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REGULAR ARTICLE

A comparative analysis of the clotting and fibrinolytic activities of the snake venom (*Bothrops atrox*) from different geographical areas in Venezuela

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Abstract Venom constitution within the same snake species can present considerable geographical variations. *Bothrops atrox* venoms were obtained from adult snakes captured at different geographical locations: Parguasa (Bolívar state); Puerto Ayacucho 1, Serranía del Cuao and Puerto Ayacucho 2 (Amazon state). The coagulant and fibrinolytic activities of these venoms were compared. Amidolytic activity of crude snake venom was measured by a micromethod designed in our laboratory. Coagulant activity on plasma and fibrinogen due to thrombin-like activity in venoms was also determined. Crude snake venom fibrinolytic activity by the fibrin plate method was assayed. Chromatographic studies were developed on Protein-Pack 300 column. Polyacrylamide gel electrophoresis was carried out under reduced conditions. After SDS-PAGE of samples, the fibrin-zymography was tested on agarose–fibrin plates.

The results demonstrated several differences among *B. atrox* venoms from different geographical areas. Chromatograms and SDS-PAGE profiles indicated that venoms from the same species presented differences in the molecular mass of their components. The procoagulant activity depended on the utilized method (amidolytic versus clotting). Parguasa and Puerto Ayacucho 2 venoms presented procoagulant activity for both methods. Furthermore, Parguasa venom had also the highest hemorrhagic activity and the lowest LD50. In relation to the fibrinolytic activity, Puerto Ayacucho 1 venom was the most active, equally for fibrin plates as for the

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amidolytic method (t-PA like). This venom had the lowest coagulant activity, which induced us to think that probably its procoagulant activity was interfered by its fibrinolytic activity.

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Introduction

Viperidae *Bothrops* snake venoms have been reported to have many biochemical and physiological effects [1–3]. Venoms from the same species of diverse geographical areas could diverge in their components and consequently in their toxicity, an important consideration since bites by specific populations may differ in their symptomatology, involving different treatments [4]. Some authors [5,6] reported that venom properties are not reliable indicators of taxonomic relationships due to intraspecific (individual) variation of snake venoms; the underlying causes of this phenomenon have never been explained. One of the parameters that can affect venom toxicity and cause variable results is the geographical setting of the snakes. For instance, Yoshida-Kanashiro et al. [7] have proposed that the rattlesnake (*Crotalus durissus cumananensis*) venom from both central Venezuelan savannas (Guárico state) and mountains (Laguneta, Miranda state) differs in their component composition and toxicity. In these two populations, the hemorrhagic components vary considerably. Others [8] have described significant individual variations of the biological properties on different *Bothrops* species venoms. However, a comparative study of enzymatic and other toxic activities of the venoms of the same species of *Bothrops atrox* captured in different Venezuelan geographical areas is still lacking. Snake venoms contain proteases, many of which act on a variety of coagulation and fibrinolytic components as well as on cells causing an imbalance of the hemostatic system [9]. The coagulant and fibrinolytic activities in *Bothrops* venom has been investigated by several groups [9–11]. These activities have been attributed to venom components such as thrombin like enzymes, proteases capable of activating factor X and prothrombin, and fibrino(genolytic) proteinases [12–15]. The result of envenoming may be from mild to fatal, depending on the species, the victim and the amount of venom injected into victim [16]. In this study, we compared the coagulant and fibrinolytic activities of the mapanare (*B. atrox*) snake from four different Venezuelan locations, suggesting that these activities reflect differences in the studied geographical areas.

Materials and methods

Reagents

Chromogenic substrates and human fibrinogen (Kabi Vitrum, Sweden) (10% w/w of plasminogen as contaminant) were obtained from Chromogenix AB (Milano, Italy). Molecular mass standards for SDS-PAGE were from Bio-Rad Laboratories Ltd. (California, USA). Purified factor Xa, bovine alpha thrombin, one chain t-PA (sctPA), Two chains u-PA (tcu-PA) and plasmin (used for controls), were obtained from American Diagnostica Inc. (Greenwich, CT, USA).

Animals

Albino Swiss National Institute of Hygiene (NIH) strain male mice of 18–22 g obtained from the National Institute of Hygiene “Rafael Rangel,” Caracas, Venezuela, were used. The investigation complies with the bioethical norms taken from the guide “Principles of Laboratory Animal Care” [17]. The study was approved by the Tropical Medicine Institute of the Universidad Central de Venezuela Ethics Committee.

Venoms

B. atrox venoms were obtained from adult snakes captured at different geographical locations (Fig. 1): Parguasa (Bolívar state); Puerto Ayacucho 1, Serranía del Cuao and Puerto Ayacucho 2 (Amazon state). Most venoms were pooled samples from the same snake. Venom was filtered through a 0.45- μ m membrane, lyophilized, divided into 30 mg samples and stored at -80°C .

Chromatographic venom profiles

Samples from *B. atrox* venom were run in a molecular exclusion chromatography on Protein-Pack 300 (300 \times 978 mm) equilibrated with 50 mM ammonium acetate pH 6.9 plus 125 mM NaCl, (equilibrium buffer) in order to compare their chromatographic profiles. Venom samples (3 mg/



Figure 1 Different geographical locations of *B. atrox* under study.

100 μ L) were dissolved in equilibrium buffer and injected into the column. The elution was carried out with the same solution at 0.4 mL/min flow rate and monitored at 280 nm.

Protein concentration determination

The protein concentration was determined by the method of Lowry et al. [18].

Determination of MHD

To determine the minimal hemorrhagic dose (MHD) for the crude venoms, the Omori-Satoh et al. [19] method was used. A series of eight dilutions were made for the snake venom, of which 0.1 mL of each dilution was intracutaneously injected into the depilated backs of mice. The mouse was sacrificed and the skin removed after 24 h. The hemorrhagic diameter on the skin was measured and the MHD determined. The MHD is defined as the amount of venom protein that causes a 10 mm hemorrhagic dot.

Determination of lethality

Venom lethality was determined in mice by intraperitoneal injection of the sample and the LD₅₀ value calculated according to the method of Spearman–Karber [20].

Amidolytic activity

Amidolytic activity of crude snake venom was measured by a micromethod standardized in our laboratory. Briefly, in 96-well polystyrene plates, a mixture of 80 μ L of the recommended buffer for each substrate, 10 μ L of the venom sample (0.1 mg/mL, 0.5 mg/mL or 1 mg/mL) and 10 μ L of substrate (final concentration) (0.6 mM S-2238, 1.2 mM S-2288, 0.8 mM S-2222, 0.52 mM, 0.80 mM S-2251 and 0.16 mM S-2444) were placed in each well. Bovine thrombin, human factor Xa, sct-PA, tcu-PA, and plasmin were used as positive controls. After incubation at 37 °C for 15 or 30 min, the absorbance at 405 nm was measured. One unit of amidolytic activity was expressed as ΔA 405 nm/min. Specific activity was calculated as units/mg of protein.

Fibrinolytic activity

Fibrinolytic activity of crude snake venom was studied by the fibrin plate method as described by Marsh and Arocha-Pinango [21]. Briefly, fibrin plates were prepared using 3-cm-diameter Petri dishes: 1.5 mL of a 0.1% plasminogen-rich fibrinogen in imidazol saline buffer, pH 7.4 was clotted by adding 75 μ L bovine thrombin (10 U/mL, in 0.025 M CaCl₂). The mixture was incubated at room temperature for 30 min. Then, a 10- μ L sample was applied over the fibrin film; after 24 h incubation at

37 °C, the diameter of lysis areas was measured. Fibrinolytic activity was expressed in lysis (mm²/μg) sample. Human plasmin, t-PA (sct-PA) and u-PA (tcu-PA) were used as positive controls.

Coagulant activity on plasma and fibrinogen

The thrombin-like coagulant activity in venoms was determined by the method adapted from Austen and Rhymes [22]. Briefly, in a borosilicate tube at 37 °C 0.1 mL of 0.05 M Tris–HCl buffer, pH 7.4 (coagulation buffer) containing 0.025 M CaCl₂ or 0.1 mL venom sample (diluted in coagulation buffer) was incubated. Then 0.1 mL of fresh citrate human plasma or 0.3% human fibrinogen solution in coagulation buffer was added. The solution was mixed thoroughly and clotting time recorded. Duplicated tests were performed and the mean clotting time calculated. All experiments and appropriate controls were repeated at least four times with essentially similar results.

SDS-PAGE analysis

Polyacrylamide gel electrophoresis was carried out following the Laemmli method [23] using a Mini-Protean II system (Bio-Rad Laboratories, Hercules, CA). Venom samples were diluted in 0.063 M Tris–HCl buffer, pH 6.8, containing 2% SDS, 5% glycerol and 0.001% bromophenol blue and then boiled for 4 min before electrophoresis at 100 V (constant). After electrophoresis, the gels were stained with 0.1% brilliant blue Coomassie R250 in acetic acid/ethanol/water (5:25:70, v/v) and then destained in the same solution.

Fibrin-zymography

Fibrin-zymography was carried out according to the Carvajal and Arocha-Pinango [24]. After SDS-PAGE of samples using 12% polyacrylamide gels and non-reducing conditions, the gels were washed twice with gentle agitation for 1 h at room temperature with 0.2% Triton X-100. After washing with distilled water for 5 min, the gels were placed on agarose-fibrin plates prepared by pouring 8.5 mL of 1.2% human fibrinogen in 0.005 M imidazol-saline buffer, pH 7.4 with 8.5 mL of 2.5% (w/w) low-melting-point agarose in water and 2 mL of 1 U/mL thrombin in 0.025 M CaCl₂ into a gel bond plate (13.5 × 9.5 cm) at 42 °C. After plates incubation at 37 °C for 18 h in a humid chamber, fibrinolytic activities of enzymes were visualized as clear bands against a black background. The tcu-PA was used as positive control.

Inhibitor effects

Amidolytic activity factor Xa-like and t-PA-Like was assayed in the presence of protease inhibitors (final concentration) (10 mm EDTA–Na; 10 mm EGTA–Na; 10 mm 1,10 phenantroline; 50 μg/mL SBTI, 10 mm benzamidine, and 100 U/mL aprotinine).

Statistical analysis

The activities were described statistically according to their mean and standard deviation (Microsoft Excel). Variance analysis and the Student's *t*-test were used to study possible differences between groups.

Results

Chromatographic venom profiles

The molecular exclusion column Protein-Pack 300 chromatographic profile from four studied venoms is shown in Fig. 2. Four main peaks with elution times of 23.25, 25.75, 33.75 and 41.25 min in all studied venoms were detected, which were classified according to their molecular mass in high (H), intermediate (I), medium (M) and lower (L). Quantitative variations among the venoms were evidenced. The peaks H and L showed the biggest intensity (more protein components) in Serranía del Cuao venom. In Parguasa and Puerto Ayacucho 1 venoms, M peak was predominant. Additionally, in both venoms, a peak that corresponds a very low-molecular-weight component was observed. Puerto Ayacucho 2 venom presented all peaks in relatively similar quantities.

Electrophoretic analysis

The SDS-PAGE patterns under reduced conditions pointed up some differences among the four studied venoms (Fig. 3). Serranía del Cuao venom showed several bands of molecular weight higher than 60 kDa. All the venoms had in common a range of bands between 50–45 kDa, 27–20 and 15 kDa. Serranía del Cuao and Puerto Ayacucho 2 venoms showed bands of lower molecular weight (<15 kDa).

Procoagulant activity

The procoagulant activity determined in the four *B. atrox* snake venoms captured in different geographical areas of Venezuela is shown in Table 1.

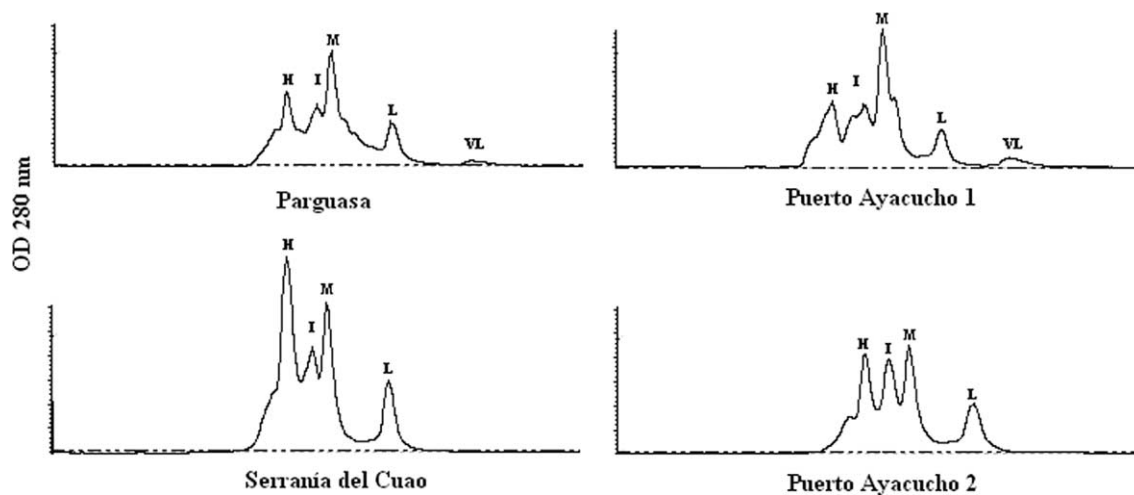


Figure 2 Chromatographic profiles of *B. atrox* venoms (3 mg/100 μ L) on Protein-Pack 300 (300 \times 78 mm) column equilibrated with 50 mM ammonium acetate buffer pH 6.9 plus 125 mM NaCl. Flow rate at 0.4 mL/min and monitored at 280 nm.

All venoms presented a procoagulant activity by both amidolytic and coagulant methods. When comparing all the venoms using amidolytic methods, it was observed that Parguasa and Serranía del Cuao venoms presented the higher activity for S-2222 substrate and to S-2238 substrate Parguasa and Puerto Ayacucho 2 showed the higher activity. However, thrombin-like activity was higher than factor Xa-like activity in all venoms.

Additionally, Puerto Ayacucho 2 venom by coagulant method was the most active ($p < 0.05$). Furthermore, Parguasa venom was significantly most active with plasma as substrate than with purified fibrinogen ($p < 0.01$). Significant differences among Puerto Ayacucho 2 and other venoms were observed. This was more active with purified fibrinogen than plasma ($p < 0.01$). These activities

presented considerable variations depending on the capture areas.

3.4. Fibrinolytic activity

Table 2 shows the fibrinolytic activity determined with chromogenic substrates and with fibrin plates. The amidolytic method demonstrated a t-PA-like activity in all venoms, being Puerto Ayacucho 1 the most active venom. The other substrates (S-2251, S-2244 y S-2302) were slightly hydrolyzed by the venoms. The fibrinolytic activity, evaluated on plasminogen-rich fibrin plates, also evidenced that all the venoms were active. Puerto Ayacucho 1 presented the higher fibrinolytic activity by both methods. However, appreciable differences were observed among the venoms from different locations.

The first fibrinolytic activity assays on plasminogen rich-fibrin plates were carried out with 10 μ g of sample (**Fig. 4**). These results showed a fibrin area without lysis amid the lysis area, an effect that was more pronounced in Parguasa and Puerto Ayacucho 2 venoms, which suggested the presence of fibrinolytic inhibitors in these venoms. To corroborate this effect, the activity was determined at different venom doses. In the results showed in **Table 3**, it is observed that a dose-response effect did not exist. Taking the smallest concentration as reference, Puerto Ayacucho 1 venom presented the higher specific activity.

The amidolytic activity present in the studied venoms was also assayed in the presence of protease inhibitors. The results show that Factor Xa-like and t-PA-like activities decreased 70% in the presence of EDTA-Na, EGTA-Na and 1,10-phenan-

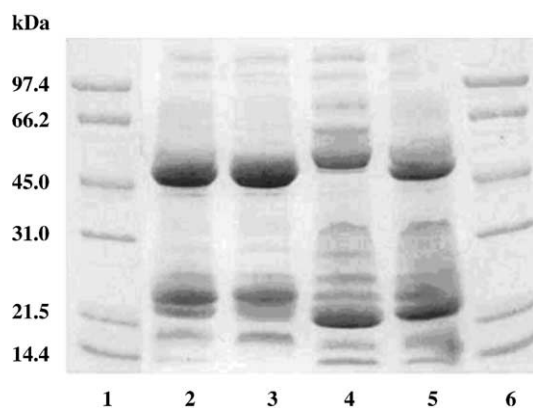


Figure 3 SDS-PAGE (12%) of *B. atrox* venoms (50 μ g) under reducing conditions. Gels were stained with brilliant blue Coomassie. Lines: (1, 6) LMW; (2) Parguasa; (3) Puerto Ayacucho 1; (4) Serranía del Cuao; (5) Puerto Ayacucho 2.

Table 1 Procoagulant activity

Venoms	Amidolytic method ^a (mUA/min/mg)		Coagulant method (IU Thrombin/mg) ^b	
	S-2222 (Factor Xa)	S-2238 (Thrombin)	Plasma	Purified Fibrinogen
Parguasa	294 ± 29 (<i>p</i> < 0.05 ^c)	438 ± 78	30.7 ± 2.0 (<i>p</i> < 0.01 ^d)	24.3 ± 0.7
Puerto Ayacucho 1	126 ± 17	190 ± 37 (<i>p</i> < 0.05 ^c)	17.5 ± 1.1	18.6 ± 0.2
Puerto Ayacucho 2	177 ± 20	473 ± 85	35.2 ± 1.5 (<i>p</i> < 0.05 ^c) (<i>p</i> < 0.01 ^d)	39.6 ± 1.4 (<i>p</i> < 0.05 ^c)
Serranía del Cuao	235 ± 39	383 ± 85	28.0 ± 1.1	25.3 ± 0.4

Data are expressed as mean ± S.D. (*n* = 4).

^a Amidolytic activity was expressed as ΔA 405 nm/min/mg protein.

^b Coagulant activity was determined by the Austen and Rhymes DEG [22]. The results expressed in thrombin-like units by plotting the clotting times against a calibration curve prepared with a thrombin standard (National Institute for Biological Standards and Control, London, England).

^c Statistical differences between groups.

^d Statistical differences between substrates (human plasma vs. purified fibrinogen).

trolin and 10% in the presence of SBTI, benzamidine/HCl, and aprotinin (results not showed).

The MDH present in Parguasa, Puerto Ayacucho 1, Puerto Ayacucho 2 and Serranía del Cuao venoms was of 2.5, 5.6, 5.6 and 4.6 μg/mouse, respectively. On the other hand, the LD₅₀ calculated for Parguasa, Puerto Ayacucho 1, Puerto Ayacucho 2 and Serranía del Cuao venoms were 4.0, 6.1, 7.9, and 8.3 mg/kg, respectively.

Fibrin-zymography of venoms

Fig. 5 shows plasminogen-rich fibrin-zymographic patterns of the venoms with sample doses giving a lysis area of 600 mm² in the fibrin plate. In Parguasa, Puerto Ayacucho 1 and Puerto Ayacucho 2 venoms, major bands of 27, 25 and 45–50 kDa, respectively, were observed. No well-defined lytic band in Serranía del Cuao venom was detected.

Discussion

Bothrops species are responsible for more human morbidity than any other group of venomous snakes. *Bothrops asper* remains the most medically

important pit viper in Central America [25] since it is responsible for approximately 50% of snakebite envenomation in this region. In South America, *B. atrox*, *Bothrops colombiensis* and *Bothrops jararaca* are clinically the most important. In Brazil and Venezuela, *Bothrops* species are responsible for more than 80% of all recorded snakebites [26]. In Venezuela, *B. atrox* species only lives to the south of the Orinoco River [26].

The existence of several clinical manifestations and hemostatic parameters may be induced by individual variability of the venom composition. Snake venoms vary in their biochemical composition and pharmacological profile, not only between different species, but also within a single species and in snakes of different ages and sex [27–29]. Snake venoms from Viperidae family contain a mixture of proteins with many biological functions, such as proteolytic activities, which can act on the hemostatic system. Generally, they are classified into several groups as follows: (a) fibrinogen clotting enzymes; (b) fibrino(geno)lytic enzymes; (c) plasminogen activators; (d) prothrombin activators; (e) factor V and factor X activators; (f) hemorrhagins; and (g) platelet aggregation inhibitors [30].

Table 2 Fibrinolytic activity

Venoms	Amidolytic method ^a (mUA/min/mg)				Fibrin plate activity ^b 10 μg sample mm ² (mm ² /μg)
	S-2251 (Plasmin)	S-2302 (Kallikrein)	S-2444 (Urokinase)	S-2288 (t-PA)	
Parguasa	10 ± 1	25 ± 7	33 ± 6	219 ± 21	100 ± 11 (10.0)
Puerto Ayacucho 1	8 ± 2	48 ± 2	21 ± 5	292 ± 44	257 ± 41 (25.7) ^c (<i>p</i> < 0.001)
Puerto Ayacucho 2	9 ± 1	27 ± 5	17 ± 7	246 ± 19	121 ± 11 (12.1)
Serranía del Cuao	16 ± 9	28 ± 1	28 ± 10	199 ± 19 ^c (<i>p</i> < 0.01)	51 ± 7 (5.1)

Data are expressed as mean ± S.D. (*n* = 4).

^a Amidolytic activity was expressed as ΔA 405 nm /min/mg protein.

^b Fibrinolytic activity was evaluated on plasminogen-rich fibrin plates using Marsh and Arocha-Pinango method [21].

^c Statistical differences between groups.

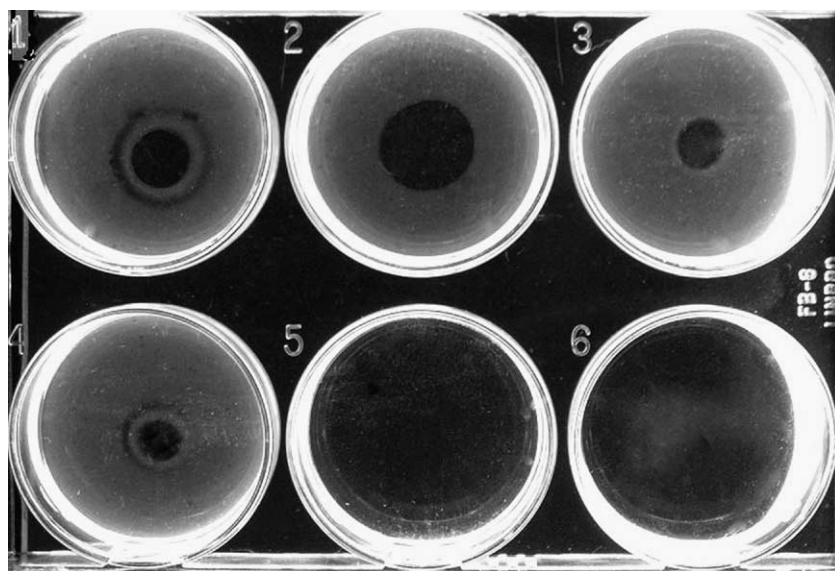


Figure 4 Fibrinolytic activity of *B. atrox* venoms (10 μg) was assayed in plasminogen-rich fibrin plates. Samples: (1) Parguasa; (2) Puerto Ayacucho 1; (3) Serrania del Cuao; (4) Puerto Ayacucho 2; (5) t-PA Standard; (6) Plasmin Standard.

Hemostatic disorders are the major signs of systemic envenoming in bothropic accidents. Systemic symptoms such as internal hemorrhage, hypotension, renal failure and shock may also occur [16,30–32]. Several studies have demonstrated that the blood coagulation disorders are frequently recorded in patients following systemic envenoming mainly by young *B. jararaca* [16] or *B. asper* [33]. Kamiguti et al. [32] suggested that true disseminated intravascular coagulation (DIC) through thrombin formation may also occur in such accidents, due to procoagulant components or a thrombin-like activity found in the venom of *B. jararaca*. These authors also reported that *in vitro* higher levels of prothrombin and factor X activators are present in the venom of juvenile *B. jararaca* compared with adults.

Snake venoms from different genera or species differ in their biochemical constitution. These variations are also observed intra-species and in snakes of different sex and maturity status [8,28,33–35].

Our observations also indicated that venoms from the same species, but from different geographical areas presented variations in the number of molecular mass electrophoretic bands (under reduced conditions) and chromatographic profile. The molecular exclusion chromatographic analysis, in relation to the proportion of the main protein bands evidenced differences among the four studied venoms. The results showed that Serranía del Cuao venom had the biggest proportion of high-molecular-mass components, while Parguasa and Puerto Ayacucho 1 venoms presented those of lowest molecular mass. These profiles also evidenced significant differences in the venoms of closed geographical localities (Puerto Ayacucho 1 and 2). These results were in agreement with previous observations carried out with the venom of *B. alcatraz* from Brazil [36].

The analysis of venom samples for fibrin-zymography showed major active bands of 27, 25 and 45–50 kDa approximately in Parguasa, Puerto Ayacucho

Table 3 Fibrinolytic activity of individual *B. atrox* venoms on plasminogen-rich fibrin plates

Venoms	Fibrin plate activity ^a mm ² (mm ² /μg sample)			
	μg sample			
	1	5	10	50
Parguasa	0	77 ± 5 (15.3)	100 ± 11 (10.0)	400 ± 34 (8.0)
Puerto Ayacucho 1	55 (55)	175 ± 21 (34.9)	257 ± 41 (25.7)	507 ± 38 (10.1)
Puerto Ayacucho 2	0	59 ± 5 (11.7)	121 ± 17 (12.1)	330 ± 25 (6.6)
Serranía del Cuao	0	40 ± 4 (8.1)	51 ± 5 (5.1)	255 ± 15 (4.5)

Effect of sample concentration on lysis area.

Data are expressed as mean ± S.D. (n=4).

Lysis area in mm² (brackets in mm²/μg sample).

^a Fibrinolytic activity was evaluated on plasminogen-rich fibrin plates using Marsh and Arocha-Pinango method [21].

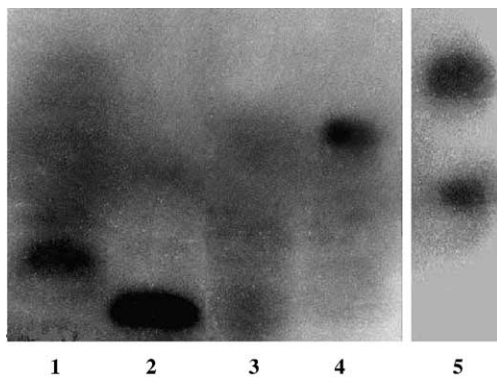


Figure 5 Fibrin-zymography of *Bothrops atrox* venoms. SDS PAGE (12%) and fibrizymography were carried out as described in Materials and Methods. Lines 1) Parguasa; 2) Puerto Ayacucho 1; 3) Serrania del Cuao; 4) Puerto Ayacucho 2; 5) tcu-PA Standard.

1 and Puerto Ayacucho 2 venoms, respectively. No well-defined lytic bands in Serranía del Cuao venom were detected. These results indicated the presence in Puerto Ayacucho 2 venoms of P-III class active metalloproteinases with molecular masses of 50 kDa. On the other hand, in Parguasa and Puerto Ayacucho 1 venoms, the fibrinogenolytic activity can be associated with a serine proteinase trypsin-like or a P-I class active metalloproteinases with molecular masses of 25 kDa. Additionally, these components could be in very low concentration in Serranía del Cuao venom.

It would be possible that geographical variation in venom composition reflects natural selection for feeding on local prey [35,37]. Other authors [38] have reported that same species of snake living in different locations differed in their venom composition and toxicity. For instance, the tiger snake (*Notechis scutatus*) venom from both Mount Gambia and Melbourne and also another population at Lake Alexandrina in South Australia had neurotoxic (notexin and scutoxin) presynaptic components, which varied in their proportional amounts.

In the present study, the procoagulant and fibrinolytic venom activities of adult specimens of mapanare (*B. atrox*) snakes from four Venezuelan locations were evaluated.

The procoagulant activity of the venoms was first evaluated by the amidolytic method. The presence of a direct thrombin-like and factor Xa-like activities in all of them was demonstrated. The results showed that Parguasa and Puerto Ayacucho 2 venoms presented the highest factor Xa-like and thrombin-like activities, respectively. Puerto Ayacucho 2 for clotting method was the most active venom. On the other hand, Parguasa venom was significantly most active on plasma compared with purified fibrinogen ($p < 0.001$) (30.65 versus 24.34

IU thrombin/mg) (Table 1). This suggested the presence of prothrombin and/or factor X activators such as it has been reported by Hofmann and Bon [16,17], since, from a procoagulant point of view, Parguasa venom may be able to produce a more intense DIC because the high factor Xa-like activity.

Procoagulant activity by both methods showed that differences between the localities also existed (Puerto Ayacucho 1 versus Puerto Ayacucho 2), which evidenced variations in the composition and proportion of the active venom components.

Fibrinolytic activity was evaluated with S-2251, S-2302, S-2444 and S-2288 chromogenic substrates, which displayed specificity for plasmin, kallikrein, urokinase, and t-PA, respectively. In all venoms, a high t-PA-like activity was observed. The fibrinolytic activity also was estimated using fibrin plates. All venoms were active on plasminogen-rich fibrin plates. Puerto Ayacucho 1 venom presented the higher fibrinolytic activity by both methods. An appreciable difference in specific activity among venoms of different areas was observed.

The activity determined on fibrin plates with 10 μ g protein was higher in Puerto Ayacucho 1 venom. To this concentration on Parguasa and Puerto Ayacucho 2 venoms, a fibrin area without lysis inside a lysis area (indicating the presence of possible fibrinolytic inhibitors) was observed (Fig. 4). These results suggested the profound effects that *Bothrops* venoms cause on hemostatic system of preys and humans, which would not depend only on the quantity of venom, age and sex [28] of the snake but also on the balance between activators and inhibitors present in the venom.

The above fibrinolysis findings could also have repercussions on the final balance of patient hemostasis. Puerto Ayacucho 2 venom could induce a more intense hemorrhagic syndrome than other studied venoms, via endogen fibrinolysis activation by t-PA-like activity.

The amidolytic activity present in the four studied venoms was also assayed in the presence of protease inhibitors. The results showed that factor Xa-like and t-PA-like activities decreased 70% in the presence of EDTA-Na, EGTA-Na and 1,10-phenantroline, and 10% in the presence of SBTI, benzamidine, and aprotinine, suggesting that metalloproteinases are among the most abundant enzymes found in these venoms.

In summary, the results demonstrated several differences between the four *B. atrox* venoms from different geographical areas. The procoagulant activity depended on the utilized method (amidolytic versus clotting). Parguasa and Puerto Ayacucho 2 venoms presented the procoagulant activity for both methods ($p < 0.05$). Furthermore, Parguasa

Table 4 Comparison of activities of venoms from four specimens of *B. atrox*

<i>B. atrox</i> venoms	Coagulant activity ^a		Amidolytic activity ^b t-PA-like (S-2888)	Fibrinolytic activity ^c (10 µg sample)	MHD ^d	LD ₅₀ ^e
	Human plasma	Purified fibrinogen				
	IU Thrombin-Like/mg		mUA/min /mg	mm ² (mm ² /µg)	µg/mouse	mg/kg
Parguasa	30.7 ± 1.9	24.3 ± 0.7 ^f (<i>p</i> < 0.001)	219 ± 21	100 ± 11 (10.0)	2.5	4.0
Puerto Ayacucho 1	17.5 ± 1.1	18.6 ± 0.2	292 ± 44	257 ± 41 (25.7) <i>p</i> < 0.001 ^g	5.6	6.1
Puerto Ayacucho 2	35.2 ± 1.4	39.6 ± 1.4 ^f (<i>p</i> < 0.001)	246 ± 19	121 ± 11 (12.1)	5.6	7.9
Serranía del Cuao	27.9 ± 1.1	25.3 ± 0.4	199 ± 19 <i>p</i> < 0.05 ^g	51 ± 7 (5.1)	4.6	8.3

Data are expressed as mean ± S.D. (*n* = 4).

^a Coagulant activity was determined by the Austen and Rhymes DEG [22]. The results expressed in thrombin-like units by plotting the clotting times against a calibration curve prepared with a thrombin standard (National Institute for Biological Standards and Control, London, England).

^b Amidolytic activity was expressed as ΔA 405 nm /min/mg protein.

^c Fibrin plate method: 10 µg sample.

^d MHD: minimum hemorrhagic dose, determined by Omori-Satoh et al. [19].

^e LD₅₀: Lethal doses 50, determined by Spearman-Kärber method (1978) [20].

^f Statistical differences between substrates (human plasma vs. purified fibrinogen).

^g Statistical differences between groups.

venom had also the highest hemorrhagic activity. These results are correlated with the lowest LD₅₀ that this venom displays (Table 4).

In relation to the fibrinolytic activity, Puerto Ayacucho 1 venom was the most active, equally for fibrin plates (*p* < 0.001) as for the amidolytic method (t-PA like) (*p* < 0.05). This venom had the lowest coagulant activity (*p* < 0.05), which induced to think that probably its procoagulant activity was interfered by its fibrinolytic activity. It is fundamental to carry out studies with purified systems trying to determine the different sites of action on coagulation mechanism and fibrinolytic system.

Venezuelan Amazonian forest environment has a high potential for genetic variations in the same species, such as body size, growth rate, as well as in venom composition. This snake distribution on different geographical areas offers a helpful model to investigate changes in components and activities in snake venoms due to the effects of natural selection pressure, a consequence of isolation or for feeding [36,37] on local prey.

This work suggest that since snakebites are inflicted by specimens from different geographical locations, pools of venoms from the same species captured at different geographical regions may be used for immunizing animals in antivenom production centers.

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