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Protein profile, morphometric analysis and toxicity of *Bothropsatrox* (viperidae) venom from Colombian Amazon

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ABSTRACT

The genus Bothrops is one of the most important in America. The main clinical effects of envenoming by botropoid venoms are local tissue damage, life threatening bleeding and blood coagulation disorders, in addition to shock and renal failure. Currently, there are no reports from specimens of B. atrox in the Colombian Amazon. Therefore, this work aims to study the preliminary protein composition, morphometric characteristics and toxicity of B. atrox venoms from specimens belonging to the Caquetá Colombian department. The results showed no differences between the morphometrical parameters of male and female snakes, meanwhile, the protein composition showed an absence of a protein fractions between 91-100 and 111-120 KDa in male specimens, which are present in females with a relative abundance, the median lethal dose found was 63.8 µg/mouse, which are in accordance with others snake venoms found in Peru, but are lower than others species like Crotalusdurissuscumanensis, M. mipartitus and M. dumerilii.

Keywords: Protein profile, toxicity of venoms, morphometrical parameters

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INTRODUCTION

The study of snake venom has increased in the last years due to the high mortality rate caused by snake-bite. The knowledge about the content and characterization of proteins and peptides in snake venoms allows the improvement in the clinical diagnosis, medical treatment and also it is considered as the “cornerstone” for basic research directed to discover new strategies to produce antivenoms and other drugs with potential clinical use.[1]

One of the most important snake genuses in America is the *Bothrops* genus, also known as “mapaná”, “four noses”, “pudridora”or “cat hair”. This snake genus comprising between 32 or 37 species [2] of Neotropicalpitvipers, commonly referred as “lanceheads”. The Neotropicalpitvipers are widely distributed in tropical Latin America, from Mexico to Argentina including parts of the lower Caribbean islands.[2]

According to statistical data, the species *Bothropsasper* is the leading cause of snake-bite mortality and morbidity in Central America.[3, 4] Another identified species, *Bothropscolombiensis*, has been reported to account for over 36% of the more than 5000 accidents caused every year by snake-bites in Venezuela,[5, 6] on the other hand, *Bothropsatrox* is responsible for more human fatalities in South America than any other American reptile.[4]

B. asper and *B. atrox* inhabit the tropical rainforest up to 1200 m east (both species) and in the case of *B. asper* also inhabit in the north and west of the Colombian Andes.[2] They inflict the 70 to 90% of the 3000 bites reported every year in Colombia,[7, 8] these species are highly adaptable and widely distributed (Figure 1). The ontogenetic and geographical variability in their venom composition and pharmacological profile have been also reported in species from *B. atrox* and *B. asper* from the Colombian departments of Meta and Antioquia, respectively (Figure 1).[8-10]

Figure 1. Geographical location of snake species *B. asper* (black spot: Antioquia/San Carlos) and *B. atrox* (gray spot: Meta/Villavicencio) studied in Colombia

The zone delineated by the arrow marks the geographical distribution of B. atroxthat extends to the southeast towards the Peruvian and Brazilian Amazon. The white spot with coordinates 75,5°25´N, 1.4°30´E located the place of collection of juvenile specimens B. atrox used in this study.

Additionally, It has been reported that the species *B. atrox* is implicated in most of the approximately 3500 human snake-bites registered in the Brazilian Amazon region,[11] and the mortality in rubber tappers and indigenous people has been estimated to be 400 of 100,000 inhabitants in some areas of the rain forest.[12] Yearly, in Ecuador near of 1200 –1400 cases of snake-bites are reported in 19 of the 21 provinces. In the East of the Andes, the principal venomous species are *B. atrox* and the two-striped forest pitviper *Bothriopsisbilineatasmaragdina. Bothropsatrox* causes almost 60% of the snake-bites, whilst *B. bilineatasmaradigma* is responsible for the 36% of the bites,[13] for this reason, *B. atrox* is considered as one of the most relevant snake species in America.

It has been reported that the botropoid venom causes multiple effects in the human health. Within the main clinical effects of envenoming by botropoid venoms are local tissue damage (myonecrosis, hemorrhage and edema), life threatening bleeding and blood coagulation disorders, in addition to shock and renal failure. Besides these effects, necrosis and bacterial infection at the site of the bike may cause permanent physical handicap.[3, 4]

Due to the differences in the symptomatology after an ophidian envenomation involving specimens of the same species in different geographical locations,[14] it is necessary a better knowledge of the chemical composition, pharmacological and immunochemical features required for making effective antivenoms. Currently there are no reports from specimens of *B. atrox* in the Colombian Amazon. Therefore, this work aims to study the preliminary protein composition, morphometric characteristics and toxicity of *B. atrox* venoms from specimens belonging to the Caqueta Colombian department, in order to tentatively determine the ontogenetic characteristics of same species in different geographic locations.

EXPERIMENTAL SECTION

Snakes

The animals were identified and classified taxonomically by the biologist Sergio Cubides of the SUA serpentarium of the University of Amazonia (UNIAMAZONIA) following key Duellman (1978).[15] The snakes handling was given under the permission of study for scientific research on biological diversity, by the resolution 049 of 25 July 2008 of regional CORPOAMAZONIA Caquetá, Colombia. The weight, length (head, tail and total length) and venom pH were determined.

Venoms

The venoms used in this study came from six young specimens of *B. atrox* in good health conditions, captured in a rural area of Florence-vía Morelia and around the Corporación de Ferias y Mataderos del CaquetáCOFEMA. The animals were kept in captivity at the serpentarium of UNIAMAZONIA (SUA), Central headquarters, Florencia, Caquetá. Ophidians remained housed in individual cages at a temperature of 24-30 °C, with water ad libitum and were fed with one or two mice (Musmusculus) every two weeks according to their size. The venom was removed manually from venom glands and immediately freezed and lyophilized, then was stored at -20 °C, until the time of use.

Protein Characterization

Protein quantification was performed using the micro-biuret method, according to Itzhaki and Gill (1964).[16] A calibration curve was determined using different concentrations of bovine serum albumin (from 1 to 100 mg/mL). The protein contents of crude *B. atrox* venom were assessed by polyacrylamide gel electrophoresis treated with ß-mercaptoethanol[17] using the chip pro260 of the ultracapilar The Experion^{TM} System of Bio-Rad Laboratories, Inc USA. Molecular mass standards of 10, 20, 25, 37, 50, 75, 100, 150 and 260 kDa were used as a reference of linear response, concentrations of 2.5 to 2000 nanograms per microliter and detection with Coomassie Brilliant Blue G250.

Biological Activity

The lethal dose (LD) was evaluated by intraperitoneal injection into mice (Balb/C strain, 18–20 g) using groups of 8–10 mice at each dose. The LD_{50} was calculated by Probit analysis.[18] Briefly, serial dilutions of a pool venom were evaluated by intraperitoneal injection (0.5 mL in saline), the animals were observed for 48 h and their clinical signs were recorded. Saline were used as a negative control.

Statistical analysis

The results obtained were expressed as the mean ±standard error of the mean (SEM) and statistically analyzed by unpaired t test. Differences with $p < 0.05$ were considered statistically significant.

RESULTS

The morphometric characteristics of all snakes in study are shown in Table 1. The greater dispersion of data in females was observed in body weight, whereas in males the head diameter was the most varied, however, we found no significant differences ($p > 0.05$) between the different variables studied between male and female.

Table 1. Morphometric characteristics of the different snakes studied

	Weight (g)	Full Length (mm)	Tail Length (mm)	Length of Head (mm)	Diameter of Head (mm)
Male	400 ± 70	1132 ± 120	151 ± 24	39 ± 9.3	74 ± 44
Female	388 ± 60	1156 ± 13	132 ± 3.5	33 ± 1.5	34 ± 0.6

Physical and chemical analysis of the venom

To obtain the venoms were made three spaced sampling by a period of 22 days and was determined: dry mass (g), pH, moisture (%) and protein concentration (mg/dL).

The average of dry mass obtained after lyophilization was 43.8±28.1 mg with a moisture content of 65.9 ±16.4% and an average protein concentration of 67.4±19.8 mg/dL for male snakes, meanwhile, the female snakes showed no differences ($P > 0.05$) compared with the male snakes (Table 2).

Table 2. Physical and chemical characteristic of the venoms obtained from the *B. atrox* snakes

	dry mass (mg)	pH	moisture (%)	protein concentration (mg/dL)
Males	43.8 ± 28.1	7.1 ± 0.3	65.9 ± 16.4	67.4 ± 19.8
Females	47.4 ± 19.7	7.1 ± 0.7	67.2 ± 14.8	70.6 ± 18.3

Polyacrylamide gel electrophoresis

The electrophoretic profiles obtained from each venom samples, it is observed in Figure 2. Each electrophoretic profile allowed to determine the number of protein fractions, the molecular weight (kDa) and the respective relative abundance (%) through the use of automated capillary electrophoresis (Table 3). The analysis of each electrophoretic profile indicated that juvenile snakes of *B. atrox* showed protein fractions with molecular weight between 9.35 to 160.99 kDa.The Table 3 shows a predominance of fractions between 21 - 50 and 101-110 kDa, while the distribution for the rest of venoms in the study ranged from 71-80 to 91-100 kDa.

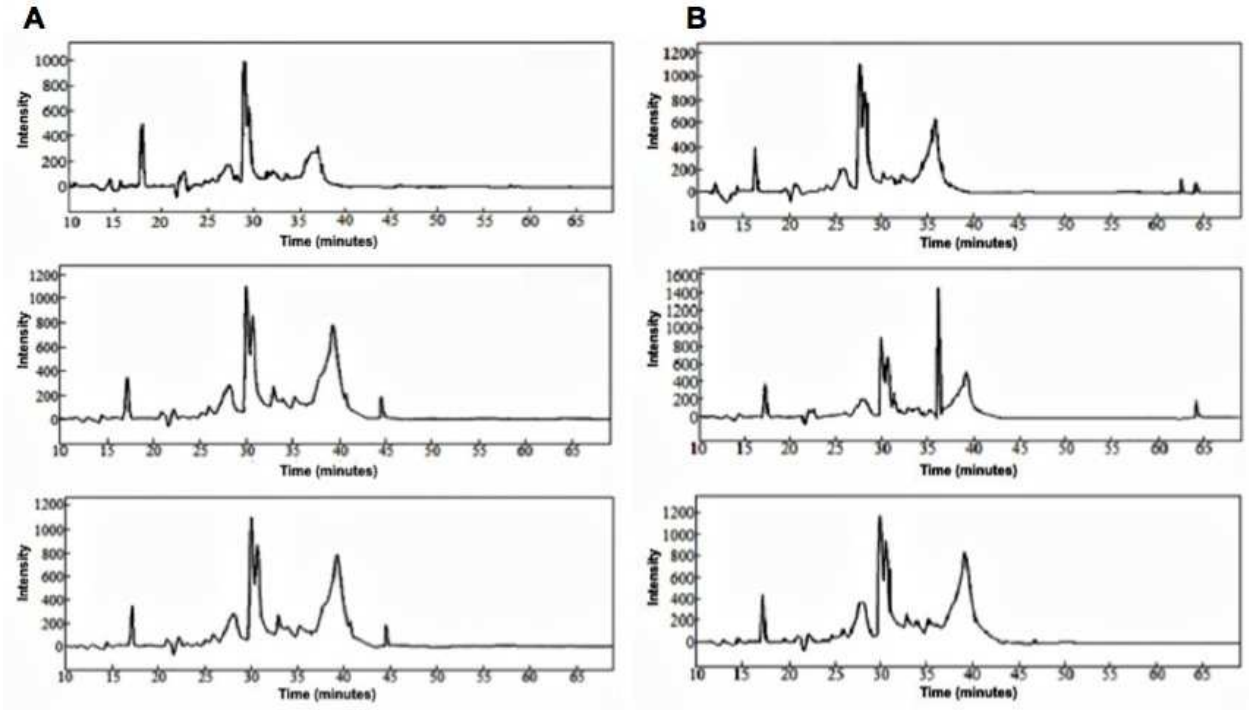

Figure 2. Electrophoretic profiles for samples obtained from juvenile venom of *B. atrox* snakes. A) electrophoretic profile of the male snakes and B) electrophoretic profile of the female snakes

Table 3. Relative abundance (%) of the different protein fractions found in the electrophoretic analysis of juvenile snake venoms of *B. atrox* (automated capillary electrophoresis)

Molecular Weight	Relative Abundance (%)	
	Males	Females
<10	0.85 ± 0.73	1.0 ± 0.9
10-20	3.1 ± 2.9	1.9 ± 0.7
21-30	16.1 ± 1.6	27.3 ± 20.1 *
31-40	22.0 ± 14.9	17.5 ± 15.8
41-50	14.7 ± 5.8	9.4 ± 9.2
51-60	2.4 ± 2.2	3.1 ± 1.7
61-70	4.3 ± 0.7	3.0 ± 0.8
71-80	2.6 ± 1.0	12.8 ± 17.3 **
81-90	10.0 ± 12.3	2.8 ± 4.8
91-100	0	10.7 ± 18.5 ***
101-110	22.2 ± 19.2	9.0 ± 15.6
111-120	0	0.8 ± 1.3
>120	0.3 ± 0.5	0 **

** p< 0.05; ** p < 0.01; *** p < 0.001*

Biological Activity

The table 4 shows the results of the determination of the LD_{50} using a venom pool of the snakes studied. The results shows that at a concentration of 70.3 µg/mL of poison, all the animals died (100% death), while at a concentration of 63.8 µg/mL 50% of the animals die (LD_{50} 63.8 µg/mL). Moreover, with a concentration of 55.1 µg / mL, no animal died (0% dead).

Table 4. Lethal Dose test of the venom obtained from the *B. atrox* snakes

Venom (µg/mL)	55.12	57.88	60.77	63.8	66.99	70.34
Dead Animals (48 h)	0/4	1/4	1/4	2/4	2/4	4/4

DISCUSSION

Snake venom contains a mixture of powerful proteins and peptides that have evolved to be targeted to receptors, ion channels, or enzymes,[19] in addition to some carbohydrates, nucleosides, lipids, and metal ions, whose functions are not all known.[20] Poisonous snakes are responsible for around 50,000 deaths among five million cases of ophidian accidents per year in the world, especially in the rural areas of tropical countries in Asia, Africa, and South America.[21]

The morphometric characteristics, the content and protein profile along with the median lethal dose of venom of six juvenile snakes (*Bothropsatrox* species) were determined. The electrophoretical profiles were similar in the snakes with the same development status with a minimal variation ($p > 0.05$) in relation to the sex of the animals. The use of automated-capillary electrophoresis combined with the analysis of electrophoretical profiles allowed the determination of the number of protein fractions, molecular weight (KDa) and its relative proportion (%) (Table 3). The results indicated that *B. atrox* juvenile snakes have a range of protein fractions comprised between 9.35 to 160.99 KDa. Regarding to female snakes, the results showed that the predominant protein fractions were between 21-50 and 71-80 KDa, these fractions represent the 67.0% of the protein mixture. In male snakes, the results showed that the predominant protein fractions were between 21-50 and 101-110 KDa, representing the 75.0% of the protein mixture. The obtained results differ with the reports of venom of *B. atrox* from Perú, Malaga et al. (2000) [22] described two common protein bands in all cases, one at 66 KDa approximately, which is not major in our samples (< 4.0%), and another band with a molecular weight of 14.3 KDa, band that was not found in the present study. In a work that studied the electrophoretical patterns of five snake venoms from Bothrops gender from Perú, including the species *B. atrox*, *B. barnetti*, *B. brazili*, *B. hyoprora* and *B. pictus*,[23] it was observed a number of 14 bands for *B. barnetti*, three of them with less than 66 KDa, while the venom from *B. bazili* had seven bands with less than 45 KDa, which agrees with the results observed in this study.

An electrophoretical analysis using SDS-PAGE in *Bothropsalternatus* reported by Gay et al., (2004) [24] showed a band with a mass range between 45 to 68 KDa, similar to the results described here and its fractions correspond to 7% of the mixture in this case. A second band, exhibited proteins in a range from 29 to 36 KDa, being one of the most abundant in our work (Figure 2, Table 3). A third band described by Gay et al., (2004) [24] at 24 KDa, was found here between 20-25 KDa and finally, a band between 14.2-20 KDa, which was a minor band in this study. A study of the proteins venom of *Bothropsjararacá*[25] showed three main bands, one between 14.2 and 20.1 KDa, which was a minor band in our study. A second band was found at 24 KDa approximately, which was similar to the band described here, and a third band between 45 and 66 KDa. The last band was comparable with a protein band reported in our work, but with a less amount (approximately, 7%). An electrophoretical profile of venom from *Bothropsvenezuelensis*[26] presented twelve protein bands with molecular weight between 10 and 61.5 KDa. Eight of them were found in the range of 10 to 15 KDa, in this work, the presence of proteins in this range was minor. Protein characterization of *Bothrops* venom in Argentina using SDS-PAGE evidenced that *Bothropsalternatus* showed 11 bands, *B. neuwiedi* expressed 9 bands, while *B. ammodytoides*, *B. jararacá* and *B. jararacassu* showed nine, eight and six bands, respectively. Three bands were characteristic in the samples with variable proportions, summarized as < 14 KDa, 20-30 KDa and 30-99 KDa.[27] These data are in concordance with the results obtained in the present study and they indicate a little variation in the protein composition of the venom according to the geographical location of the studied animals.

Available information about *B. atrox* venom proteins shows a variable range of molecular sizes. According to the intervals presented in Table 3 and online information (www.uniprot.org), of the first molecular weight interval (<10 KDa) it have been described some proteins as venom prothrombin activator (www.uniprot.org) and other proteins with hemagglutinin activity, like C-type lectingalatrox.[28] Snake venom metalloproteinase Batx-1 exhibited haemorrhagic activity [29] while disintegrinbatroxostatin inhibits fibrinogen interaction with platelets.[30, 31] In the next protein interval, from 10 to 20 KDa, it have been described enzymes like L-amino-acid oxidase [32, 33] and phospholipase A2 homolog 1.[34] In the range of 21-30 KDa it has been reported proteins of the venom of *Bothropsatrox* having enzymatic activity, some of these proteins are cathelicidin-like peptides,[35] thrombin-like enzyme batroxobina[36] and snake venom metalloproteinase atroxlysin-1.[37] In the range from 41 to 50 KDa, it have been reported some metalloproteases, known as zinc metalloproteinase-disintegrin-like batroxstatin-1, 2 and 3.[38]

On the other hand, proteolitic and coagulant activity have a wide range of distribution (30-100 KDa) in the venom of *Bothrops* species, while phospholipase activity is closer in these species (10-20 KDa), except in *B. alternatus* that has a higher molecular weight range (35-50 KDa).[39] Characterization of *B. atrox* venom proteins from Ecuador, Peru, Brazil and Colombia evidenced an identical molecule K49-PLA2 with 13,826 Da. It has been described that the molecular weight of *B. atrox* venom in those countries varies from 6 to 97.4 KDa.[40] An investigation about *B.*

colombiensis venom in Venezuela showed six protein bands between 23.0 and 40.3 KDa. The bands at 26.5 and 27.5 KDa expressed a potent fribrinolitic activity,[41] in this work, similar protein fractions were found.

The results show that at a concentration of 70.34 µg/mL all inoculated mice died (4/4), thus showing that the median lethal dose is 63.8 µg/mouse. The LD_{50} found in this study is less potent that the reported by Artunduaga M., 2008 [42] for juvenile snakes of the same species (44.0 µg/mouse) and for the same geographic region. According to the above, the pool of venoms used here has little effectiveness in evaluating an antivenom. Similar studies on acute toxicity in snake venoms of *Bothropsasper* and *Porthidiumnasutum* (formerly *Bothropsnasutus*) of Antioquia and Choco, registered values of 66.2 µg/mouse (49.5 - 88.6 µg/mouse) and 109 µg/mouse (82-131 µg/mouse), respectively.[43] Another study of the LD_{50} for the *B. atrox* venom reported 67.87 µg/mouse,[44] a similar result to that obtained here.

The average weight of a mouse used in this study was 19 g, therefore the LD_{50} would be equivalent to 3.36 mg/kg, which is a higher value compared to the registered (0.210 mg/kg) for the rattlesnake *Crotalusdurissuscumanensis* present in the town of Porshoure, Venezuelan Guajira, indicating that the latter is highly toxic.[45] In *B. atrox* has been reported LD_{50} values of 3.33 mg/kg (2.79 to 3.96 mg/kg),[46] very similar to the value reported in this study.

Other reports of LD_{50} are (mg/kg): *B. asper*2.5 ±0.4, *Ath. Nummifer* 7.0 ±1.7, *C. d. durissus* 5.7 ±0.8, *C. basilisks* 10.6 ±2.8, *C. atrox* 2.4 ±3.0, *C. scutulatus* 2.3 ±0.5, *Agk. Bilineatus* 5.8 ±1.0 and *M. nigrocinctus*2.3 ±0.5,[47] which are similar to those in this study. Compared with the LD_{50} values (µg/mouse) of snakes of the genus *Micrurus*: *M. surinamensises* (13.56 µg/mouse); *M. mipartitus* (5.53 µg/mouse); *M. isozonus* (28.92 µg/mouse); *M. dumerilii* (8.83 µg/mouse) and *M. medemi* (47.2 µg/mouse) shows that *B. atrox* venom is less potent

CONCLUSION

The snakes distribution from different geographical areas offers a helpful model to investigate changes in their components and activities of its venoms. The knowledge of the protein profile of snake venoms is a cornerstone for the development of antivenoms more efficient and specific, taking into account the evolution of venoms system of different geographical localizations. The proteome of both female and male juvenile specimens of *B. atrox* from the Caquetádepartment is very similar, the only difference to note is the absence of protein fractions between 91-100 and 111-120 KDa in male specimens, which are present in females with a relative abundance of 10.7 and 0.8%.

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