

Original Research Paper

Identification of Venom-Specific Fingerprints of Clinically Relevant Uruguayan Snakes by MALDI-TOF

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Abstract: Snakebite envenomation is a particular public health problem in many areas of the world, including extensive areas of Latin America. In particular, in Uruguay, snakebite accidents are caused by two snakes of the genus *Bothrops*: *Bothrops alternatus* and *Bothrops pubescens*. The only effective treatment available is the administration of the specific antivenom. Although there is an ELISA that allows the determination of venom in serum samples, the technique does not allow discrimination between the two species and can also generate uncertainty in the case of foreign snakes, highlighting the need for alternative analytical methods that ensure species identification. The aim of this work was to use of Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) for the identification and discrimination of *B. pubescens* and *B. alternatus* venoms and evaluate the suitability for diagnostic use. The two venoms were compared by SDS-PAGE and MALDI-TOF MS. The results showed similar protein profiles for both venoms with differences in the distribution of bands by SDS-PAGE densitometry. However, the use of the MALDI-TOF MS technique allowed discrimination between these two venoms, providing characteristic “fingerprints” that are proposed as a new tool suitable for clinical diagnosis.

Keywords: Snake Venom, Densitometry, Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS)

Introduction

Snakebite envenomation is a neglected tropical disease and a particular public health problem in many areas of the world, including extensive areas of Latin America (Theakston *et al.*, 2003; WHO, 2010). Recently the World Health Organization (WHO) estimate in about 2.7 million cases of envenoming and 81 000–138 000 deaths every year (WHO, 2019).

In Uruguay, an average of 65 snakebite accidents are reported each year (Morais *et al.*, 2006; Morais *et al.*, 2012). All are caused by snakes in the genus *Bothrops*: *B. Pubescens* (BP) and *B. Alternatus* (BA). Accidents generally occur during the warm months of the year, coinciding with the period of greater biological and metabolic activity of the snake. Snakebite accidents are almost exclusive to suburban and rural areas, occurring predominantly in rural workers. The toxic effect of *Bothrops* venom is mainly characterized by alterations in coagulation and important local damage that can lead to amputation of the affected limb in severe cases (Markland, 1998; Matsui *et al.*, 2000). These toxic

effects are caused by several protein families including metalloproteases, serinoproteases, phospholipases A₂, among others (Calvete *et al.*, 2007; Calvete, 2011a; Gay *et al.*, 2015). The envenomation diagnosis is based on clinical evidence and coagulation parameter analysis, but it is not possible to distinguish between the two species.

Antivenom constitutes the only effective treatment for snake bite envenomation (Chippaux and Goyffon 1998; Theakston *et al.*, 2003). Antivenoms are constituted by antibodies purified from immunized animals against snake venom. Every snake specie have a particular profile of toxins showing immunological differences and thus the antivenom is specific against the snake specie (WHO, 2010).

Numerous countries have many snake species that can cause severe envenoming so it is important to discriminate the specie to know what type of antivenom should be used (WHO, 2019). The snake antivenom used in Uruguay is bivalent (neutralize the venom of the two species), so the discrimination of the two types of venom

is not important for patient treatment, but it could be useful for the determination of the physiopathological differences that can exist between them.

Additionally, the entry of foreign specimens as pets for private collections has recently introduced a situation in the country that is not completely regulated and could generate an accident involving confusion between a foreign and a native snake; consequently, the antivenom treatment could be unsuccessful. For doubtful cases, an Enzyme-Linked Immunosorbent Assay (ELISA) is available and can detect venom antigenaemia with a very good sensitivity (10 ng/mL), but it is only effective for native venoms (Morais *et al.*, 2012).

The use of heterologous proteins for human treatment might activate the immune system and cause adverse reactions, including mild symptoms such as chills, nausea and fever or more serious problems such as bronchospasm and anaphylactic shock and the rational use of antivenom is thus very important (Otero-Patiño *et al.*, 1998; Morais and Massaldi, 2009; Morais, 2018)

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) technique is highly developed for the diagnosis of microorganisms, with important databases of profiles for the identification of pathogens (Pranada *et al.*, 2015). The technique allows the generation of characteristic peptide and protein profiles that serve as a "fingerprint" for the identification of the species, which are becoming a very important tool in diagnostic material. The robustness of the technique has been demonstrated for a wide range of organism (Demirev and Fenselau, 2008; Qian *et al.*, 2008; Fenselau, 2013).

Furthermore, the technique can quickly identify proteins present in unknown cellular suspensions or from complex mixtures (Liang *et al.*, 1996; Pineda *et al.*, 2000). The ability of this technique to rapidly and accurately discriminate for bacterial species has become a revolution in the clinical microbiology and has demonstrated the power of this tool (Demirev and Fenselau, 2008; Hrabák *et al.*, 2013; Karimi and Amanati, 2016).

In the case of snake venoms, MALDI-TOF MS is commonly used to identify the composition of a particular snake venom or for identification of snake species in crude venom but not for diagnostic purpose in patients biological samples (Chapman *et al.*, 2003; Calvete *et al.*, 2007; Calvete, 2011b).

The aim of this work was to use of MALDI-TOF MS for the identification and discrimination of *B. pubescens* and *B. alternatus* venoms and evaluate the suitability for diagnostic use.

Materials and Methods

Venom of *Bothrops Pubescens* (BP) and *Bothrops Alternatus* (BA) were obtained from the Serpentarium at the Institute of Hygiene, Uruguay. MALDI matrix 3, 5-

Dimethoxy-4-hydroxy-cinnamic acid (sinapinic acid) and peptide calibration standard mix were purchased from Bruker Daltonics (Billerica, MS, USA).

SE 260 mini vertical gel electrophoresis unit and Coomassie Brilliant Blue were purchased from Amersham Bioscience and Sigma, USA respectively.

SDS-PAGE Analysis

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli (1970). Non-reducing samples of venom (50 µg) or reduced samples (plus 0.1 M DTT) were loaded onto a 12.5% acrylamide gel and run in an SE 260 mini vertical gel electrophoresis unit at 20 mA for 1½ hour. The gels were then stained with Coomassie Brilliant Blue.

Densitometry Analysis

Band quantification was performed using Gel-Pro Analyzer 3.1 software (Media Cybernetics). The densitometry analysis was performed on the resulting images by a desktop scanner and digital photographic machine. The band selection was performed manually, the same filter was applied to eliminate noise in all cases and the quantification was determined by the relative percentage of the band in relation to the amount loaded in the lane.

MALDI-TOF Analysis

MALDI measurements were conducted on a Microflex LR MALDI-TOF (Bruker Daltonics, Billerica, MS, USA) with a 337 nm nitrogen laser operated in positive ion linear mode with delayed extraction and optimized in the m/z range of 0 to 20 kDa. Calibrations were performed with a peptide calibration standard mix (Bruker Daltonics). The laser was fired 100 times at each of ten locations for each sample well on a 96-well plate for a cumulative 1000 shots per sample well taken at 30 % intensity.

At the time of analysis, 1 µL of each of the samples was mixed with 1 µL of matrix solution Sinapinic Acid (SA) 10 mg/ml in sterile water with 1% TFA or 2, 5 Dihydroxybenzoic Acid (DHB) at a ratio of 1:1. The sample and matrix mix was spotted onto a 96-well stainless steel plate and allowed to air dry for 15 min at room temperature.

Results

All the venoms patterns were analyzed by SDS gel electrophoresis and by a mass spectrometry. The matrix selected for the identification of the venoms proteins were SA and DHB, these organic matrices demonstrated to provide an effective ionization of other organisms. The spectra patterns obtained were evaluated on the

basis of signal strength, resolution and reproducibility. These criteria are all essential for effective fingerprinting (Krishnamurthy *et al.*, 2000; Fenselau, 2013)

Figure 1 shows the protein profiles of both venoms obtained by SDS-PAGE electrophoresis. High similarity of the protein location bands of the two venoms was observed. However, the densitometry of the bands shows important differences in concentration (Table 1). The most important band of BA is located at 49-51 kDa (62 kDa under reducing conditions), corresponding to approximately 32% of the total venom. The counterpart of BP corresponds to only 6% of the total venom. On the other hand, the most important band of BP is located at 18-20 kDa, corresponding to 33% of the total venom, while in BA, it corresponds to 10%.

Figures 2 and 3 show the mass profiles of both venoms with SA and DHB matrices, respectively. The highest sensitivity achieved with SA was 1 ng (1000 ng/mL), whereas the use of DHB matrix allowed an increase in the sensitivity to 100 pg (100 ng/mL). The presence of plasma in the venom sample did not interfere with the peak visualization.

In the case of *Bothrops alternatus* with a concentration of 1 ng/mL, the protein profile (Fig. 2) showed three outstanding peaks at approximately m/z 2638, m/z 3821, m/z 7712, whereas for *Bothrops pubescens*, the peaks were at approximately m/z 8869 and m/z 13771.

Table 1: The relative concentration of most abundant components of both venoms

Band (MW)	<i>Bothrops alternatus</i>	<i>Bothrops pubescens</i>
49-51 KDa	32 %	6 %
26 KDa	12 %	2 %
23 KDa	9 %	20 %
18-20 KDa	9 %	33 %

Figure 3 shows the protein profile of 100 ng/mL of *Bothrops alternatus* venom with three outstanding peaks at approximately m/z 2274, m/z 3543, m/z 4884 and of *Bothrops pubescens* with three outstanding peaks at approximately m/z 4825, m/z 6830 and 13766.

Discussion

Envenomation resulting from snakebites is an important public health problem, particularly in South and Central America. The distinct clinical manifestations and lethality in snakebite are the result of different types of venom, which are complex mixtures of biologically active components (WHO, 2019). In particular, the venoms of many snake species cause important alterations in the coagulation system and local tissue damage (WHO, 2019). The presence of these alterations is, in fact, the indication that the patients were envenomed by snake venom, increasing the need for access to antivenom treatment via the health system (Morais *et al.*, 2012).

In Uruguay, envenomation by *Bothrops* snakes shows similar clinical manifestations, making it impossible to discriminate between the two species. Nevertheless, the identification of the species is irrelevant for clinical purposes due to the bivalent nature of the antivenom (Morais *et al.*, 2012). Recently, however, many people in the country have acquired foreign snake species as pets. Although many of the snakes of the Viperidae family generate similar clinical manifestations, it becomes very relevant to identify the snake species in case of a possible accident involving a foreign snake because the clinical manifestations might be similar, but the antivenom cross neutralization could fail.

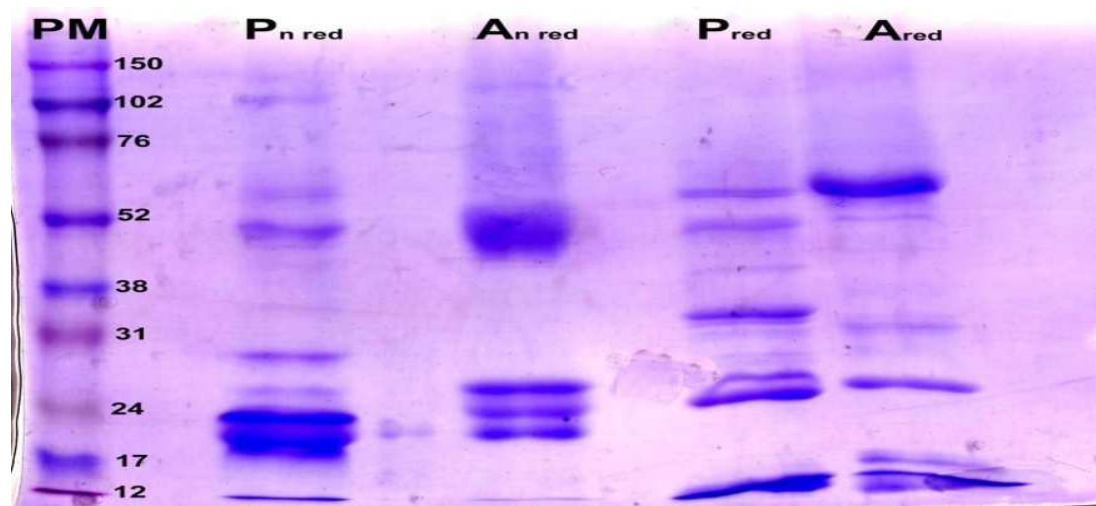


Fig. 1: Proteins profiles of *B. alternatus* (A) y *B. pubescens* (P) by SDS PAGE. Lane (1) MW; Lane (2) BP not reduced. Lane (3) BA not reduced. Lane 4 BP reduced. Lane 4) BA reduced

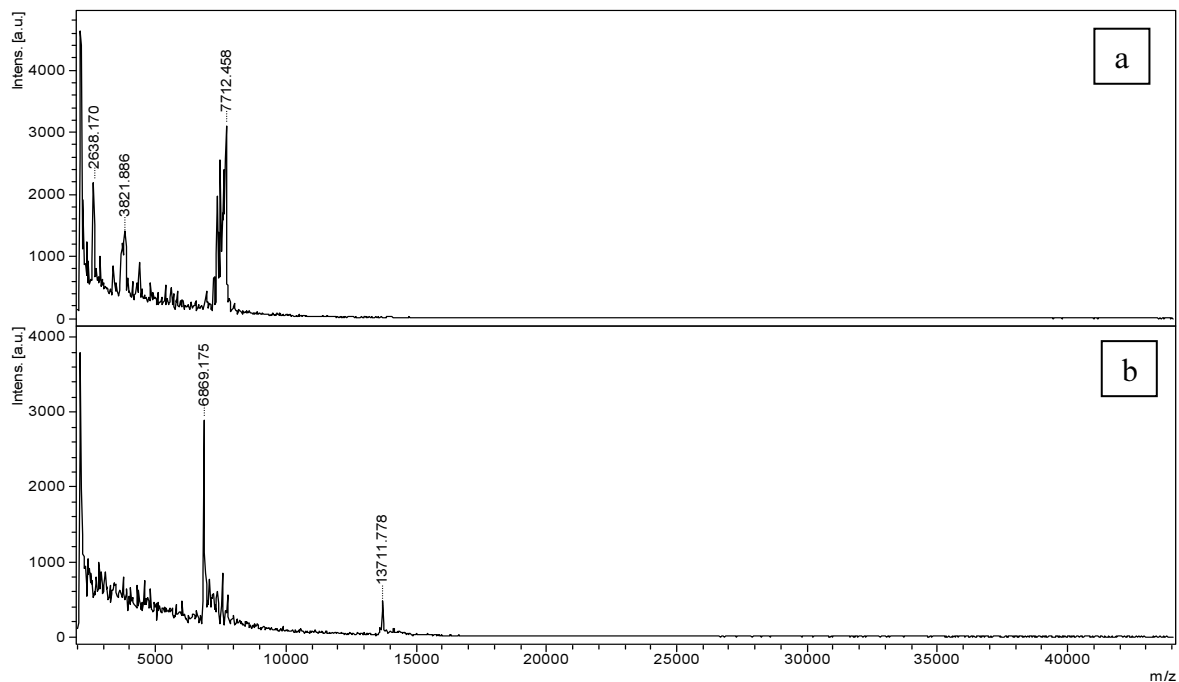


Fig. 2: Representative MALDI-TOF spectra of *Bothrops alternatus* and *Bothrops pubescens* Comparative MALDI-TOF mass spectra of 1 ng of (a) *Bothrops alternatus* (b) *Bothrops pubescens* showing three outstanding peaks at around m/z 2638, m/z 3821, m/z 7712 and in *Bothrops alternatus* profile; and two outstanding peaks at around m/z 8869 and 3711 in *Bothrops pubescens* profile. The products were detected as [M+Na]⁺ and/or [M+K]⁺ ions

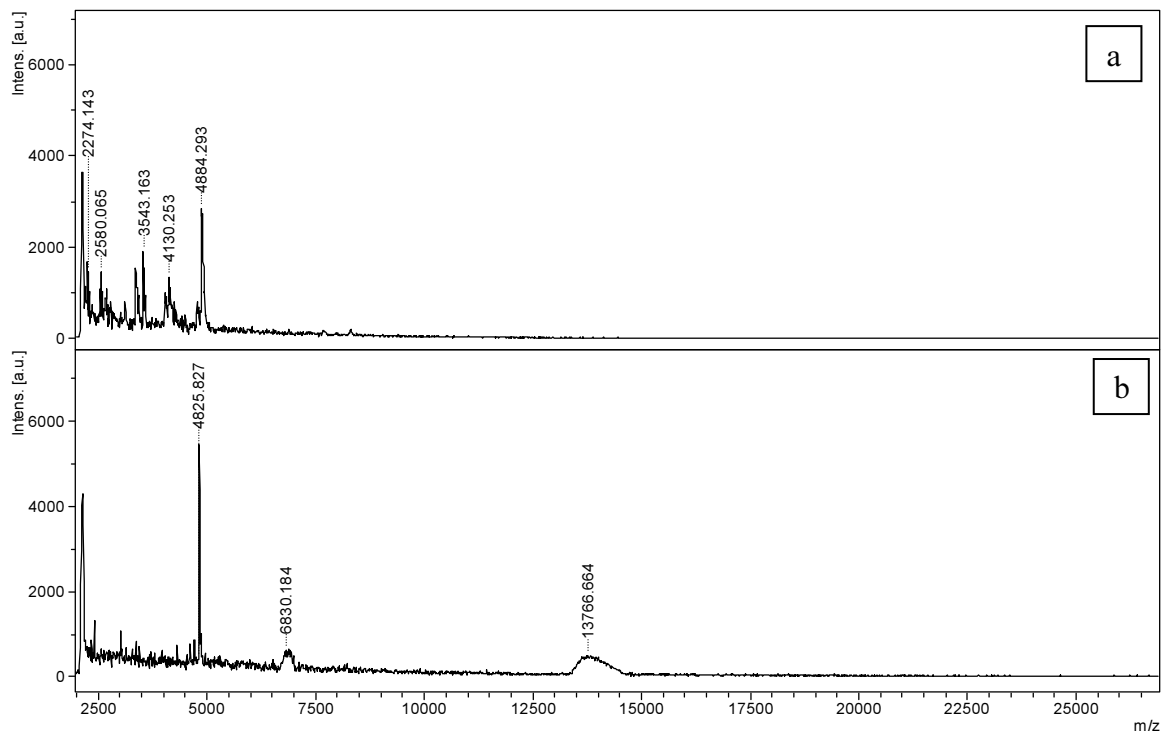


Fig. 3: Representative MALDI-TOF spectra of *Bothrops alternatus* and *Bothrops pubescens* Comparative MALDI-TOF mass spectra of 100 ng of (a) *Bothrops alternatus* (b) *Bothrops pubescens* showing three outstanding peaks at around m/z 2274, m/z 3543, m/z 4884 and in *Bothrops alternatus* profile; three outstanding peaks at around m/z 4825, m/z 6830 and 13766 in *Bothrops pubescens* profile. The products were detected as [M+Na]⁺ and/or [M+K]⁺ ions

Based on this complexity, for both clinical and scientific purposes, the identification of the species associated with a snake venom accident becomes a real challenge.

The venom antigen concentration in blood depends on several factors, including the snake species and is detected by different types of ELISA (O'Leary *et al.*, 2006; Kulawickrama *et al.*, 2010). Therefore, in all cases that use ELISA, we refer to venom antigenaemia or venom antigen levels instead of venom concentration because only the venom compounds that react with antivenoms are detected.

The results reported in the present work showed a sensitivity of 0.1 ng of venom of the *Bothrops* genus, corresponding to a concentration of 100 ng/mL, obtained by MALDI TOF analysis. In Uruguay, the venom antigenaemia values found for *Bothrops* genus were between 20 and 140 ng/mL (Morais *et al.*, 2012). These results were similar to the venom antigenaemia found by Bucher *et al.* (1997) with *Bothrops lanceolatus* bites in Martinique, with values ranging from 6 to 80 ng/mL (Bucher *et al.*, 1997).

In comparison, regarding other snakes of the *Bothrops* genus, França *et al.* (2003) studied the venom level antigens in patients bitten by *Bothrops jararaca* and found a mean value of venom antigenaemia of 224 ng/mL, although when they measured the venom level in patients, more than 60% had venom levels lower than 200 ng/mL. Additionally, patients bitten by *Bothrops atrox* in Colombia were found to have a mean of 67 ng/mL of serum venom antigens before antivenom treatment (Otero *et al.*, 1996).

The high sensitivity and reproducibility achieved by mass spectrometry suggest that the technique is suitable to detect venom in patient serum samples. In some cases, it could be necessary to concentrate the venom present in blood to obtain adequate sensitivity in all patients, especially in mild envenomations. Clinical assays will be necessary to clarify this point.

Conclusion

MALDI TOF-MS analysis is a rapid and cost-effective tool that should be further explored because it may contribute to the development of a snake venom database of samples from patients bitten by snakes, similar to the case of bacterial identification.

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Author's Contributions

Victor Morais: Participated in the conception and design of the study, carried out part of the experimental work and assisted in drafting the manuscript.

Norma Suárez: Participated in the conception and design of the study, carried out part of the experimental work and assisted in drafting the manuscript.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and no ethical issues involved.

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