificus* venom antigens, horse-antivenom, human IgG and IgM antibodies evaluated in serum and in cerebrospinal (CSF) fluid from envenomed patients.

PATIENTS AND METHODS

PATIENTS

Sixteen patients, 13 males and 3 females, aged 13 to 63 years (mean 33±15) bitten by *Crotalus durissus terrificus* were studied. The patients were admitted to the Tropical Diseases Clinic at the School of Medicine of Botucatu (São Paulo State University - UNESP). Patients included in the study were those who had either brought the dead snake with them to the Hospital or who were clinically suspected of *C. d. terrificus* envenoming. The patients were asked about the time elapsed between the bite and medical attention as well as about previous administration of antivenom. After clinical evaluation, all patients were submitted to specific i.v. antivenom therapy according to the severity of each case. The severity of envenomations was classified as follows: severe, moderate or mild according to several authors (**4**,**20**). Patients received the following doses of specific anti-*Crotalus* venom produced by the Butantan Institute: mildly envenomed patients received 100 mg, moderately envenomed patients 200 mg and severely envenomed patients 300 mg. Blood samples were collected without anticoagulant into 5-ml plastic tubes before specific treatment and then at different periods ranging from 1 to 90 days. The blood was centrifuged, the serum was allowed to separate and stored at -20° C until assayed for the presence *C. d. terrificus* venom, specific human IgG and IgM antibodies and horse antivenom levels. Thirteen patients had their cerebrospinal fluid collected by lumbar puncture 24 h after the envenomation for the assessment of venom and horse-antivenom levels.

Seric crotalic venom, IgG and IgM human antibodies against venom and specific horse-antivenom levels were assessed using ELISA. For the standardization of this technique, blood samples of 8 healthy individuals, blood donors at the University Hospital, aged 18 to 45 years were collected and used as the control group.

METHODS

VENOM, ANTIVENOM AND ANIMALS: Venom was extracted from adult *C. d. terrificus* snakes and kept at the Serpentarium of **"The Center for the Study of Venoms and Venomous Animals"** - CEVAP - UNESP, Botucatu, São Paulo State University, Brazil. The venom of each snake was obtained by manual compression of the venomous glands, then pooled, centrifuged, filtered, lyophilized and stored at -20° C before use.

Hyperimmune horse anti-*Crotalus* venom was liquid, pepsin-digested, purified equine anti-*Crotalus* products, (**24**) which were prepared using basically identical techniques that were expected to result in a predominantly F(ab)2 preparation produced by the Butantan

Institute.

Adult male rabbits (*Oryctolagus cuniculus*, Norfolk 2001 strain) weighing around 2.0-2.5 kg were used. All animals were maintained at the animal facilities of the Institute of Biosciences of Botucatu - UNESP - São Paulo State University, where they received water and food under controlled environmental conditions.

PREPARATION OF RABBIT SPECIFIC IgG ANTI-*Crotalus durissus terrificus* VENOM*:* Three rabbits were immunized over a 40-day period with crude venom at a concentration of 100 µg/ml. The venom was mixed with 1 ml of Freund's complete adjuvant (v/v) and 0.25 of the emulsion was injected s.c. in 8 different sites. This same inoculation was repeated three times at 10 day-intervals. Seven days after the last antigen injection, the animals were bled by cardiac puncture and antivenom antibodies were measured by ELISA using 10 µg/ml of crude venom for coating the plates. The IgG fraction anti-*C. d. terrificus* was isolated by affinity chromatography on protein A-Sepharose.

SANDWICH-ELISA FOR VENOM DETECTION: Linbro Titertek (Flow Laboratories, Inc., Mclean, VA, U.S.A.) 96-well plates were coated overnight at 4° C with 100 µl of 1:8000 dilution F(ab)2 fraction of horse anti-C. d. terrificus venom in carbonate buffer 0.2M pH 9.6. The plates were washed with phosphate buffer saline pH 7.4 containing 0.05% of Tween 20 (T-PBS). At this and all other steps, the wells were washed six times with 200 ml of T-PBS. The blocking solution (5% casein in PBS) was added and the plates were incubated for 30 min at 37° C and washed six times with T-PBS. Both sera and cerebrospinal fluid from patients and sera from healthy controls were tested. A volume of 100 ml of samples and a series of venom standards (ranging from 500 ng to 0.1 ng/ml diluted in normal serum) were then added in duplicate to the wells and incubated for 60 minutes at 37° C. After washing the plates, a volume of 100 µl of 1:400 dilution of rabbit IgG anti-whole C. d. terrificus venom was used in each well, and the plates were incubated 60 min at 37°C. The wells were subsequently washed and 100 µl of goat anti-rabbit IgG conjugated to peroxidase (Sigma Chemical Co., USA) in 1:10000 dilution was added. The plates were incubated for 60 min at 37°C. The wells were then washed with T-PBS, and 100 µl of an enzimatic substrate containing 0.4 mg/ml and 2 µl/ml of hydrogen peroxide in citrate-phosphate buffer 0.1M pH 5.2 was added. After incubation of the plates at room temperature in the dark, the reaction was stopped after 15 min by addition of 50 µl of sulfuric acid 2M, and absorbance values were measured at 492 nm using an ELISA reader (Multiskan Spectrophotometer, EFLAB, Helsinki, Finland)*.*

DETERMINATION OF IgG AND IgM ANTI-Crotalus durissus terrificus VENOM ANTIBODIES: Sera of patients were titrated for their antibody content against a 10 µg/ml crude venom solution in a Linbro-Titertek 96-well plates according to the technique described by Theakston *et al*. (**25**). Test samples and positive and negative control sera diluted from 1:10 to 1:1280 in incubation buffer (T-PBS) were added in duplicate to the wells. A 1:1000 dilution of rabbit anti-human IgG conjugated to peroxidase (Sigma Chemical Co. U.S.A.) and 1:2000 dilution of rabbit anti-human IgM conjugated to peroxidase (Sigma Chemical Co., U.S.A.) were used. Plates were read using an ELISA reader and the titers determined as the reciprocal of the highest dilution that showed an absorbance higher than 0.050 at 492 nm, since non-specific reactions were observed below this value.

DETERMINATION OF HORSE-ANTIVENOM ANTIBODY LEVELS: For assay of therapeutic horse anti-*C. d. terrificus* antibodies in serum of treated patients, Linbro 96-wells microplates were coated with 10 µg/ml of crude venom solution as described above. Serum samples of patients and appropriate positive and negative control sera diluted 1:2 in incubation buffer (T-PBS) were added to the wells. After the plates were washed, a 1:10000 dilution of rabbit anti-horse IgG conjugated to peroxidase (Sigma Chemical Co., U.S.A.) was added to the wells and the plates were incubated for 60 min at 37°C. The addition of the enzymatic substrate and the remainder of the assay procedure was done as described above. Admission samples from the patients (zero antivenom) and healthy control sera served as controls for the antivenom assay.

RESULTS

Of the 15 patients studied, 6 had already received anti-*Crotalus* venom before their admission at the Tropical Diseases Clinic. Three of them presented severe clinical features

and had received 20, 100 and 150 mg of antivenom, respectively. Two of them developed mild envenoming and were treated with 100 and 200 mg of antivenom, respectively. The sixth patient presented moderate clinical features and was treated with 200 mg of antivenom. None of the 6 patients showed detectable seric venom levels as shown in **Figure 1**. Of the other 9 patients, who had not received antivenom previously, one developed mild envenoming, two presented moderate clinical features and 6 showed severe envenoming. The highest seric venom levels were observed in patients with severe clinical aspects as shown in **Figure 2**. Venom levels ranged from 2 to 108 ng/ml.

FIGURE 1. Seric venom antigen concentrations of 15 patients: 6 had previously received antivenom, 9 has not.

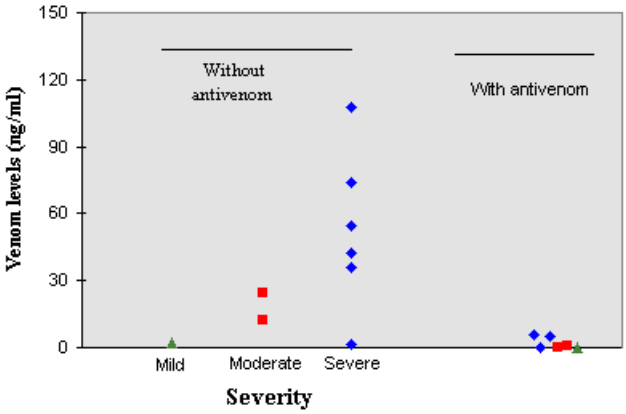


FIGURE 2. Time elapsed between the bite and specific treatment in 9 patients according to envenomation severity and venom levels.

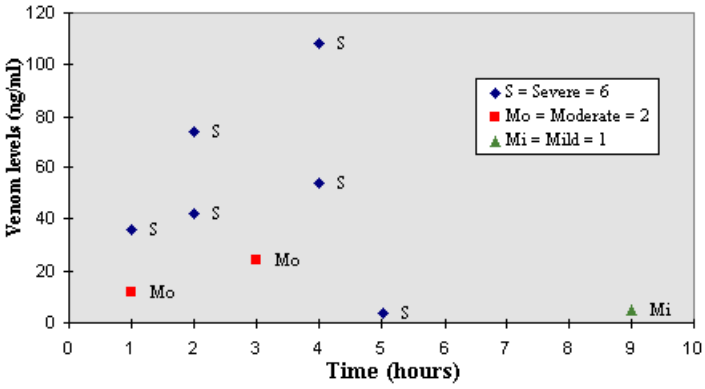
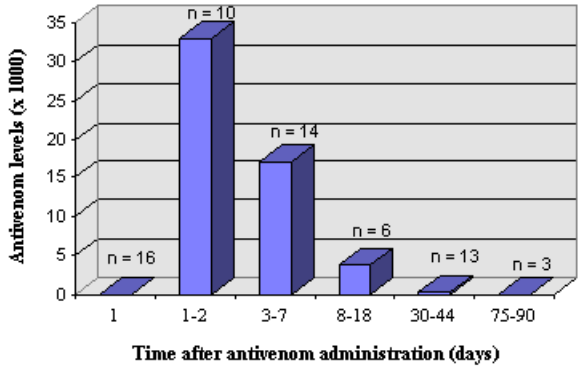


Figure 2 shows the relationship between seric venom levels observed in 9 patients who had not received antivenom previously and the time elapsed between the bite and medical attention. Of these 9 patients, 5 presented seric venom levels after specific treatment as follows: after 24 h two patients presented 4 and 12.8 ng/ml, respectively; after 48 h one patient presented 3.6 ng/ml; after 72 h one patient presented 3.8 ng/ml and after 96 h one patient presented 2.4 ng/ml.

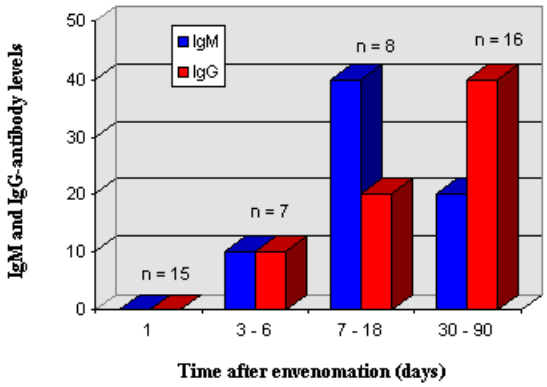
Figure 3 shows the distribution of horse antivenom seric levels studied in 16 patients regarding the time after collection. Forty-four days after specific antivenom treatment anti-*Crotalus* venom was observed.

FIGURE 3. Horse-antivenom levels in patients bitten by *Crotalus durissus terrificus* according to time after administration. Results are reported as medians.



IgM human antibodies against *Crotalus* venom were observed up to 18 days after specific treatment as shown in **Figure 4**. The highest levels were seen between 7 and 18 days after treatment.

FIGURE 4. IgM and IgG-human antibodies against *Crotalus durissus terrificus* venom according to time after envenomation. Results are reported as medians.



IgG human antibodies against *Crotalus* venom were observed up to 90 days, as shown in Figure 4. The highest levels were observed between 30 and 90 days after specific treatment.

None of the 13 patients who had their cerebrospinal fluid collected presented either venom or antivenom levels.

DISCUSSION

A successful treatment for snake-bitten patients is achieved when the offending species is correctly identified, which is only possible when the biting snake is brought for identification. If the patient does not know the snake species, the medical practitioner has to rely on clinical diagnosis. However, in many cases, clinical diagnosis does not help very much. In certain regions of Brasil, for instance, namely the Amazon region, there are different species which can cause the same clinical features of envenoming (**4**,**20**). Thus, venom detection and identification in body fluids or in bite site might be useful to confirm clinical diagnosis as well as to select the appropriate treatment.

In the present study, the authors used the ELISA technique described by Theakston *et al.*

(**25**) for specific and sensitive detection of both antigens and antibodies. High venom levels were observed only in those patients who had not received antivenom previously. Those patients who had already been submitted to serotherapy, even those who received lower doses than those usually recommended, did not show detectable circulating venom levels. The patients with severe clinical manifestations presented the highest circulating venom levels. In addition, 5 patients showed low circulating venom levels some days after specific treatment. These results are consistent with those of other authors (**10**,**24**). According to Theakston *et al*. (24), who studied patients bitten by *Bothrops* snakes, one possible explanation to the persistence of low levels of venom in patients up to 3 days after the antivenom administration may be the failure of Brazilian antivenoms to neutralize all antigens. On the other hand, the absence of circulating venom, even in those patients who had already received low doses of antivenom, suggests that the doses of antivenom administered in the treatment of crotalic envenoming may be higher than they should.

Venom absence in cerebrospinal fluid may be explained by at leat two hypotheses, as follows: 1) late collection of cerebrospinal fluid around 24 h after envenomation would allow enough time for the venom neutralization by the antivenom; 2) the venom is not capable of crossing the blood-brain barrier. The absence of antivenom in the cerebrospinal fluid corroborates this latter hypothesis.

Antivenom levels were detectable in blood circulation of bitten patients up to 44 days after specific treatment. These results agree with those by Theakston *et al.* (**24**) who detected the presence of bothropic antivenom up to 40 days after specific treatment. These findings as well as those of Theakston *et al.* (24) allow us to suggest that the doses administered in the treatment of crotalic envenoming may be too high. The high levels of horse IgG might contribute to an unacceptably high incidence of early anaphylatic and late (serum sickness) type reactions (24).

The evaluation of the humoral response by the determination of human IgM antibody levels showed that these are soon detected and disappear around 30 days . On the other hand, human IgG antibody levels were high between 30 and 90 days after envenomation. These results are in accordance with those by Domingos *et al.* (**14**) who studied patients bitten by *Bothrops jararaca*. These authors (14) reported that IgG antibodies were detected mainly between 50 and 70 days after treatment The high antibody levels probably play a protective role since Theakston (**22**) have observed that Nigerian victims of snakebite showed some resistance against subsequent snakebites. In addition, Theakston *et al.* (**26**) showed that sera from Ecuadorian indians bitten more than once by a venomous snake were able to neutralize a lethal dose of the same venom. On the other hand, Barraviera *et al.* (**8**) showed in an indirect manner the interaction between the venoms and the immune system in patients bitten by *Bothrops* and *Crotalus* snakes.

In conclusion, the evaluation of seric and cerebrospinal fluid levels of *Crotalus durissus terrificus* venom and antivenom enables us to suppose that neither the venom nor the antivenom is capable of crossing the blood-brain barrier. Conversely, the production of specific human IgM and IgG antibodies suggests that when the venom, even in the presence of the antivenom, is presented to the immune system cells, an immune response is prepared.

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