

**KINETICS OF VENOM AND ANTIVENOM SERUM LEVELS, CLINICAL
EVALUATION AND THERAPEUTIC EFFECTIVENESS IN DOGS INOCULATED
WITH *Crotalus durissus terrificus* VENOM**

**SANGIORGIO F. (1), SAKATE M. (1), NOGUEIRA R. M. B. (2), ARAÚJO JR J. P.
(3), CHAVEZ-OLORTEGUI C. (4)**

(1) Department of Small Animal Medical Clinic and Toxicology, School of Veterinary Medicine and Animal Husbandry, São Paulo State University, UNESP, Botucatu, São Paulo State, Brazil; (2) Department of Small Animal Medical Clinic, University of Western São Paulo, UNOESTE, Presidente Prudente, São Paulo State, Brazil; (3) Department of Microbiology and Immunology, Botucatu Institute of Biosciences, UNESP, Botucatu, São Paulo State, Brazil; (4) Department of Biochemistry and Immunology, Institute of Biological Sciences, Federal University of Minas Gerais-UFMG, Belo Horizonte, Minas Gerais State, Brazil.

ABSTRACT: This work evaluated the clinical and therapeutic aspects as well as serum levels of venom and antivenom IgG by enzyme-linked immunosorbent assay (ELISA) in experimental envenomation of dogs with *Crotalus durissus terrificus* venom. Twenty-eight mixed breed adult dogs were divided into four groups of seven animals each, Group I: only venom; Group II, venom + 50 ml of anti-bothropic-crotalic serum (50mg) + fluid therapy; Group III, venom + 50 ml of anti-bothropic-crotalic serum + fluid therapy + urine alkalination; Group IV, 50 ml of anti-bothropic-crotalic serum. The lyophilized venom of *Crotalus durissus terrificus* was reconstituted in saline solution and subcutaneously inoculated at the dose of 1mg/kg body weight. The dogs presented clinical signs of local pain, weakness, mandibular ptosis, mydriasis, emesis and salivation. The venom levels detected by ELISA ranged from 0 to 90ng/ml, according to the severity of the clinical signs. Serum antivenom ranged from 0 to 3ug/ml and was detected for up to 138h after treatment. ELISA results showed the effectiveness of the serum therapy for the venom neutralization.

KEY WORDS: *Crotalus durissus terrificus*, dogs, clinical signs, ELISA.

CONFLICTS OF INTEREST: There is no conflict.

CORRESPONDENCE TO:

FABÍOLA SANGIORGIO, Rua Henrique Dias, 580, apto 502, Londrina-PR, Brasil.
Phone: 65 32513513 or 65 84195727. Email: fsangiorgio@ig.com.br.

INTRODUCTION

There are, worldwide, about 3,000 snake species, from which 10% to 14% are considered venomous. The World Health Organization (WHO) estimates that 1,250,000 to 1,665,000 accidents with venomous snakes occur annually with 30,000 to 40,000 deaths (21).

Ophidic accidents are a serious problem for humans and animals, specially in tropical areas (19). Venomous snakes existent in Brazil belong to the genera *Bothrops*, *Crotalus*, *Lachesis* and *Micrurus*, which are responsible for more than 20,000 accidents annually notified to the Ministry of Health. Most accidents are caused by snakes of the genera *Bothrops* (90.5%), followed by *Crotalus* (7.7%), *Lachesis* (1.4%) and *Micrurus* (0.4%) (8).

Crotalic venom is considered highly toxic in comparison with that of the genus *Bothrops* (7) and has three main actions: neurotoxic (25, 26), myotoxic (3, 4, 18) and coagulant (2). Accidents caused by *Crotalus durissus* lead to death in 74% of untreated and 12% of treated patients (21).

The clinical features of the crotalic accident are characterized by local and systemic manifestations. The local alterations are discreet, which distinguishes this type of accident from the bothropic and lachetic ones. On the other hand, systemic manifestations are severe (17).

The treatment of choice to neutralize the ophidic venom action is the use of heterologous serum therapy. Also, supportive care such as fluid therapy and use of sodium bicarbonate is needed to alkalize the urine, since acid urine potentiates the intratubular myoglobin precipitation (5).

The selection of specific antivenom for the treatment of snakebite victims is based on the identification of the snake and on the clinical signs presented by the patient. However, in some cases, the clinical signs of envenomation are not sufficient to determine the genus responsible for the accident, which makes immunodiagnostic tests useful to determine the involved snake species (11). Laboratory confirmation of the accident can be made by using crotalic venom antigens, which may be detected in the blood using ELISA (21). This technique presents vantages over other tests because of its simplicity, rapidity, sensitivity and specificity for the detection of low concentrations of venom in the serum and urine (6). It is considered the ideal method

to detect and quantify the venom and antibodies in the serum of humans and animals (16).

In several studies involving ELISA assays, cross reaction among antigens of several snake species was observed, mainly due to the use of partially purified polyclonal antibodies. Based on that, there is a need to prepare species-specific antibodies to obtain reliable results. Selvanayagam & Gopalakrishnakone (22) and Chávez-Olorteghi (11) used high affinity chromatography to eliminate cross-reaction of equine hyperimmune serum immunoglobulins, obtaining species-specific IgG for the detection of bothropic or crotalic venom using ELISA.

The present study aimed at evaluating the clinical aspects of dogs experimentally envenomed with crotalic venom as well as at using ELISA to quantify serum levels of crotalic venom and antivenom.

MATERIALS AND METHODS

This experiment was approved by the Ethics Committee of São Paulo State University-UNESP, Botucatu, São Paulo State, Brazil.

Animals

Twenty-eight young adult dogs, male and female, were used. They had no defined breed, were clinically healthy (13) and weighed between 4 and 7kg.

Experimental Groups

Four experimental groups (Groups I, II, III and IV) were constituted of seven animals each. Group I was inoculated with *Crotalus durissus terrificus* venom; Group II received the venom and six hours after inoculation was treated with 50ml (50mg) of anti-bothropic-crotalic serum (Vencofarma®), intravenously, associated with fluid therapy with sodium chlorate (0.9% NaCl, dose 50ml/kg); Group III was inoculated with venom and six hours after inoculation was treated with 50ml (50mg) of anti-bothropic-crotalic serum, intravenously, and fluid therapy (0.9% NaCl, dose 50ml/kg) containing 8.4% sodium bicarbonate (dose 4mEq/kg); Group IV was inoculated with 50ml (50mg) of anti-bothropic-crotalic serum, intravenously.

Venom

Crotalus durissus terrificus venom was provided by the Center for the Study of Venoms and Venomous Animals, CEVAP-UNESP, Botucatu, São Paulo State, Brazil.

It was obtained by compressing the venom glands and was further lyophilized and stored at -20°C, being dissolved in sterile saline solution at the moment of administration to obtain the concentration of 40mg/ml. The dose of 1mg/kg was administered to the middle third of the lateral face of the thigh, after local trichotomy and antisepsis.

The ELISA technique used in this study was developed by Chávez-Olortegui *et al.* (11).

Venom Quantification by ELISA

ELISA was carried out in flat bottom microplates (Nunc-Maxisorp®) sensitized with 100µl of 0.8µg/ml anti-crotalic IgG diluted in 0.05M carbonate-bicarbonate buffer, pH 9.6, and incubated at 4°C for 12h. After three washes with phosphate-buffered saline (PBS), pH 7.2, containing 0.5% of Tween 20 (PBST), 200µl of blocking solution (PBS containing 10% of nonfat dry milk powder) were added to each well and the plate was incubated at 37°C. After one hour, the plate was washed and 100µl of dog serum were added to each well, diluted (v/v) in PBS containing 10% nonfat dry milk powder and 0.5% Tween 20 (PBSTL), in duplicate. The standard curve with crotalic venom in dilutions between 250 and 1.95ng/ml was also determined. After incubation at 37°C for one hour and new washes, 100µl of anti-*Crotalus durissus terrificus* serum marked with peroxidase conjugate in a 1:1000 dilution in PBSTL were added and the plate was again incubated at 37°C for one more hour. After washes, the reaction was revealed by adding 100µl of substrate and chromogen (100µl of 10mg/ml tetramethylbenzidine and 10µl of a 30% H₂O₂ solution in 10ml acetate-citrate buffer, 0.05mol/l, pH 5.0) remaining 15 minutes at room temperature, when it was interrupted by adding 100µl of 1M HCl per well. Reading was carried out in an ELISA automatic reader (Multiskan EX – Labsystems®) using a 450nm filter. In order to determine the quantity of venom, the standard curve linear tendency of each plate with correlation coefficient $R^2 > 0.97$ was used.

Antivenom (Horse IgG) Quantification by ELISA

ELISA was carried out in flat bottom microplates (Nunc-Maxisorp®) sensitized with 100µl of crotalic venom at 1µg/ml diluted in 0.05M carbonate-bicarbonate buffer, pH 9.6, and incubated at 4°C for 12h. After three washes with PBST, 200µl of blocking solution (phosphate-buffered saline containing 10% of nonfat dry milk powder) were added to each well and the plate was incubated at 37°C. After one hour, the plate was washed and 100µl of dog serum were added to each well in a 1:3 dilution in PBSTL, in duplicate. The standard curve with anti-*Crotalus durissus terrificus* IgG in dilutions ranging from 10 to 0.07µg/ml was also determined. After incubation at 37°C for one hour and new washes, 100µl of equine anti-IgG marked with peroxidase conjugate in a 1:1500 dilution in PBSTL were added and the plate was again incubated at 37°C for one more hour. After washes, the reaction was revealed by adding 100µl of substrate and chromogen (100µl of 10mg/ml tetramethylbenzidine and 10µl of a 30% H₂O₂ solution in 10ml of acetate-citrate buffer, 0.05mol/l, pH 5.0) remaining 15 minutes at room temperature, when it was interrupted by adding 100µl of HCl 1M per well. The reading was carried out in an ELISA automatic reader (Multiskan EX – Labsystems®) using a 450nm filter. In order to determine the quantity of anti-*Crotalus* IgG, the standard curve logarithmic tendency of each plate with correlation coefficient $R^2 > 0.97$ was used.

ELISA and clinical evaluations were performed at different moments.

Clinical Evaluation

M0: control moment; M1: 6h after venom inoculation; M2: 24h after venom inoculation and 18h after treatment; M3: 48h after venom inoculation and 42h after treatment; M4: 72h after venom inoculation and 66h after treatment; M5: 144h after venom inoculation and 138h after treatment (Table 1).

Venom Quantification by ELISA

Venom was quantified at six moments in Groups I, II and III; M0: control moment; M1: 2h after venom inoculation; M2: 6h after venom inoculation; M3: 8h after venom inoculation and 2h after treatment; M4: 24h after venom inoculation and 18h after treatment; M5: 48h after venom inoculation and 42h after treatment (Table 1).

Antivenom (horse IgG) Quantification by ELISA

Antivenom was quantified at six moments in Groups II, III and IV; M0: control moment; M1: 8h after venom inoculation and 2h after treatment; M2: 24h after venom inoculation and 18h after treatment; M3: 48h after venom inoculation and 42h after treatment; M4: 72h after venom inoculation and 66h after treatment; M5: 144h after venom inoculation and 138h after treatment (Table 1).

Statistical Analysis

The parametric variables were interpreted using analysis of variance of the differences among moments and the differences among groups at the same moment. The average values were compared using the Tukey test and the significance level of $p < 0.05$ was adopted.

RESULTS

Clinical Effects of *Crotalus durissus* Venom in Dogs

After crotalic venom inoculation, signs of discomfort were noticed in all animals. Discreet and unapparent edema was verified at the venom inoculation site. The envenomed dogs presented ataxia which progressed to paralysis and moderate sedation, ophthalmoplegia and mandibular ptosis. Sialorrhea (Figure 1) was noticed in some animals from 6 to 24h after crotalic envenomation. Other clinical signs such as bilateral mydriasis reactive to light and episodes of emesis and diarrhea occurred in animals from Groups I, II and III from 6 to 48h after venom inoculation (Figure 2).

In animals that did not receive treatment (Group I), the recovery of the clinical signs ataxia and sedation took about 24h longer than that in animals that were subjected to treatment. However, no deaths occurred.

In treated animals, there was no difference in the clinical recovery between those that were subjected to urine alkalination and those that received only serum therapy associated with fluid therapy without urine alkalination.

Venom Quantification by ELISA

Serum concentration of crotalic venom ranged between 5 and 90ng/ml. The maximum serum levels of venom were noticed 2h after envenomation in animals from Groups I, II and III. Venom concentrations were equal to those at the control

moment 8h after envenomation. Group I animals, which did not receive treatment, presented higher concentrations, between 8 and 48h after intoxication, in comparison with those from other groups (Table 2).

Antivenom (horse IgG) Quantification by ELISA

IgG serum concentrations in animals from Groups II, III and IV ranged between 0 and 3 μ /ml. The IgG levels reached maximum concentrations 2h after serum therapy, remaining high for 66h in the animals from all three treated groups, i.e. Groups II and III, which received crotalic venom and antivenom, and Group IV, which only received antivenom. In animals from Groups III and IV, IgG serum levels kept high up to 138h after treatment in comparison with the control moment. Group IV animals presented, at this moment, the highest IgG concentrations, compared with those of the other groups (Table 3).

Table 1. Moments of clinical evaluation, venom quantification and antivenom quantification.

	MOMENTS														
	C	AV	AT	AV	AT	AV	AT	AV	AT	AV	AT	AV	AT	AV	AT
	0	2h	-	6h	-	8h	2h	24h	18h	48h	42h	72h	66h	144h	138h
Clinical Evaluation	x			x				x	x	x	x	x	x	x	x
Direct ELISA	x	x		x		x	x	x	x	x	x				
Indirect ELISA	x					x	x	x	x	x	x	x	x	x	x

C: control; AV: after venom inoculation; AT: after treatment.

Table 2: Average values of venom (ng/ml), obtained by ELISA, in dogs at control and different moments after crotalic venom inoculation.

Groups	Moments					
	M0	M1	M2	M3	M4	M5
I	6.35±5.70 Ac	63.27±35.63 Aa	39.47±23.12 Aab	25.24±12.26 Abc	12.12±7.25 Abc	5.06±2.31 Ac
II	7.26±7.79 Abc	89.52±60.36 Aa	50.75±37.26 Aab	1.45±1.32 Bc	2.23±1.99 Bc	1.08±1.38 Bc
III	4.40±2.86 Ab	81.55±55.37 Aa	49.95±36.89 Aa	4.18±2.68 Bb	2.81±1.71 Bb	0.56±1.07 Bb

Capital letters compare moments in each group. Lowercase letters compare groups at every moment. Means followed by the same letters do not differ significantly ($p < 0.05$).

Table 3. Average values of antivenom IgG ($\mu\text{g/ml}$), obtained by ELISA, in dogs at control and different moments after venom inoculation.

Groups	Moments					
	M0	M1	M2	M3	M4	M5
II	0.09±0.04 Ac	2.19±0.67 Ba	2.04±0.69 Aa	1.57±0.61 Aab	1.41±0.58 Bab	0.91±0.33 Bbc
III	0.08±0.33 Ad	3.01±0.45 Aa	2.09±0.65 Ab	1.93±0.37 Ab	1.89±0.43 ABb	1.16±0.29 Bc
IV	0,08±0 Ad	2.56±0.42 ABa	2.34±0.26 Aab	2.05±0.31 Abc	2.08±0.31 Abc	1.82±0.29 Ac

Capital letters compare moments in each group. Lowercase letters compare groups at every moment. Means followed by the same letters do not differ significantly. ($p < 0.05$)



Figure 1. Animal from Group III presenting sialorrhea 6h after inoculation of crotalic venom.

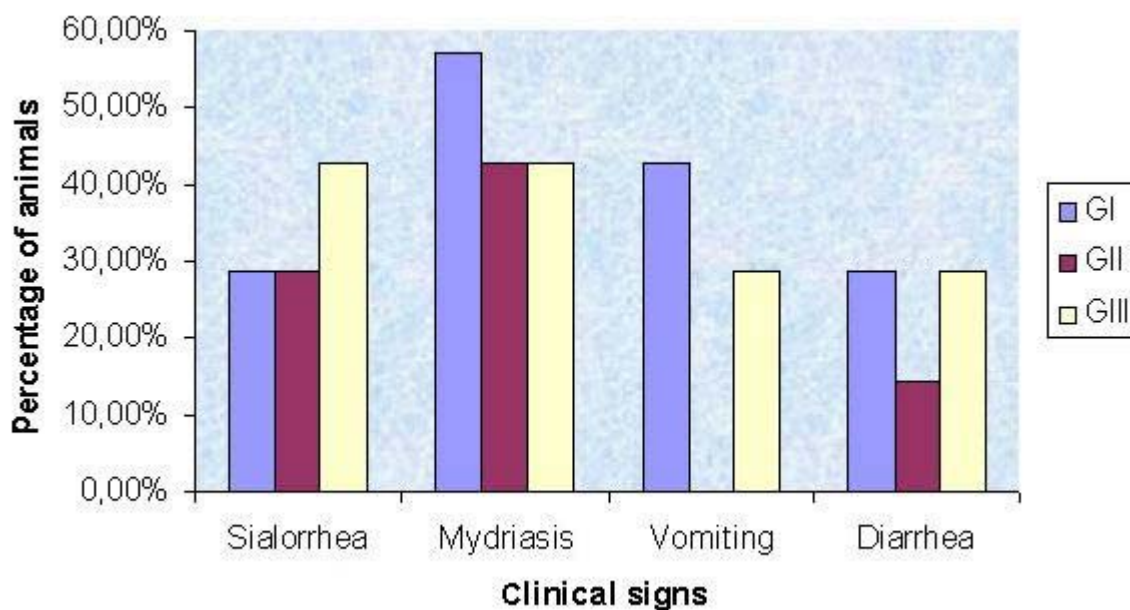


Figure 2. Percentage of animals that presented sialorrhea, mydriasis, vomiting and diarrhea at different moments after inoculation of crotalic venom.

DISCUSSION

ELISA has been widely used in clinical and experimental studies to quantify venom and antivenom in human and animal patients due to its high sensitivity and specificity and low cost (9, 10, 14, 16, 23, 24). The present study shows the clinical alterations caused by crotalic venom and their correlation with serum levels of venom and antivenom IgG, proving the effectiveness of specific serum therapy associated or not with urine alkalination.

The clinical effects noticed in this study were similar to those reported in previous ones (12, 15, 20). The results of direct ELISA were correlated with the clinical manifestations shown by the animals in each group. Between two and six hours after envenomation, crotalic venom levels were higher and the animals from all groups presented clear clinical signs. At moments when the quantity of venom was similar to that at the control moment, the animals no longer presented signs of crotalic envenomation.

Amaral *et al.* (1) quantified crotalic venom in 37 patients bitten by snakes of the genus *Crotalus* and noticed that the longer the time interval spent between the bite and the blood collection, the lesser the quantity of venom detected by ELISA. They also found that the venom serum levels became undetectable in most patients when the time spent from the accident to the blood collection was longer than eight hours. These remarks are similar to the results of our experiment, in which eight hours after envenomation, the values of venom were similar to those at the control moment, even for untreated animals.

The levels of antivenom IgG could be detected for up to 138h after serum therapy in the animals from Groups III and IV and for up to 66h after serum therapy in animals from Group II. These results partly agree with those reported by Amaral *et al.* (1), who collected blood samples of victims of crotalic accidents during 24h after serum therapy and detected high titers of antivenom IgG over this period.

In animals from Groups II and III, which were inoculated with crotalic venom and treated with specific serum therapy, there was an interaction between IgG and the venom antigens, which explains the lower levels of circulating IgG in these two groups 138h after treatment, relative to animals from Group IV, which were not envenomed and therefore did not presented antigen-antibody binding.

CONCLUSION

ELISAs are of great utility for the conclusive diagnosis of *Crotalus durissus terrificus* envenomation and for the kinetic assessment of the venom and antivenom in dog serum, also allowing the assessment of the effectiveness of different therapeutic protocols.

ACKNOWLEDGEMENTS

The authors are grateful to Master's Program at UNOESTE; Master's Program at School of Veterinary Medicine and Animal Husbandry, São Paulo State University - UNESP, Botucatu; and Center for the Study of Venoms and Venomous Animals, CEVAP-UNESP, Botucatu.

REFERENCES

- 1 AMARAL CFS., CAMPOLINA D., DIAS MB., BUENO CM., CHAVEZ-OLORTEGUI C., PENAFORTE CL., DINIZ CR., REZENDE NA. Time factor in the detection of circulating whole venom and crotoxin and efficacy of antivenom therapy in patients envenomed by *Crotalus durissus*. *Toxicon*, 1997, 35, 699-704.
- 2 AMARAL CFS., REZENDE NA., PEDROSA TMG., SILVA OA., PEDROSO ERP. Afibrinogenemia secundária a acidente ofídico crotálico (*Crotalus durissus terrificus*). *Rev. Inst. Med. Trop. São Paulo*, 1988, 30, 288-92.
- 3 AZEVEDO-MARQUES MM., CUPO P., COIMBRA TM., HERING SE., ROSSI MA., LAURE CJ. Myonecrosis, myoglobinuria and acute renal failure induced by South American rattlesnake (*Crotalus durissus terrificus*) envenomation in Brazil. *Toxicon*, 1985, 23, 631-6.
- 4 AZEVEDO-MARQUES MM., HERING SE., CUPO P. Evidence that *Crotalus durissus terrificus* (South American rattlesnake) envenomation in humans causes myolysis rather than hemolysis. *Toxicon*, 1987, 11, 1163-8.
- 5 BARRAVIERA B. Acidentes por serpentes dos gêneros "*Crotalus*" e "*Micrurus*". In: BARRAVIERA B. Coord. *Venenos Animais: uma visão integrada*. Rio de Janeiro: Publicações Científicas, 1994: 281-95.

- 6 BARRAVIERA B., SARTORI A., SILVA MFP., KANENO R., PERAÇOLI MTS. 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. *J. Venom. Anim. Toxins.*, 1996, 2, 14-27.
- 7 BELLUOMINI HE. Conhecimentos sobre as serpentes brasileiras e medidas de prevenção de acidentes. *Rev. Bras. Saúde Ocup.*, 1984, 12, 82-96.
- 8 BRASIL. Ministério da Saúde. Fundação Nacional de Saúde. *Manual de diagnósticos e tratamento de acidentes por animais peçonhentos*. 2.ed. Brasília: Ministério da Saúde, 2001. 131p.
- 9 CHÁVEZ-OLORTEGUI C., FONSECA SCG., CAMPOLINA D., AMARAL CFS., DINIZ CR. ELISA for the detection of toxic antigens in experimental and clinical envenoming by *Tityus serrulatus* scorpion venom. *Toxicon*, 1994, 32, 1649-56.
- 10 CHÁVEZ-OLORTEGUI C., LOPES CS., CORDEIRO FD., GRANIER C., DINIZ CR. An enzyme linked immunosorbent assay (ELISA) that discriminates between *Bothrops atrox* and *Lachesis muta muta* venoms. *Toxicon*, 1993, 31, 417-25.
- 11 CHÁVEZ-OLORTEGUI C., PENAFORTE CL., SILVA RR., FERREIRA AP., REZENDE NA., AMARAL CFS., DINIZ CR. An enzyme linked immunosorbent assay (ELISA) that discriminates between the venoms of Brazilian *Bothrops* species and *Crotalus durissus*. *Toxicon*, 1997, 35, 253-60.
- 12 COLLICCHIO RC., SAKATE M., BALARIN MRS., HATAKA A., KLEIN RP., VIANA L., NOGUEIRA FS. Relato de caso: Alterações clínicas e laboratoriais conseqüentes à picada de cascavel (*Crotalus durissus terrificus*) em uma cadela gestante. *Clín. Vet.*, 2002, 40, 45-8.
- 13 FEITOSA FLF. *Semiologia Veterinária*. São Paulo: Roca, 2004. 807p.
- 14 HO M., WARREL MJ., WARREL DA., BIDWELL D., VOLLER A. A critical reappraisal of the use of enzyme-linked immunosorbent assays in the study of snake bite. *Toxicon*, 1986, 24, 211-21.
- 15 HUDELSON S., HUDELSON P. Pathophysiology of snake envenomation and evaluation of treatments-Part II. *Comp. Contin. Educ. Pract. Vet.*, 1995, 17, 1035-40.
- 16 JÁCOME D., MELO MM., SANTOS MM., HENEINE LG. Kinetics of venom and antivenom serum and clinical parameters and treatment efficacy in *Bothrops alternatus* envenomed dogs. *Vet. Hum. Toxicol.*, 2002, 44, 334-72.

- 17 JORGE MT., RIBEIRO LA. Epidemiologia e quadro clínico do acidente por cascavel sul-americana (*Crotalus durissus*). *Rev. Inst. Med. Trop. São Paulo*, 1992, 34, 347-54.
- 18 MAGALHÃES RA., RIBEIRO MMF., RESENDE NA., AMARAL CF. Rabdomiólise secundária a acidente crotálico (*Crotalus durissus terrificus*). *Rev. Inst. Med. Trop. São Paulo*, 1986, 28, 228-33.
- 19 NETTO DP., CHIACCHIO SB., BICUDO PL., ALFIERI AA., NASCIMENTO N. Humoral response and neutralization capacity of sheep serum inoculated with natural and cobalt 60-irradiated *Crotalus durissus terrificus* venom (LAURENTI, 1768). *J. Venom. Anim. Toxins*, 2002, 8, 297-314.
- 20 NOGUEIRA RMB., SAKATE M. Clinical and hematological alterations in dogs during experimental envenomation with *Crotalus durissus terrificus* venom and treated with antiophidic serum. *J. Venom. Anim. Toxins incl. Trop. Dis.*, 2006, 12, 285-96.
- 21 PINHO FMO., PEREIRA ID. Ofidismo. *Rev. Assoc. Med. Bras.*, 2001, 47, 2-13.
- 22 SELVANAYAGAM ZE., GOPALAKRISHNAKONE P. Tests for detection of snake venoms, toxins and venom antibodies: review on recent trends (1987-1997). *Toxicon*, 1999, 37, 565-86.
- 23 THEAKSTON RDG., LLOYD-JONES MG., REID HA. Micro-ELISA for detecting and assaying venom and venom-antibody. *Lancet*, 1977, 2, 639-41.
- 24 THEAKSTON RDG., REID HA., LARRICK JW., KAPLAN J., YOST JA. Snake venom antibodies in Ecuadorian Indians. *J. Trop. Med. Hyg.*, 1981, 84, 199-202.
- 25 VITAL BRAZIL O. Neurotoxins from the South American rattlesnake venom. *J. Formos. Med. Assoc.*, 1972, 71, 394-400.
- 26 VITAL BRAZIL O. Venenos ofídicos neurotóxicos. *Rev. Assoc. Med. Bras.*, 1980, 26, 212-8.