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A study on the venom of *Bothrops alternatus* Duméril, Bibron and Duméril, from different regions of Argentina

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ABSTRACT

We report a comparison of toxic and enzymatic activities of *Bothrops alternatus* venoms collected from specimens of different regions of Argentina with a pool of these same venoms. We determined lethal, hemorrhagic and pro-coagulant (plasma and fibrinogen) doses, as well as indirect hemolysis, hydrolysis of gelatin and fibrinogen. The electrophoretic pattern of the venoms and that of fibrinogen incubated with each of the venoms was studied by SDS-PAGE. The toxic activities were consistent with those reported for these venoms but with noticeable differences between samples which do not seem related to geographical or climatic characteristics. In some cases the pooled venom showed values close to the individual samples, but it was not representative since over half of the venoms (5/9) exhibited very different patterns of toxicity. The potency of some activities in the individual venoms ranged from much higher to much lower than those determined in the pooled venom (0.1 to 3 fold). Not all venoms exhibited thrombin-like activity but all hydrolyzed α and β chains of fibrinogen; however, degradation patterns were not always similar. Variation in activities and toxicities do not seem to be related to geographical or climatic characteristics.

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1. Introduction

Bothrops alternatus ("yará grande", "víbora de la cruz", "urutú", "viriogaká curussú", "crucera") is one of the snakes of widest distribution in Argentina, Bolivia, Brazil, Paraguay and Uruguay (Hoge and Romano, 1972). This snake is considered a monotypic species (Barrio and Miranda, 1966), and is distributed from the Center to the entire North of the country (approximately 38°–22° S and 70°–54° W), with some fragmented localities in the province of Buenos Aires (Barrio and Miranda, 1966; Ministerio de Salud, 2007). This species inhabits forested, semidesertic and highland zones, even at high altitudes.

Although this species is not as aggressive as other *Bothrops*, it is of great medical and veterinary importance in

Argentina, mainly due to its size (an adult female can surpass 1.5 m in length and weight over 2 kg), the large amount of venom that it can deliver (de Roodt et al., 1998b) and because it can be found close to human dwellings even in the most populous cities of Argentina (de Roodt et al., 1996, 2006a).

B. alternatus venom in Argentina is endowed with the toxic activities described for the bothropic venoms (Sanchez et al., 1992) and exhibits a strong immunochemical relationship to venoms from other species of *Bothrops* (de Roodt, 2002). Barrio and Miranda (1966) described some immunochemical differences in samples of venom of *B. alternatus* from different regions of Argentina but there are no comparative data on differences in toxicity of the venom of this species from different regions of the country, although it is reasonable to presume that these differences exist.

This intraspecific variation can be of ontogenetic, geographic or seasonal origin and, in addition, individual

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differences can be observed in single specimens of a genus (Chippaux et al., 1991). Occasionally, the distinct composition of a venom from a single species can be related to different clinical manifestations of envenomation (Glenn et al., 1983; Saravia et al., 2002; Flight et al., 2006) and may be of importance for the adequate diagnosis, treatment and antivenom production (Chippaux and Goyffon, 1998).

In Argentina, although *B. alternatus* and *B. neuwiedi* are the snakes of greatest medical importance (Martino et al., 1979; de Roodt, 2002; Ministerio de Salud, 1980, 2007), the geographic variation of their venom has not been systematically studied. As a first step to remedy this scarcity of information we decided to study some toxic and biochemical characteristics of different populations of adult *B. alternatus* from different geographic regions of Argentina.

2. Materials and methods

2.1. Experimental materials

2.1.1. Venoms

Venoms used in this study were obtained from captive adult *B. alternatus* specimens from Buenos Aires (localities of San Nicolás, Baradero, Dock Sud, and Olavarría) and Entre Ríos (localities of Concordia and Gualeguay) from the Serpentarium of the National Institute for Production of Biologicals (INPB), those from the province of Corrientes were from the Serpentarium Palo Seco (Mortypeil Co., Monte Caseros, Corrientes), those from Córdoba were from the Centro de Zoología Aplicada (Center of Applied Zoology), National University of Córdoba (Córdoba), and those from Misiones were from the Centro Zootoxicológico de Misiones (Zootoxicological Center of Misiones) (Oberá, Misiones) (Fig. 1). In each case the snakes were captured in the region of the serpentariums from which the samples were obtained.

The maximal number of available snakes was used to generate the regional venom samples. The number of snakes used to obtain the different samples of venom were as follows: San Nicolás, 7; Baradero, 8; Dock Sud, 5; Concordia, 18; Gualeguay, 12; Misiones, 24; Corrientes, 20; Olavarría, 9 and Córdoba, 30. In each case similar amounts of venom from each individual snake were used to generate the regional samples of venoms. The venom pool representative of all the regions studied was obtained by mixing equal amounts of each regional venom sample.

Venoms were obtained by manual milking, vacuum-dried immediately, and stored at -20°C until used. In all cases, previous to use, venoms were redissolved in 0.15 M NaCl.

2.1.2. Animals

Mice (CF-1, 18–22 g) and rats (Wistar, 180–220 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 accordance with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Research Council (2002).

2.2. Experimental methods

2.2.1. Determination of lethal potency

Different doses of each venom were injected by the intraperitoneal (i.p.) route in CF-1 mice (18–22 g, 6 mice per dose level) according conventional techniques (WHO, 1981). The number of deaths 48 h after injection was recorded and the lethal potency was expressed as Median Lethal Dose (LD₅₀), which was defined as the dose of venom that kills the 50% of the mice expressed in $\mu\text{g}/\text{mouse}$. The plot of mortality versus venom dose was analyzed by non-linear regression using the software package Prism 3 (GraphPad, CA, USA).

2.2.2. 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 to 400 μg), were injected intradermally in Wistar rats, using 3 points per dose level. After 24 h, the skin was excised and the major perpendicular diameters of the hemorrhagic haloes were measured from the dermal face with a digital caliper. The MHD was defined as the amount of venom (μg) that produces a mean hemorrhagic halo of 1 cm of diameter. All the experiments were in triplicate.

2.2.3. Pro-coagulant activity

Pro-coagulation was studied in normal human plasma and in bovine fibrinogen, according to Theakston and Reid (1983) with some modifications. Briefly, normal plasma or bovine fibrinogen (2 g%, en NaCl 0.15 M) were treated with different doses of the samples of venom and the time of clot formation was recorded. 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). The results are the mean of triplicate determinations.

2.2.4. Indirect hemolytic activity

This activity was studied in liquid medium as described by Al-Abdulla et al. (1991) with the following modifications. Samples containing 10% of rabbit red blood cells, 10 mM of CaCl_2 and 5% egg yolk in a final volume of 1 ml in PBS pH 7.4, were incubated 30 min. at 37°C with different venom concentrations diluted in 100 μl of PBS pH 7.4. As positive control, we used 100 μg of *Bothrops neuwiedi diporus* venom in the same conditions. Negative controls received 100 μl of PBS pH 7.4. After incubation, the reaction was stopped by adding 100 μl of EDTA 0.1 M. After centrifugation, the absorbance of the supernatants was read at 550 nm. Determinations were in triplicate. The hemolytic dose (HD) was defined as the venom dose that produced the 50% of hemolysis when compared with the positive control. The results were analyzed by non-linear regression using the software Prism 3.02 (GraphPad, CA, USA).

2.2.5. SDS-PAGE

Separation of 10 μg of each venom by SDS- PAGE was done in 15% acrylamide/bisacrylamide (Bio Rad) gels under reducing and non-reducing conditions, according to Laemmli (1970). After fixation gels were stained with

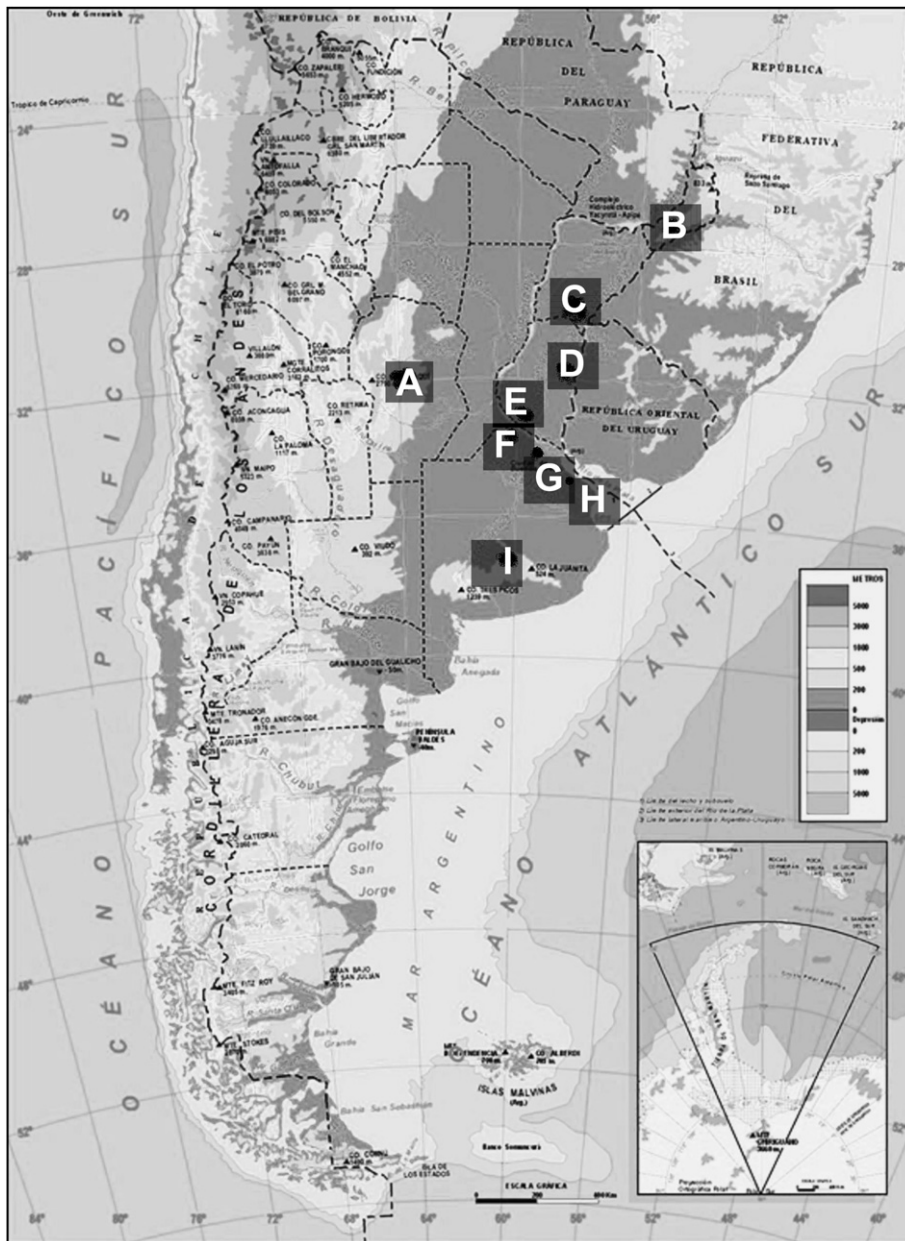


Fig. 1. Geographic localization of *Bothrops alternatus* specimens used to obtain the different pool of venoms: (A) Córdoba, 31°24'S, 64°11'W, 477 m over sea level, temperate grassland climate; (B) Oberá, 27°28'S, 55°07'W, 298 m over sea level, subtropical without dry station climate; (C) Monte Caseros, 30°15'S, 57°37'W, 58 m over sea level, subtropical without dry station climate; (D) Concordia, 31°28'S, 58°01'W, 32 m over sea level; (E) Gualeguay, 33°09'S, 59°20'W, 18 m over sea level; (F) San Nicolás, 33°19'S, 60°18'W, 13 m over sea level; (G) Baradero, 33°48'S, 59°31'W, 11 m over sea level; (H) Dock Sud, 34°39'S, 58°19'W, at sea level; (I) Olavarría, 36°53'S, 60°20'W, 180 m over sea level. The weather of the regions in Buenos Aires and Entre Ríos provinces (from D to I) have Pampean humid temperate climate.

Coomassie brilliant blue (Bio Rad). The relative amounts of venom proteins were analyzed by densitometry using the software Gel-Pro Analyzer (Media Cybernetics, MA, USA).

2.2.6. Hydrolysis of fibrinogen

Samples of each venom (1 mg), were incubated with 20 mg of 2% bovine fibrinogen (Sigma) in NaCl 0.15 M by 6 h at 37 °C. The fibrinolytic activity was determined in two

ways: (a) After the incubation samples were precipitated with 20% Trichloroacetic acid at 4 °C for 1 h and centrifuged for 5 min. The supernatants were collected and read at an absorbance of 280 nm in a spectrophotometer. The results are expressed as A_{280nm}/mg. (b) The pattern of fibri(noge)n hydrolysis of the samples was studied by SDS-PAGE (de Roodt et al., 2006b). Briefly, ten micrograms of each sample were run in a 10% acrylamide/bisacrylamide gels

under reducing conditions. Gels were stained with Coomassie blue R-250 (Bio Rad). Broad range molecular weight markers (Promega) were used as reference.

2.2.7. Hydrolysis of gelatin

Venoms were tested for their capacity to hydrolyze gelatin. Briefly 50 to 1000 µg of each venom were incubated in a solution containing 20% of gelatin (Molecular Biology grade, BioRad) in PBS pH 7.4 in a final volume of 2 ml for 6 h at 37 °C and then 2 ml of 40% of Trichloroacetic acid were added. Samples were thoroughly homogenized in a vortex for 60 s, stored overnight at 4 °C and centrifuged at 9000 g. Absorbance of the supernatants was read at 280 nm. The results were analyzed by non-linear regression using the software Prism 3.0 (Graph Pad, CA, USA) and expressed as the dose of venom that produced half of the maximum A_{280} .

2.2.8. Statistics

All data are expressed as mean ± SD (standard deviation) or with the 95% confidence interval (c.i.) in brackets. Linear and non-linear regression analyses and Student *t*-test were performed by using the combined Prism 3.0 software (Graph Pad, CA, USA). A multivariate statistical method such as Principal Component Analysis (PCA) was performed to explore the pattern of variation in toxicity of the venoms from different localities. In this analysis we used the following variables: MHD, MCD-P, MCD-F, IH, and LD₅₀. We used the triplicate values obtained for all localities and for the pool and, in the case of LD₅₀, the minimum and maximum values (95% c.i.) were included in the analysis. The result of PCA is a new set of variables (Principal Components) that are linear combinations of the original variables and are uncorrelated (Pla, 1986; Manly, 1986). Thus, the new variables generated are smaller in number and account for the inherent variation of the data to the maximum possible extent. We carried out a PCA using the correlation matrix of the standardized data by way of Statistica 6.0 (StatSoft, 2001). We obtained the first two principal components of the PCA in order to describe the pattern of variation in toxicity among localities.

3. Results

3.1. Lethal potency

Lethal potencies (LD₅₀) are shown in Table 1. Lethal potency of the venoms from Corrientes, Olavarría, Gualeguay, and Concordia were not significantly different from the pool (e.g. Olavarría *t* = 0.98; *p* > 0.05). Venoms from Misiones, Dock Sud, and Baradero were more potent than the pool (e.g. Misiones *t* = 6.08; *p* < 0.05). Samples from Córdoba and San Nicolás were significantly less toxic than the pool (e.g. San Nicolás *t* = 5.94; *p* < 0.05). Lethal potencies ranged from 5.7 to 27.8 LD₅₀/mg of venom (Table 1).

3.2. Hemorrhagic activity

Hemorrhagic activities (MHD) are shown in Table 1. Venoms from Baradero, Córdoba, Olavarría, and Misiones were not significantly different from the pool (e.g. Baradero

Table 1 Toxic potencies of venoms of *Bothrops alternatus* from different regions of Argentina. The potencies are expressed in their correspondent units and deviations or confidence intervals (left columns) and as the number of doses contained in 1 mg of venom (right columns).

VENOM	Lethal Potency		Hemorrhagic Potency		Coagulant Potency		Fibrinogen		Indirect hemolytic activity	
	LD ₅₀ ^a	LD ₅₀ /mg	MHD ^b	MHD/mg	MCD-P ^c	MCD-P/mg	MCD-F ^d	MCD-F/mg	IH ^e	IH/mg
Córdoba	134.9 (125.3–145.3)	7.4	52.2 ± 14.0	19.2	4.9 ± 0.8	204.1	101.0 ± 29.4	9.9	3.8 (3.3–4.2)	263.2
Misiones	35.9 (34.5–37.4)	27.9	102.8 ± 46.9	9.7	2.9 ± 0.2	344.8	391.1 ± 30.7	2.6	5.5 (5.0–5.9)	181.8
Gualeguay	71.4 (64.0–79.5)	14.0	145.0 ± 31.0	6.9	4.2 ± 0.2	238.1	1085.0 ± 30.3	0.9	26.5 (24.6–28.5)	37.7
Concordia	66.9 (63.9–69.9)	14.9	118.8 ± 21.5	8.4	2.9 ± 0.2	344.8	152.7 ± 5.5	6.5	11.5 (9.9–13.5)	87.0
Corrientes	91.6 (71.5–117.2)	10.9	233.9 ± 34.5	4.3	10.9 ± 1.4	91.7	180.3 ± 7.1	5.6	25.2 (29.5–29.7)	39.7
San Nicolás	174.1 (156.5–193.6)	5.7	292.3 ± 74.58	3.9	4.5 ± 0.3	222.2	244.3 ± 9.8	4.1	39.7 (36.7–43.0)	25.2
Baradero	56.4 (50.4–63.1)	17.7	50.6 ± 12.4	19.8	2.9 ± 0.6	344.8	251.6 ± 9.4	4.0	17.1 (14.3–20.4)	58.5
Dock Sud	49.9 (44.7–55.6)	20	321.0 ± 77.62	3.1	7.6 ± 0.4	131.6	>1000	<1.0	7.3 (6.8–7.9)	137.0
Olavarría	82.3 (79.7–84.9)	12.2	78.3 ± 46.0	2.8	5.1 ± 1.4	196.1	151.3 ± 8.7	6.6	3.8 (3.3–4.2)	263.2
Pool	90.4 (76.1–107.5)	11.1	48.8 ± 11.2	20.5	8.1 ± 0.3	123.5	133.35 ± 1.6	7.5	10.7 (10.2–11.3)	93.5
Mean ^f	84.8 ± 43.9	14.5	155.0 ± 102.5	9.8	5.1 ± 2.6	235.4	395.3 ± 376.8	4.6	15.6 ± 2.6	121.5

^a DL₅₀ (Median Lethal Dose) = it is defined as dose of venom that kills the 50% of the challenged and it was expressed in µg/mice, 95% confidence interval is expressed into brackets.

^b MHD (Minimal Hemorrhagic Dose) = it is defined as the amount of venoms (µg) ± Standard Deviation (SD) that produces a mean hemorrhagic halo of 1 cm.

^c MCD-P (Minimal Coagulant Dose on Plasma).

^d MCD-F (Minimal Coagulant Dose on Fibrinogen) = dose of venom that clots human plasma or a solution of bovine fibrinogen in 60 seconds. Doses were expressed in mg/ml ± SD.

^e IH (Indirect Hemolytic Potency) = it was defined as the venom dose that produced the 50% of hemolysis when compared with positive controls. The 95% confidence intervals are expressed into brackets.

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$t = 0.29$; $p > 0.05$). Venoms from Concordia, Gualeguay, Corrientes, San Nicolás, and Dock Sud were of lower hemorrhagic potency than the pool (e.g. Dock Sud $t = 5.86$; $p < 0.05$).

Hemorrhagic potencies ranged from 3.1 to 19.2 MHD/mg of venom, and the pool was the most potent (20.5 MHD/mg of venom) (Table 1).

3.3. Pro-coagulant activity

Values of MCD-P are presented in Table 1. Venom from Dock Sud showed no significant differences from the pool ($t = 1.84$; $p > 0.05$). Venom from Corrientes showed MCD-P less than pool ($t = 3.37$; $p < 0.05$), while venoms from the other localities showed MCD-P values significantly greater than the pool (e.g. San Nicolás $t = 109$; $p < 0.05$).

Values of MCD-F are presented in Table 1. Thrombin-like activity in the venoms from the different regions was statistically different from the pool. The venom from Gualeguay showed a very low MCD-F activity ($t = 14.29$; $p < 0.05$). MCD-F values for venom from Córdoba showed major activity than the pool, while the venoms from the other localities showed minor thrombin like potency (MCD-F major values than pool). In our hands, this activity was undetectable in the venom of snakes from Dock Sud.

Measurable pro-coagulant potencies on plasma and fibrinogen ranged from 91.7 to 344.8 MCD-P/mg of venom and from 0.9 to 9.9 MCD-F/mg of venom (Table 1).

3.4. Indirect hemolytic activity

Values of IH are presented in Table 1. Venom from Concordia showed no significant differences from the pool ($t = 0.49$; $p > 0.05$). The IH values of venoms from Córdoba, Misiones, Dock Sud, and Olavarría were greater than that of the pool (e.g. $t = 23.89$; $p < 0.05$). Venoms from Gualeguay, Corrientes, San Nicolás, and Baradero had a lower IH potency than that of the pool (e.g. $t = 79.73$; $p < 0.05$). The indirect hemolytic potency ranged from 25.2 to 263.2 IH/mg of venom (Table 1).

3.5. SDS-PAGE

Analysis of the individual venoms by SDS-PAGE under non-reducing showed very similar patterns although some bands were absent or weaker (Fig. 2a). The analysis of the gels with the software showed that most of the material in all cases was between 47 and 32 kDa and 25 and 16 kDa. Venom from Baradero showed an important band between 16 and 6.5 kDa, which if present, was very slight in the other venoms.

Additional bands around 30 kDa were apparent in the venoms from Baradero, Corrientes and Concordia, which were not observed or weakly stained in the other samples. All the samples contained weakly stained material around 6.5 and 16 kDa. The samples from Baradero showed one more strongly stained band of ~10 kDa, which can be observed in the samples from Olavarría, Misiones and Corrientes but with lower intensity.

The samples from Mesopotamia (Concordia, Gualeguay, Corrientes and Misiones), showed very similar patterns

although one of the bands around 10 kDa, present in the other two venoms, was absent in the venoms from Concordia and Corrientes (Fig. 2b). All venoms showed a strongly stained band around 47 kDa with the exception of that from Corrientes, which showed a strongly stained band under this molecular mass. The sample from Baradero showed an additional band around 30 kDa, absent or very lightly stained in the other samples. The venom from Olavarría was the most different. In this case, instead of the band around 40–47 kDa present in all samples, two main bands around 35 and 47 kDa are apparent. The venom from Córdoba in general resembled those of the province of Buenos Aires.

3.6. Hydrolysis of fibrinogen

- a) All venoms showed hydrolytic activity on fibrinogen, hydrolyzing α and β chains. The products of degradation observed in the different samples were very similar although not identical. Some bands could not be observed and others showed different intensity. The most divergent pattern was that produced by the venom from Dock Sud snakes (Fig. 3).
- b) The capacity to hydrolyze the fibrinogen was very similar, ranging around 0.8 to 1.0 $A_{280\text{nm}}$ /mg in all cases. The potencies were $0.8 \times \text{mg}^{-1}$ for San Nicolás, Olavarría, Concordia, Gualeguay and Córdoba, $0.9 \times \text{mg}^{-1}$ for Baradero, $1.0 \times \text{mg}^{-1}$ for Dock Sud, Corrientes and Misiones.

3.7. Hydrolysis of gelatin

All venoms hydrolyzed gelatin. The doses of venom that produced 50% of the A_{280} were: Dock Sud 36.0 (22.6 to 49.4), Olavarría 40.6 (32.8 to 48.9), Concordia 40.9 (28.9 to 52.8), Misiones 47.0 (40.7 to 53.2), Córdoba 53.1 (44.9 to 61.2), Pool 62.2 (48.5 to 76.0), Gualeguay 62.6 (41.9 to 83.4), Corrientes 62.7 (47.1 to 78.2), Baradero 74.2 (55.5 to 92.9), San Nicolás 81.2 (62.1 to 100.2).

3.8. Principal component analysis

The first three principal components of PCA explained 87.6% of the total variability. The variables contributing more to separation the localities along the principal components were: IH with high and negative correlation on PC 1; MCD-F with high and negative correlation on PC 2; and MCD-P with high and positive correlation on PC 3. The plot of PC 1 vs. PC 2 (Fig. 4) shows that there are three groups with considerable separation, indicating differences among the venoms belonging to different localities. One of these groups is constituted by venoms from San Nicolás and Corrientes, the second one by Gualeguay and Dock Sud, and the third one by the venoms of the other five localities and the pool. Thus, San Nicolás and Corrientes are high IH and small MCD-F (on negative values of PC 1 and on positive values of PC 2); Gualeguay and Dock Sud are high IH and high MCD-F (on negative values of both PC 1 and PC 2); and the other five localities and the pool are small IH

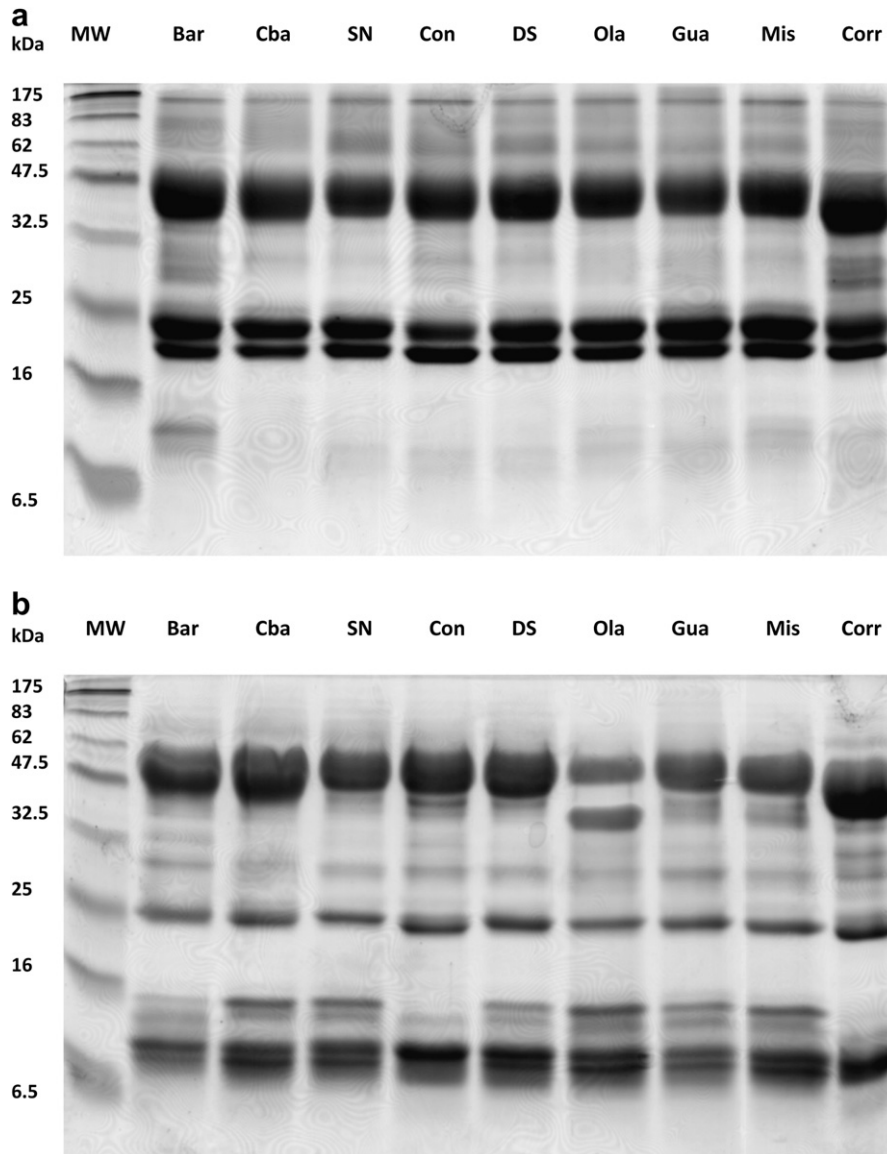


Fig. 2. a. SDS-PAGE at 15% in non reducing conditions of *B. alternatus* venom from different regions of Argentina. In the left the molecular masses of the markers are expressed in kDa. Bar: Baradero, Cba: Córdoba; SN: San Nicolás; Con: Concordia; DS: Dock Sud; Ola: Olavarría; Gua: Gualaguay; Mis: Misiones; Corr: Corrientes. All the samples showed the major components around 35–50 kDa and around 20–25 kDa. Venoms from Baradero, Corrientes and Concordia showed in addition bands around 30 kDa, which are absent or weakly stained in the other samples. All the lanes showed material among the 6.4–10 kDa but weakly stained. The sample of Baradero shows a strong stained band in the order of 10 kDa which is observed in the venoms from Olavarría, Misiones and Corrientes but with lower intensity. b. SDS-PAGE at 15% A/B, in reducing conditions of *B. alternatus* venom from different regions of Argentina. In the left the molecular masses of the markers are expressed in kDa. Bar: Baradero, Cba: Córdoba; SN: San Nicolás; Con: Concordia; DS: Dock Sud; Ola: Olavarría; Gua: Gualaguay; Mis: Misiones; Corr: Corrientes. The samples from Mesopotamia (Concordia, Gualaguay, Corrientes and Misiones), showed very similar patterns although the venoms from Concordia and Corrientes did not showed one of the bands around 10 kDa present in the other two venoms. All the venoms showed a strongly stained band around 47 kDa with the exception of that from Corrientes that showed a strongly stained band with a relative moiety under 47 kDa. The sample from Baradero showed a band around 30 kDa absent or very slight stained in the other samples. The venom from Olavarría was the venom that showed the biggest differences. In this case instead the band around 40–47 kDa present in all the samples, were observed two principal bands around 35 and 47 kDa. The venom from Córdoba in general resembled the pattern from those of the province of Buenos Aires.

and small MCD-F (approximately on positive values of both PC 1 and PC 2). The plot of PC 2 vs. PC 3 (not shown) indicates major overlap among venoms belonging to different populations, except for those from Dock Sud which are high MCD-F and high MCD-P (on negative values of PC 2 and positive values of PC3).

4. Discussion

Variability of snake venoms, even at the level of a single specimen, is well known (Chippaux et al., 1991). It has been described in Crotalidae (Cavinato et al., 1998; Aguilar et al., 2007; Girón et al., 2007) and Elapidae (Shashidharamurthy

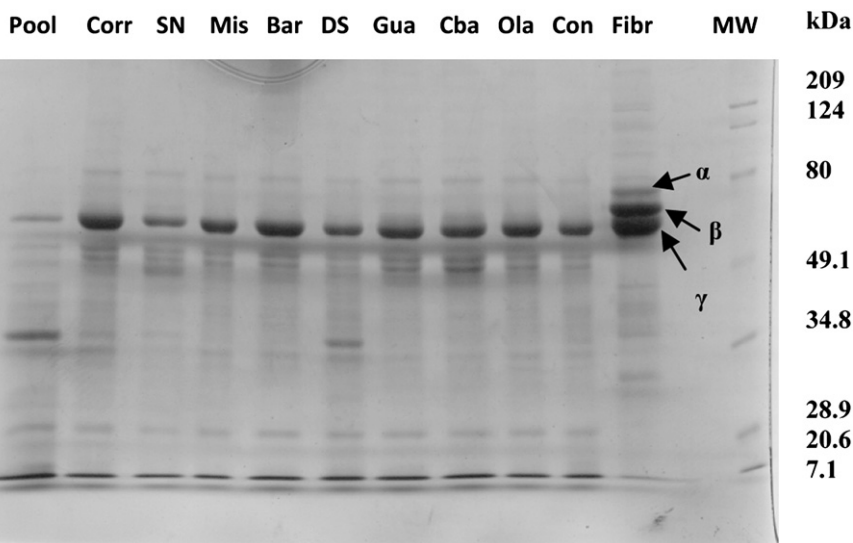


Fig. 3. SDS-PAGE at 10% A/B in reducing conditions of fibrinogen digestion by venoms of *B. alternatus* from different regions of Argentina. In the right the molecular masses of the markers are expressed in kDa. Bar: Baradero; Cba: Córdoba; SN: San Nicolás; Con: Concordia; DS: Dock Sud; Ola: Olavarría; Gua: Gualeguay; Mis: Misiones; Corr: Corrientes; Fibr: fibrinogen. Arrows indicate the α , β and γ chains. The chains α and β are totally digested by all the venoms. The patterns of degradation are not identical in the samples. The lane of the fibrinogen treated with venom from San Nicolás showed products of degradation different to the rest. The most conspicuous degradation products can be observed among 40–60, 30–35 kDa, 20 y 28 kDa and below 20 kDa.

et al., 2002; Chanhom et al., 2009). More recently, transcriptomic and proteomic approaches have been applied to the study of venom variation in several additional species of vipers, such as neurotoxicity of *Vipera aspis* venom (Ferquel et al., 2007), variation in *Bothrops asper* (Alape-Giron et al., 2008) and *Lachesis* (Sanz et al., 2008) among others. In this work we studied toxicological and enzymatic variation of *B. alternatus* from different regions of Argentina.

The electrophoretic patterns of the venoms of *B. alternatus* were similar to those described for that species in Argentina (de Roodt et al., 1998a; de Roodt, 2002) (Fig. 2a and b). The most notorious difference was observed under reducing conditions in the venom from Olavarría (Fig. 2b), which is a geographically fragmented region (Tandilia and Ventania systems) where Barrio and Miranda

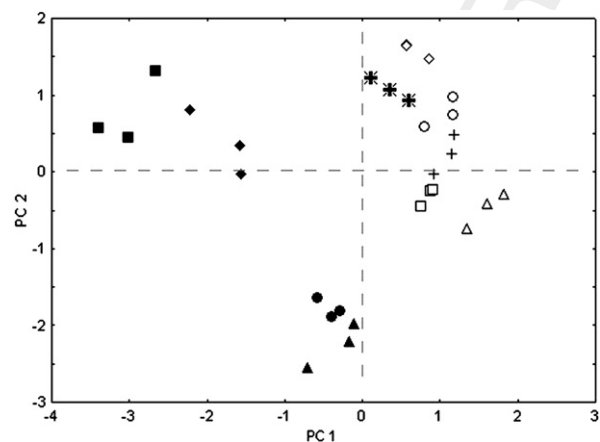


Fig. 4. Plot of PC 1 vs PC 2 of the Principal Component Analysis. (\diamond) Córdoba, (Δ) Misiones, (\bullet) Gualeguay, (\square) Concordia, (\blacklozenge) Corrientes, (\blacksquare) San Nicolás, (\boxplus) Baradero, (\blacktriangle) Dock Sud, (\odot) Olavarría, and (\boxplus) Pool.

(1966) reported both immunochemical differences in the venom and distinct morphological characteristics.

The toxic activities were those described for the venoms of *B. alternatus* from different regions from Brazil and Argentina (Sanchez et al., 1992; de Roodt, 2002) and their potencies were within the previously reported ranges (Ferreira et al., 1992; Sanchez et al., 1992; de Roodt et al., 1998a, de Roodt, 2002; Araujo et al., 2008).

Hemorrhagic and pro-coagulant activity on plasma, proteolytic activity on fibrinogen or gelatin, and phospholipase activity were detected in all samples, although potencies varied. Thrombin-like activity was evident in most samples, but values for venom from Gualeguay were very low and this activity was undetectable in venom from Dock Sud (Table 1).

All venoms hydrolyzed gelatin and fibrinogen and showed hemorrhagic activity. There was no correlation between these enzymatic or toxic activities and the region from which the venom was obtained. In agreement with previous results, there was no correlation between lethal potency and the other toxic activities (de Roodt, 2002). For example, venoms from Dock Sud were among the most lethal but had the lowest hemorrhagic activity and the lowest (or absent) thrombin-like activity. Conversely, venoms from Córdoba exhibited some of the lowest lethal potencies but one of the highest hemorrhagic activities (Table 1).

All venoms hydrolyzed α and β chains of fibrinogen, according with previous results made with a pool of *B. alternatus* venom from different regions of Argentina (de Roodt, 2002). This activity was observed even in venoms with low or undetectable thrombin-like activity, indicating that although these venoms are incapable of transforming fibrin into fibrinogen, they are able to hydrolyze fibrin and/or fibrinogen (Fig. 3).

Principal Components Analysis showed three clearly separated groups of localities based on IH and MCD-F of venoms (Fig. 4). In spite of relatively small geographic separation, the venoms from San Nicolás and Baradero were quite different in terms of toxic activities. The same situation is apparent between venoms from the province of Corrientes (locality of Monte Caseros) and Concordia (in the province of Entre Ríos). However, venoms from Dock Sud appear to be related to venoms from Gualeguay, and this in turn be related to the floods of the Paraná River, which carry snakes among the vegetation and are thought to be the main vehicle for colonization of snakes to the River Plate shore (de Roodt et al., 1996).

When comparing the activities of the regional samples and the venom pool, wide differences in several cases were observed. This may be due to synergism and/or antagonism between different toxic components, or the action of toxin isoforms that may contribute to these significant differences, which can vary several fold depending on the activity being measured (Table 1).

Variation in toxic/enzymatic activities may be associated to the diet available (Daltry et al., 1996). Although we reported biochemical ontogenetic variation in the venom of these snakes (de Roodt, 2002), toxicologically this variation does not seem to be of the magnitude observed in other species of *Bothrops* that as juveniles feed on amphibians or reptiles, like *B. jararaca* or *B. moojeni* (Andrade and Abe, 1993, 1999; Furtado et al., 1991). *B. alternatus* feeds on mammals throughout its life (Martins et al., 2002; Zelanis et al., 2008).

The variability of venom toxicity in addition can be related with the geographical, climatic and genetic characteristics of the snakes in the different regions (Chippaux et al., 1991). Nevertheless, in this work, venoms from geographically similar regions showed rather different patterns of toxicity, as in the case of samples from several provinces of the North East (region called “Mesopotamia” between the Paraná and Uruguay rivers) like Gualeguay, Concordia, Corrientes and Misiones, the north of the Province of Buenos Aires (in the “Pampa húmeda” = humid Pampa) like San Nicolás, Baradero, Dock Sud, Baradero and San Nicolás, or even from mountainous areas as Córdoba and Olavarría.

Envenomation symptoms due to snakebites by individuals belonging to the same genus and even species are not necessarily similar, as reported for different species and subspecies of *Crotalus* (Saravia et al., 2002). However, differences in *Bothrops*, although present, are not as marked (Ministerio de Salud, 2007). Differences in toxic activities could indicate that, even in the same species, physiopathological mechanisms of toxicity are not identical, independently of the similarity of clinical manifestations of envenomation. Although different mechanisms of envenomation have been described in the bite by some species of *Bothrops*, such as local damage caused by *B. alternatus* venom when compared to *B. jararacussu* venom (Queiroz and Petta, 1984), it is not yet clear in envenomations by *B. alternatus* from different regions or by animals of different characteristics. However, although the potency of the toxic activities seems to be different and this could influence the course of the envenomation, the

differences in the clinical manifestations can be caused in addition by the amount of venom injected. *B. alternatus* is a large snake (some large specimens may measure over 1.65 m in length and weigh over 3 kg), and can inject from a few tens to hundreds of milligrams of venom (de Roodt et al., 1998a; Sabbatini et al., 2004). This species, unlike other species of *Bothrops* from Argentina (such as the *B. neuwiedii* complex), seems to be monotypic and differences in specimens from different regions do not warrant consideration of variants as different subspecies (Barrio and Miranda, 1966). If these differences are influenced by geographic, climatic factors, diet or are related with the genetics of closed populations must be investigated.

Comparison of toxic potencies between individual venoms and the pool revealed important differences. The results of Principal Components Analysis showed that a pool is not necessarily representative of the range of all venoms included (Fig. 4). Although the need of pooling venoms for immunization protocols is unquestionable, our results show that almost the half of the venoms (5/9) displayed a very different pattern of toxic activities.

When the values of the toxic activities of individual venom samples are inspected carefully and compared to the values of the pool and the theoretical values (that is, considering the arithmetic means of the toxic activities), it is clear that only lethal potencies correlate well (Table 1). Comparison of the regional samples to the pool (toxic activity/mg of venom) showed that lethality (as LD₅₀) varied from 0.4 to 2.5 fold; the pool showed the highest hemorrhagic activity, coagulation on plasma varied from 0.7 to 3 fold, thrombin-like activity from 0.1 to 1.3 fold and indirect hemolytic activity from 0.3 to 2.8 fold. This variation in toxic activities, even in a monotypic species like *B. alternatus* from different regions, must be taken into account when pools of venom are used to study the neutralizing potency of antivenoms and underscore the necessity of more studies on venom variation in these snakes. The results suggest that not only the origin of the snakes used to obtain the venom but the toxicological characteristics of these venoms should be considered for antivenom production.

Important variability in some enzymatic and toxic activities of the venoms from individual specimens of *B. alternatus* from Brazil has been reported (Rocha and Furtado, 2005). This individual variation seems to be greater than variation between the venoms from different regions. Studies on the individual variability and regional variability of the venom of this snake in Argentina are in course.

Uncited reference

Bradford, 1976.

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Appendix. Supplementary information

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.toxicon.2010.01.015.

Conflict of interest

There are not any types of conflicts of interest in the content of this work.

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