Toxicity of *Bothrops neuwiedi* complex ("yarará chica") venom from different regions of Argentina (Serpentes, Viperidae)

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A B S T R A C T

We report a study of toxic and enzymatic activities of *Bothrops neuwiedi* complex venoms collected from specimens of different regions of Argentina and a pool of these same venoms. Were determined lethal, hemorrhagic and pro-coagulant (plasma and fibrinogen) doses and the neutralization of these activities by a bivalent antivenom. The electrophoretic pattern of different regions venom was studied by SDS–PAGE. All samples exhibited lethal potencies, hemorrhagic and coagulant (plasma and fibrinogen) activities with potencies concordant with previous studies. The only conspicuous difference in the toxicological pattern of *Bothrops diporus* venoms was the low-thrombin-like activity found in one sample. The antivenom used in this study could neutralize all the toxic activities tested and the neutralizing potency of the antivenom was comparable for all samples. Despite the wide distribution of *B. neuwiedi* complex throughout Argentina and the evident morphological variation between *B. diporus* (*B. neuwiedi* complex), this study establishes a remarkably similar toxicity profile throughout its range. This is the first systematic study on the regional variation of enzymatic and toxic activities of venom from species belonging to the *B. neuwiedi* complex, one of the snakes of highest sanitary importance in South America and their neutralization by the type of antivenom most commonly used in the South of South America.

1. Introduction

The *Bothrops neuwiedi* (Wagler, 1824) complex ("yarará chica", "cabeza candado", "yarará overa", "jararaca pintada", "yarará-î") is, at present, composed of seven species: *Bothrops diporus*, *Bothrops mattogrossensis*, *Bothrops pubescens*, *B. neuwiedi*, *Bothrops lutzi*, *Bothrops pauloensis* and *Bothrops marmoratus* (Silva, 2008). In Argentina, the systematics of the group is being reviewed and the species belonging to this complex are *Bothrops neuwiedii diporus*, *B. neuwiedii bolivianus* according to Lavilla et al. (2000), and *B. diporus*, *B. mattogrossensis* and *Bothrops paranaensis* according to Scrocchi (2009). Silva (2008) proposed that the species present in Argentina are *B. diporus*, *B. mattogrossensis* and *B. neuwiedi*. For the present work, we adopted the nomenclature put forth by Silva (2008) until the systematics of these pit vipers is clarified.

B. diporus (Cope, 1862; Silva, 2008) can be found in Paraguay and Brazil inhabiting the chaco, semitropical deciduous forests and the pampa (Silva, 2004). It is widely distributed in Argentina in the provinces of La Rioja, La Pampa, Córdoba, San Luis, Mendoza, Neuquén, Catamarca, Santiago del Estero, Tucumán, Jujuy, Salta, Formosa, Chaco, Santa Fé, Entre Ríos, Corrientes and Misiones (covering a territory of 1.595.687 km^2, approximately 60% of the

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country). This snake is small compared with other Argentinean *Bothrops* (with the exception of *Bothrops ammodytoides*) (de Roodt *et al.*, 1998b), with males having an average length of 60–70 cm and females measuring up to 110 cm (Ministerio de Salud, 2007). *B. mattogrossensis* (Amaral, 1925, 1930; Silva, 2008) is found in Peru, Bolivia, Paraguay, Argentina (Provinces of Jujuy and Formosa) and Brazil, inhabiting the savanna, pantanal and chaco biomes. This species measures around 70–80 cm although it may reach 130 cm in length (Silva, 2004). *B. neuwiedi* is distributed in Peru, Bolivia, Paraguay, Brazil and in Argentina is found principally in the province of Misiones. This snake inhabits tropical and semi-tropical deciduous forest, temperate forest and dry rocky areas (Silva, 2008). The most aggressive species of *Bothrops* in Argentina, and posses the greatest medical threat, being responsible for an important part of envenomations by snakebite in Argentina (de Roodt, 2002). The distribution of these snakes is shown in Fig. 1.

In view of the biological and medical importance of venom variation (Chippaux et al., 1991), we studied some biochemical and toxic characteristics of *B. neuwiedi* complex venoms from several provinces of Argentina as well as their neutralization by the antivenom of widest distribution in Argentina.

2. Materials and methods

2.1. Venoms

Venoms used in this study were from adult specimens of *B. neuwiedi* complex from five different regions of Argentina. Samples from the provinces of Entre Ríos, Catamarca and Formosa were obtained from animals located in the Serpentarium of the National Institute for the Production of Biologicals (INPB). Venom from Misiones was a gift of Dr. Alejandro U. Vogt from Zootoxicological Centre of Misiones (Oberá, Misiones). Those from province of Córdoba were a gift of B.Sc. Gustavo Reati from the Center of Applied Zoology of the National University of Córdoba. The number of snakes used to obtain the different samples of venom were: Entre Ríos, 4; Catamarca, 7; Misiones, 15; Formosa, 5 and Córdoba, 20. In each case similar amounts of venom from each individual snake were used to generate the regional sample of venoms. A Pool of samples was made by mixing equal amounts of each regional venom mix. In all cases, venoms were extracted by manual milking, immediately vacuum dried and stored at −20 °C until use. Venoms were redissolved in NaCl 0.15M prior to use.

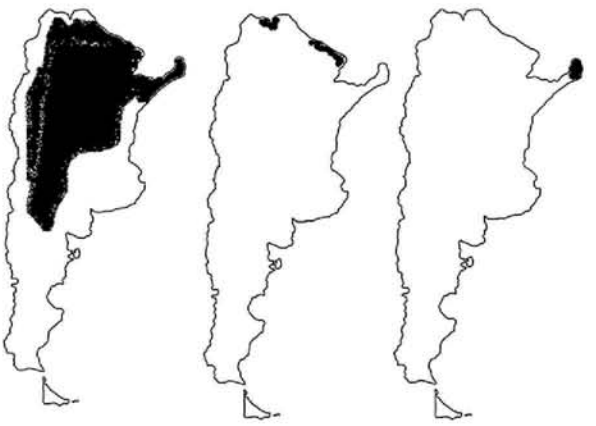

Fig. 1. Distribution of *Bothrops diporus* (left), *Bothrops mattogrossensis* (center) and *Bothrops neuwiedi* (right) in Argentina.

2.2. Experimental animals

Mice (CF-1, 18–22 g) and rats (Wistar, 200–250 g) were provided by the Animal Facility of the INPB. Animals were kept under controlled environmental conditions, with dark/light cycles of 12 h and received commercial rodent food and water *ad libitum*. All the experiments carried out with animals were in accordance with ethical standards of the INPB, in agreement with the Guide for Care and Use of Laboratory Animals published by the U.S. National Research Council (2002).

2.3. Antivenom

The Bivalent anti-bothropic antivenom produced by INPB (batch 264, expiration date May 2009) was used for the neutralization experiments. This antivenom is widely distributed in Argentina and produced using venom of *Bothrops alternatus* and *B. neuwiedi* as immunogens. It is composed of $F(ab')_2$ fragments of equine immunoglobulins.

2.4. Determination of lethal potency

Different amounts of the different venoms were injected by intraperitoneal (i.p.) route in CF-1 mice (5 per dose level), according to the conventional techniques (Theakston and Reid, 1983). The number of deaths was recorded 48 h after injection and the lethal potency was calculated as the Median Lethal Dose (LD_{50}) by non-linear regression (sigmoid dose–response curve) of the plot of mortality versus venom dose (Casasola et al., 2009) using the software package Prism 4.0 (GraphPad Inc., CA, USA). The LD_{50} was expressed in μg/mouse.

2.5. Determination of hemorrhagic activity

Hemorrhagic activity was determined as described by Theakston and Reid (1983). To determine Minimal Hemorrhagic Dose (MHD), different doses of each venom (6.25–200 μg) were injected intradermally in Wistar rats, using at least 3 points per dose level. After 24 h, the skin was excised and the major perpendicular diameters of the hemorrhagic haloes were measured on the dermal face. The MHD was defined as the amount of venom (μg) that produces a mean hemorrhagic halo with a diameter of 1 cm, and it was determined by plotting the values of the average diameter of the hemorrhagic haloes versus the dose, analyzing the dose–response curve by non-linear regression (sigmoidal dose–response curve with variable slope). The values were obtained from each experiment and the MHDs were expressed as the mean and standard deviation. When necessary, for statistical comparison

individual values of each curve analyzed to obtain the MHD and its standard deviation, were analyzed by *t* test.

All the experiments were at least in triplicate.

2.6. Determination of pro-coagulant activity

Pro-coagulation was studied in normal human plasma and in bovine fibrinogen (2 g% in NaCl 0.15M). The venom dose that produced an evident clot in 60 s (at 37 °C) was considered as the minimum coagulant dose in plasma (MCD-P) or in fibrinogen (MCD-F). The doses were expressed in mg/ml (Laing et al., 1992). It was determined by plotting the values of clotting time versus the dose, analyzing the dose–response curve by non-linear regression (exponential decay). The doses corresponding to a clotting time at 60 s were determined. These values were used to obtain the mean values and the standard deviation. For statistical comparison by *t* test, the individual values of each curve analyzed to obtain the coagulant doses and its standard deviation, were considered.

All the experiments were at least in triplicate.

2.7. SDS–PAGE

Separation of 10 μg of each venom sample by SDS–PAGE (12.5%, acrylamide/bis-acrylamide) was done under non-reducing conditions (Laemmli, 1970) along with broad range molecular weight markers (prestained, Bio Rad, control 310002875). After fixation gels were stained with Coomasie brilliant blue, scanned and analyzed with the software Gel-Pro Analyzer (Media Cybernetics, MA, USA).

2.8. Neutralization of toxic activities

2.8.1. Neutralization of lethal potency

Different antivenom doses were incubated with 4 LD_{50} of each venom for 30 min at 37 °C. After incubation, the samples were injected intraperitoneally in mice (5 mice per dose) in a final volume of 0.5 ml in NaCl 0.15M. Deaths were recorded 48 h after injection and the median effective dose (ED_{50}) was calculated as the antivenom dose giving a statistical survival of 50% by non-linear regression (Casasola et al., 2009). Positive controls were injected with 4 LD_{50} in 0.5 ml of NaCl 0.15M. The challenge doses and the time of inspection were those indicated to test the potency of antivenoms of Argentina. Antivenom potency was defined using the formula: $[(n-1)/ED_{50}] \times LD_{50}$, where $n-1$ represents the number of lethal doses of the challenge minus one (theoretically the dose of venom that killed half the mice) (Ministerio de Saúde, 1996). As the ED_{50} is expressed in μl and the LD_{50} in μg, the final result is μg/μl adjusted to mg/ml. This value of potency indicates the milligrams of venom neutralized by 1 ml of antivenom. In addition the potency was expressed as LD_{50}s neutralized by 1 ml of venom.

2.8.2. Neutralization of hemorrhagic activity

Wistar rats were injected intradermally in the back with a mixture containing 2 MHD of the different venoms diluted in NaCl 0.15M incubated with different doses of antivenom for 30 min at 37 °C in a final volume of 100 μl. After 24 h, rats were sacrificed using ether and hemorrhagic haloes were measured. The neutralizing capacity (HED_{50}) was described as the dose of antivenom that diminished the hemorrhagic areas by 50% when compared with the positive control. The results were analyzed by non-linear regression using the software package Prism 4 (GraphPad Inc., CA, USA).

2.8.3. Neutralization of coagulant activity

Tubes containing 0.4 ml of human plasma were treated with 8 MCD-P of the different venoms, previously incubated for 30 min at 37 °C with different doses of the antivenom (1–100 μl) in a final volume of 150 μl in NaCl 0.15M. After addition of the venom–antivenom mix on plasma, coagulation time was measured. As positive controls, we used 0.4 ml of plasma treated with the same venom doses diluted in 150 μl of NaCl 0.15M. The neutralizing capacity of the antivenom was expressed as the minimal dose of antivenom that delayed coagulation time more than 5 min when compared with the positive control.

2.8.4. Neutralization of thrombin-like activity

Tubes containing 0.4 ml of bovine fibrinogen 2.0 g% in NaCl 0.15M were treated with 1.0 MCD-F of the different venoms, previously incubated for 60 min at 37 °C with different doses of the antivenom (1–100 μl) in a final volume of 150 μl in NaCl 0.15M. After the addition of the venom–antivenom mix, coagulation time was measured. As positive controls, we used 0.4 ml of fibrinogen treated with the same venom doses in 150 μl of NaCl 0.15M. The neutralizing capacity of the antivenom was expressed as the dose of antivenom that delayed coagulation time more than 5 min when compared with the positive controls.

2.9. Statistics

All data are expressed as mean ± SD (standard deviation) or with the 95% confidence interval (c.i.) in brackets. Linear, non-linear regression analyses and Student's *t* test were done with the software package Prism 4.0 (GraphPad Inc., CA, USA).

3. Results

3.1. Lethal potency

Lethal potencies (LD_{50}) are shown in Table 1. There were no significant differences in the LD_{50} values ($p > 0.05$), which ranged from 51.8 to 82.6 μg/mouse.

3.2. Hemorrhagic activity

Hemorrhagic activities (MHD) are shown in Table 1. All venoms showed no differences in their hemorrhagic potency values expressed as MHD ($p > 0.05$), ranging from 288.2 to 462.6 μg.

3.3. Pro-coagulant activity

Values of MCD-P are presented in Table 1. Venom from Entre Ríos, Misiones and Catamarca showed no statistical

Table 1
Toxic activities of *Bothrops neuwiedi* of different regions of Argentina.

Venom	Lethal potency		Hemorrhagic potency		Coagulant potency			
					Plasma		Fibrinogen	
	LD_{50} (μg/mouse)	LD_{50}/mg	MHD (μg)	MHD/mg	MCD-P (μg/ml)	MCD-P/mg	MCD-F (μg/ml)	MCD-F/mg
Formosa	82.6 (61.3–111.1)	12.1 (9.0–16.3)	288.2 ± 111.4	3.5 ± 1.3	20.0 ± 1.0	50.0 ± 2.5	1047 ± 28.0*	1.0 ± 0.0*
Entre Ríos	73.8 (49.8–109.5)	13.5 (9.13–20.1)	367.7 ± 85.9	2.7 ± 0.6	13.8 ± 1.0*	72.5 ± 5.3*	214.8 ± 20.5*	4.7 ± 0.4*
Catamarca	55.4 (37.5–81.8)	18.0 (12.2–26.6)	389.2 ± 58.6	2.6 ± 0.4	16.3 ± 0.5*	61.3 ± 1.9*	644.3 ± 41*	1.6 ± 0.1*
Córdoba	76.8 (51–115.6)	13.0 (8.6–19.6)	367.7 ± 43.1	2.7 ± 0.3	35.0 ± 5.0	28.6 ± 4.1*	590.5 ± 13.4*	1.7 ± 0.0*
Misiones	51.8 (43.0–62.5)	19.3 (16–23.3)	462.6 ± 206.8	2.2 ± 1.0	16.5 ± 0.8*	60.6 ± 2.9*	322.3 ± 0.0*	3.1 ± 0.0*
Pool	61.84 (59.8–63.8)	16.2 (15.6–16.7)	313.9 ± 50.0	3.2 ± 0.5	23.0 ± 1.3	43.5 ± 2.5	537 ± 13.4	1.9 ± 0.1

LD_{50} = median lethal dose in μg/mouse. The 95% confidence interval is in parentheses. It was obtained by the study of dose–response curve of mice (CF-1 mice; 5 per dose level), injected intraperitoneal (i.p.) with different amounts of venom; MHD = minimal hemorrhagic dose in μg ± standard deviation (SD). I was obtained by measuring the hemorrhage produced by intradermal (i.d.) injection of different amounts of venom in Wistar rats. MCD-P = minimal coagulant dose on plasma in mg/ml ± SD; MCD-F = minimal coagulant dose on fibrinogen in mg/ml ± SD. (*) = values with statistically significant differences compared to the values obtained with the pool.

differences ($p > 0.05$) and the highest MCD-P activity regarding the other samples ($p < 0.05$). Venoms from Formosa and the Pool showed no significant differences among their MCD-Ps (p 0.17; t 1.672). Venom from Misiones showed no significant differences from Catamarca ($p > 0.18$; t 1.482). The highest MCD-P (the lowest potency) was that of the venom from Córdoba ($p < 0.05$; $t > 4$). Values of MCD-P of samples from Entre Ríos, Catamarca and Misiones showed statistical differences regarding Pool value ($p < 0.05$).

Values of MCD-F are presented in Table 1. Thrombin-like activity was observed in all venoms and the potencies showed significant differences in almost all cases ($p < 0.05$). The most potent activity was observed in the venom from Entre Ríos, followed by Misiones (p 0.001, t 8.693), and followed by Catamarca and Córdoba venoms with no differences between MCD-F ($p > 0.05$; t 2.514), followed by the Pool (p 0.009; t 4.670). Venom from Formosa showed the lowest potency ($p < 0.05$; $t > 14$). Values of MCD-F in all the cases showed statistical differences regarding Pool value ($p < 0.05$).

3.4. SDS–PAGE

Under non-reducing conditions (Fig. 2), the most strongly stained material was between 40 and 56 kDa, 22 and 30 kDa, and between 13 and 22 kDa, although some bands were absent or weakly stained in some of the samples. Venoms from Formosa, Catamarca and the Pool showed three strongly stained bands between 40 and 54 kDa, but in the case of Córdoba and Misiones, those bands were very light. Venom sample from Entre Ríos only showed two weakly stained bands in the range of 49–55 kDa. Venoms from Formosa, Catamarca, Córdoba and the Pool contained similar bands between 22 and 25 kDa, although these were more strongly stained in the sample from Formosa. Venom from Misiones exhibited the same bands and a heavier one at 32 kDa. In this range, the sample from Entre Ríos only showed one band at 22 kDa. Venoms from Formosa, Entre Ríos, Misiones and the Pool contained bands between 13 and 22 kDa that were absent in Catamarca and Córdoba samples.

3.5. Neutralization of toxic activities

Values of neutralization of different toxic activities are presented in Table 2. Venom from Misiones was that required the lower doses of antivenom to be neutralized ($p < 0.05$). The neutralization on the pool, considering the ED_{50}s, required lower dose of antivenom regarding the venoms from Córdoba, Entre Rios and Formosa ($p < 0.05$) and higher regarding the venom from Misiones ($p < 0.05$). When the venom milligrams neutralized by ml of antivenom was considered, venom from Entre Ríos did not showed statistical differences regarding the Pool ($p > 0.05$).

There were no observed differences in the neutralization of the hemorrhagic activity (Table 2).

Coagulation was neutralized at the lowest doses in plasma or fibrinogen in all samples.

4. Discussion

The electrophoretic patterns of the regional samples showed similarities with those previously described, with the major groups of material in the ranges over 40 kDa,

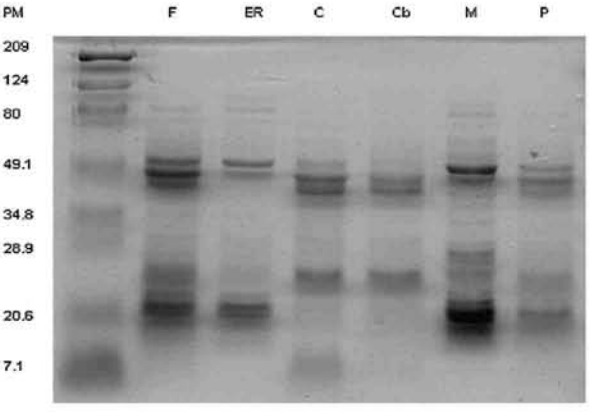

Fig. 2. SDS–PAGE at 12.5% A/B (acrylamide/bis-acrylamide) in non-reducing conditions of *Bothrops neuwiedi* from different regions of Argentina. Left-most lane, molecular weight markers in kDa. F: Formosa; ER: Entre Ríos; C: Catamarca; Cb: Córdoba; M: Misiones and P: Pool. The major components were between 30 and 50 kDa in all samples.

Table 2
Neutralization of toxic potencies of venoms of *Bothrops neuwiedi* from different regions of Argentina.

Venom	Neutralization of the lethal potency			Neutralization of hemorrhagic activity		Neutralization of coagulation	
	ED_{50} (μl)	Potency (mg/ml)	LD_{50}/ml	HED_{50} (μl)	HED/ml	Plasma (μl)	Fibrinogen (μl)
Córdoba	32.1* (29.2–35.2)	7.1* (6.5–7.9)	124.7* (113.5–136.8)	45.8 (25.3–83.2)	43.7 (24.2–79.4)	25	100
Misiones	12.3* (11.6–13.1)	12.6* (11.8–13.4)	324.9* (305.0–346.0)	43.0 (33.3–55.6)	46.5 (36.0–60.0)	12.5	100
Formosa	21.0* (20.0–22.0)	11.8* (11.2–12.4)	190.7* (181.6–200.3)	21.7 (17.2–27.4)	92.3 (73.0–116.5)	12.5	12.5
Catamarca	29.0 (14.1–59.7)	5.7 (2.8–11.7)	137.7 (66.92–281.6)	34.9 (28.3–43.0)	57.4 (46.5–70.7)	12.5	50
Entre Ríos	32.8* (22.8–47.3)	6.7 (4.7–9.7)	127.7* (88.8–183.9)	20.9 (17.6–24.9)	95.5 (80.1–113.7)	12.5	50
Pool	19.2 (19.2–19.3)	9.64 (9.63–9.65)	207.9 (207.6–208.1)	18.18 (9.6–34.5)	110.0 (58.0–208.6)	12.5	100

ED_{50}: median effective dose in μl (95% c.i.) potency: milligrams of venom neutralized per millilitre of antivenom (95% c.i.). LD_{50}/ml: median lethal doses neutralized per ml of antivenom (95% c.i.). HED_{50}: hemorrhagic median effective dose (95% c.i.). MHD/ml: minimal hemorrhagic doses neutralized per ml of antivenom. (*) = values with statistically significant differences compared to the values of neutralization on the pool.

between 20 and 40 kDa and under 20 kDa (de Roodt, 2002; de Roodt et al., 1998a) (Fig. 2).

The toxic activities were similar to those described for the bothropic venoms from different regions from Brazil and Argentina (Sanchez et al., 1992; Ferreira et al., 1992; de Roodt, 2002; Queiroz et al., 2008). All samples exhibited hemorrhagic, and coagulant (plasma and fibrinogen) activities with potencies concordant with previous studies (Ferreira et al., 1992; Sanchez et al., 1992; de Roodt et al., 1998a; de Roodt, 2002; Araujo et al., 2008; Lanari et al., 2010).

The lethal potencies showed no significant differences among the samples of the different provinces ($p > 0.05$) (Table 1), ranging from 12.1 LD_{50}/mg (Formosa) to 19.3 LD_{50}/mg of venom (Misiones).

These venoms exhibited low hemorrhagic activity by comparison to other species of *Bothrops*, in accordance with previous studies in mice and rats (de Roodt et al., 2000, 2003).

The most potent coagulant activities on plasma were those from Entre Ríos, Catamarca and Misiones ($p < 0.05$) while venoms from Córdoba and Pool, were the samples with lower coagulant activity ($p < 0.05$). See Table 1.

All samples had thrombin-like activity; however, the venom sample from Formosa had very low potency (1047 μg/ml, less than a coagulant dose by mg of venom) ($p < 0.05$). Venoms from Entre Ríos (214.8 μg/ml, over four coagulant doses by mg of venom) and Misiones (322.3 μg/ml, over three coagulant doses per mg of venom) had the highest thrombin-like activity among all the samples ($p < 0.05$) (Table 1).

The systematic of this group of pit vipers is under revision (Silva, 2004, 2008). Despite the wide distribution of *B. neuwiedi* complex throughout Argentina and the evident morphological variation, this study establishes a remarkably similar toxicological profile throughout its range. In the case of the monophyletic species *B. alternatus*, geographic variation is much greater (Lanari et al., 2010). The only conspicuous difference in the toxicological pattern of *B. diporus* venoms was the low-thrombin-like activity found in the Formosa sample.

The antivenom used in this study could neutralize all the toxic activities tested (Table 2). Nevertheless, differences regarding the neutralizing potencies in several cases were observed, despite the very close lethal and hemorrhagic potencies of the venoms tested. In the case of the neutralizing potency of the lethal activity, over the half of the sample of the provinces showed differences regarding the doses required for neutralizing the pool.

When the ED_{50}s were considered, the venom most easily neutralized, was that from Misiones ($p < 0.05$). Venoms from Córdoba, Formosa and Entre Ríos were more difficult to neutralize regarding the venom of Misiones or the Pool (Table 2). The only venom that did not show significant differences regarding the Pool was the venom from Catamarca ($p > 0.05$). When the LD_{50}/ml was considered, again, venoms from Córdoba, Formosa and Entre Ríos were more difficult to neutralize regarding the Pool ($p < 0.05$) and the venom from Misiones easier ($p < 0.05$) (Table 2).

However, it is important to note that statistical differences regarding neutralization values may be different according to how neutralizing potency is expressed. We can observe that the neutralization of the venom from Entre Ríos did not show differences when expressed in mg of venom neutralized by ml of antivenom regarding the values of the pool, but showed differences ($p < 0.05$) when the ED_{50} or LD_{50}/ml are considered (Table 2).

If the volume of antivenom necessary to neutralize 1 mg of venom is considered, it can be seen that the venoms from Misiones and Formosa were neutralized at doses of 79 μl and 85 μl per mg of venom respectively and 1 mg of the venom from Córdoba was neutralized by 141 μl of antivenom. If neutralization of the Pool (104 μl of antivenom/mg of venom) is taken as reference, neutralization of the venom from Córdoba would be underestimated since to neutralize 1 mg of this venom would require almost 35% more antivenom. In the other hand, to neutralize the venoms from Formosa or Misiones, regarding the Pool it would be required a minor volume of the antivenom, around of 20%. These observations suggest that neutralizing capacity of antivenoms determined using of Pool of venoms must be carefully interpreted when it comes to neutralization of individual samples of venom.

In general, no significant differences ($p > 0.05$) were observed in the neutralization of hemorrhagic activity and the hemorrhagic activity of the Pool was neutralized as easily as that of the individual venom samples (Table 2).

Neutralization of coagulant activities in the venom sample from Córdoba required twice the dose of antivenom necessary to neutralize the coagulant activity on plasma. This datum is interesting considering that this venom was the one with lowest MCD-P. It is possible that, in this case, the neutralizing antibodies against the enzymes responsible for coagulation were qualitatively or quantitatively deficient when comparing the neutralization of these

factors in the other venoms. The thrombin-like activity was readily neutralized at low doses in the venom from Formosa, consistent with the fact that this venom was the one with the lowest MCD-F.

The antivenom used in this study efficiently neutralized all toxic activities tested. This is important since this viper is one of the most medically important snakes throughout Southern South America (WHO, 2010) and the antivenom tested is the most widely used in Argentina (Ministerio de Salud, 2007). Antivenom for treatment of bothropic envenomation in Southern South America is produced with *B. diporus* and *B. alternatus* venoms (Pino Cheroni, 1994; Carreira et al., 2006; Ministerio de Salud, 2007). Usually, venom pools generated from specimens from different regions of Argentina are used, although this is not always possible. Usually the Pool of venoms of *B. diporus* is produced with venom from animals of only two or three provinces (generally from the North and North East of the country) and some isolated animals from other regions (de Roodt, unpublished data). Cross-neutralization by the use of *B. alternatus* venom as immunogen could interfere with the interpretation of the neutralization assays since paraspecific neutralization by specific *B. alternatus* antivenom on *B. neuwiedi* venom has been reported (Dias Da Silva et al., 1989). Nevertheless, this combination of venoms is used in countries in the region (Dias Da Silva et al., 1989; Pino Cheroni, 1994; de Roodt et al., 1998a; Carreira et al., 2006) and this type of antivenom is the most used in the case of bites by *B. neuwiedi* complex in Southern South America.

This is the first systematic study on the regional variation of toxic activities of venom of species belonging to the *B. neuwiedi* complex, one of the snakes of highest sanitary importance in South America (WHO, 2010) and their neutralization by the type of antivenom most commonly used in the South of South America.

Conflict of interest

The authors have no conflicts of interest to declare.

References

Amaral, A.D., 1925. A general considerations of snake poisoning and observations on neotropical pit vipers. Contributions from Harvard Institute of Biology and Medicine 2, 1–64.

Amaral, A.D., 1930. A new race of *Bothrops neuwiedi*. Studies of tropical Ophidia XXV. Bulletin of the Antivenin Institute of America 4, 65–67.

Araujo, H.P., Bourguignon, S.C., Boller, M.A., Dias, A.A., Lucas, E.P., Santos, I.C., Delgado, I.F., 2008. Potency evaluation of antivenoms in Brazil: the national control laboratory experience between 2000 and 2006. Toxicon 51, 502–514.

Carreira, S., Negrin, A., Tortorella, M.N., Pino, A., Menéndez, C., 2006. Ofidismo en Uruguay. CID-CEUR, Montevideo, 79 pp.

Casasola, A., Ramos-Cerrillo, B., de Roodt, A.R., Carbajal Saucedo, A., Chippaux, J.P., Alagón, A., Stock, R.P., 2009. Paraspecific neutralization of the venom of African species of cobra by an equine antiserum against *Naja melanoleuca*: a comparative study. Toxicon 53, 602–608.

Chippaux, J.-P., Williams, V., White, J., 1991. Snake venom variability: methods of study, results and interpretation. Toxicon 29, 1279–1303.

Cope, E.D., 1862. Catalogues of the reptiles obtained during the explorations of the Parana Paraguay, Vermejo and Uruguay rivers by Capt. Thos. J. Page, U.S.N.; and of those procured by Lieut. N. Michier, U.S. Top Eng., Commander of the Expedition conducting the survey of the Atrato River. I. The Paraguay collection. Proceedings of the Academy of Natural Sciences of Philadelphia, 346–359.

de Roodt, A.R., 2002. Estudio inmunobiológico del veneno de serpientes de importancia sanitaria de Argentina. Tesis Doctoral de la Facultad de Farmacia y Bioquímica de la Universidad de Buenos Aires, 313 pp.

de Roodt, A.R., Dolab, J.A., Dokmetjian, J.Ch., Litwin, S., Segre, L., Vidal, J.C., 2000. A comparison of different methods to assay the hemorrhagic activity of *Bothrops* venom. Toxicon 38, 49–62.

de Roodt, A.R., Litwin, S., Vidal, J.C., 2003. Hemorrhagic activity of *Bothrops* venoms determined by two different methods and relationship with proteolytic activity on gelatin and lethality. Toxicon 41, 949–958.

de Roodt, A.R., Dolab, J.A., Fernández, T., Segre, L., Hajos, S.E., 1998a. Cross reactivity and heterologous neutralization of crotaline antivenoms used in Argentina. Toxicon 36, 1025–1038.

de Roodt, A.R., Dolab, J.A., Galarce, P.P., Litwin, S., Gould, E., Dokmetjian, J. C., Segre, L., Vidal, J.C., 1998b. A study on the venom yield of venomous snake species from Argentina. Toxicon 36, 1949–1958.

Dias Da Silva, W., Guidolin, R., Raw, I., Higashi, H.G., Caricatti, C.P., Morais, J.F., Lima, M.L., Yamaguchi, I.K., Nishikawa, A.K., Stephano, M. N., Marcelino, J.R., Pinto, J.R., Santos, M.J., 1989. Cross-reactivity of horse monovalent antivenoms to venoms of ten *Bothrops* species. Memórias do Instituto Butantan 51, 153–168.

Ferreira, M.L., Moura-da-Silva, A.M., França, F.O., Cardoso, J.L., Mota, I., 1992. Toxic activities of venoms from nine *Bothrops* species and their correlation with lethality and necrosis. Toxicon 30, 1603–1608.

Laemmli, U.K., 1970. Cleavage of structural during the assembly of the head bacteriophage T4. Nature 227, 680–685.

Laing, G.D., Theakston, R.D.G., Leite, R.P., Dias Da Silva, W., Warrell, D.A., BIASG, 1992. Comparison of the potency of three Brazilian *Bothrops* antivenoms using in vivo rodent and in vitro assays. Toxicon 30, 1219–1225.

Lanari, L.C., Rosset, S., González, M.E., Liria, N.C., de Roodt, A.R., 2010. A study on the venom of *Bothrops alternatus* Duméril, (Bibron and Duméril) from different regions of Argentina. Toxicon 55, 1415–1424.

Lavilla, E.O., Richard, E., Scrocchi, G.J., 2000. Categorización de los anfibios y reptiles de la República Argentina. Asociación Herpetológica Argentina, 97 pp.

Ministerio de Salud, 2007. Guía de prevención, diagnóstico, tratamiento y vigilancia epidemiológica de los envenenamientos ofídicos. Ministerio de Salud, Buenos Aires, 48 pp.

Ministerio de Saúde, 1996. Normas Técnicas de Fabricaçao e Controle de Qualidade dos Soros Antiofídicos, Antitóxicos e Antirrábico aprovada pela vigilancia sanitaria. Secretaría de Vigilancia Sanitaria Ministerio de Saúde.

National Research Council, 2002. Guía para el cuidado y uso de los animales de laboratorio. Institute of Laboratory Animal Resources, Commission of Life Sciences. Academia Nacional de Medicina, México DF.

Pino Cheroni, A., 1994. Producción de suero antiofídico en Uruguay. Revista Médica del Uruguay 10, 147–154.

Queiroz, G.P., Pessoa, L.A., Portaro, F.C., Furtado, M.D.E.F., Tambourgi, D.V., 2008. Interspecific variation in venom composition and toxicity of Brazilian snakes from *Bothrops* genus. Toxicon 52, 842–851.

Sanchez, E.F., Freitas, T.V., Ferreira-Alves, D.L., Velarde, D.T., Diniz, M.R., Cordeiro, M.N., Agostini-Cotta, G., Diniz, C.R., 1992. Biological activities of venoms from South American snakes. Toxicon 30, 95–103.

Scrocchi, G., 2009. Venomous snakes fromArgentina. In: Montero, R., Autino, A. (Eds.), Sistemática y Filogenia de los Vertebrados con énfasis en La fauna Argentina. Segunda Edición, Tucumán, Argentina, 414 pp.

Silva, V.X., 2008. Taxonomic revision of the *Bothrops neuwiedi* complex (Serpentes, Viperidae) with description of a new species. Phyllomedusa 7, 45–90.

Silva, V.X., 2004. The *Bothrops neuwiedi* complex. In: Campbell, J.A., Lamar, W.W. (Eds.), The Venomous Reptiles of the Western Hemisphere, vol. 2. Cornell University Press, Ithaca, New York, pp. 410–422.

Theakston, R.D.G., Reid, H.A., 1983. Development of simple standard assay procedures for the characterization of snake venoms. Bulletin of the World Health Organization 61, 949–956.

Wagler, J., 1824. Serpentum brasiliensium species novae, ou histoire naturelle des espèces nouvelles de serpens. In: Jean de Spix, Animalia nova sive species novae (Natrix bahiensis 27), Monaco, Typis Franc. Seraph. Hübschmanni, VII 75 pp.

World Health Organization, 2010. WHO Guidelines for the Production, Control and Regulation of Snake Antivenom Immunoglobulins. WHO, Geneva, 134 pp.