Partial in vitro analysis of toxic and antigenic activities of eleven Peruvian pitviper snake venoms

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ABSTRACT

This work used eleven Peruvian snake venoms (*Bothrops andianus*, *Bothrops atrox*, *Bothrops barnetti*, *Bothrops castelnaudi*, *Bothriopsis chloromelas*, *Bothrocophias microphthalmus*, *Bothrops neuwiedi*, *Bothriopsis oligolepis*, *Bothriopsis peruviana*, *Bothrops pictus* and *Bothriopsis taeniata*) to perform in vitro experimentation and determine its main characteristics. Hyaluronidase (HYAL), phospholipase A2 (PLA2), snake venom metalloproteinase (SVMP), snake venom serine protease (SVSP) and L-amino acid oxidase (LAAO) activities; toxicity by cell viability assays using MGSO3, VERO and HeLa cell lineages; and crossed immunoreactivity with Peruvian (PAV) and Brazilian (BAV) antibothropic polyvalent antivenoms, through ELISA and Western Blotting assays, were determined. Results show that the activities tested in this study were not similar amongst the venoms and each species present their own peculiarities, highlighting the diversity within Bothrops complex. All venoms were capable of reducing cell viability of all tested lineages. It was also demonstrated the crossed recognition of all tested venoms by both antivenoms.

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1. Introduction

Of all accidents with venomous animals taking place in Peru, ophidism is the main cause of death (Martinez-Vargas, 2004). Most of the reported accidents occur within forest regions, since Peruvian Amazonia forest takes 58% of the country's territory, but areas within coast regions and Andes Mountains also present endemic medically relevant species. The great diversity of venomous snakes in Peru represents a great potential risk of ophidic accidents. In 2013, 2143 accidents with venomous snakes were recorded, according to the Peruvian National Institute of Health. Envenoming cases often occur in secluded areas, posing difficulties in registering properly the actual incidence and morbidity/mortality rates. Therefore, available epidemiological data may be underestimated

(Ministerio de Salud de Peru, 2003). In addition, many native venomous snake species remain poorly studied, in spite of the acknowledged richness of Peruvian herpetofauna. The National Institute of Health of Peru (INS) has been collecting venom from those native snakes, to encourage further research.

The Bothrops complex (following taxonomy classification proposed by Carrasco et al., 2012; comprising *Bothrops*, *Bothrocophias*, *Bothriopsis*, *Bothropoides e Rhinocerophis* subgroups) is responsible for the vast majority of the accidents involving venomous snakes in Peru (Fig. 1). Classic bothropic envenoming involves local and systemic effects. Locally, edema, hemorrhage and necrosis can lead to tissue loss and permanent disability. Systemic hemorrhage and intravascular coagulopathy can lead to acute kidney injury and cardiovascular shock and are the principal systemic complications (White, 2005; Albuquerque et al., 2013). The mentioned effects result from the integrated action of several venom components, such as metallo proteases (known as Snake Venom Metallo Proteases — SVMP), serine proteases (Snake Venom Serine Proteases — SVSP), phospholipases A2 (PLA2), L-amino acid oxidase (LAAO),
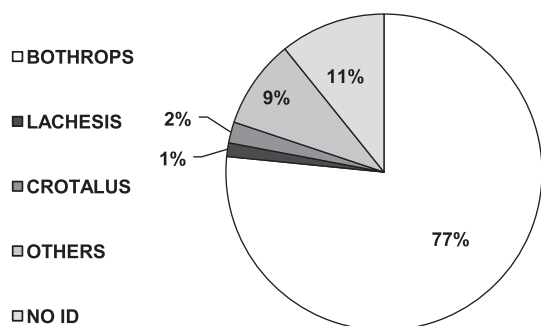
Fig. 1. Distribution of ophidic accidents in Peru, according to snake genus. Reports of accidents to the National Institute of Health from Peru (INS) in the year of 2013.

hyaluronidases and others (Calvete, 2010). Although these families of molecules are well characterized as the main bothropic venom components, their relative abundance and specificity within different species venoms (or even individuals) are subjected to variations according to different parameters, such as age and geographic localization (Calvete et al., 2011).

Studying venom properties of less known snakes is scientifically relevant to improve medical management of accidents and to drive antivenom production (Calvete et al., 2014). Moreover, snake venoms are rich in molecules with interesting pharmacological actions, being a valuable source for research in drug discovery (King, 2011).

In vitro methods for venom characterization are available and can provide relevant information. In addition of being more ethical, in vitro tests can be cheaper, faster, easily standardized and are not subjected to the inherent variation present in living animals. Animal-free approaches in venom characterization studies, although less frequent than research involving *in vivo* tests, have been performed (Lopes-de-Sousa et al., 2015) and must be pursued by scientific community, following the principle of the 3R (Replacement, Reduction and Refinement) in animal experimentation (Flecknell, 2002).

Here, we present the characterization of six venom snakes of the genera Bothrops (*Bothrops andianus*, *Bothrops barnetti*, *Bothrops castelnaudi*, *Bothrops neuwiedi*, *Bothrops pictus* and *Bothrops atrox*), four species of genera Bothriopsis (*Bothriopsis chloromelas*, *Bothriopsis oligolepis*, *Bothriopsis peruviana*, and *Bothriopsis taeniata*) and one species of genera Bothrocophias (*Bothrocophias microphthalmus*), using an animal-free approach.

2. Materials and methods

2.1. Venoms and antivenoms

Crude venoms from *B. andianus*, *B. atrox*, *B. barnetti*, *B. castelnaudi*, *B. chloromelas*, *B. microphthalmus*, *B. neuwiedi*, *B. oligolepis*, *B. peruviana*, *B. pictus* and *B. taeniata* were provided by the Instituto Nacional de Salud (INS), located in Lima, Peru and crude venom from *Bothrops jararaca*, used as control, was provided by Fundação Ezequiel Dias (FUNED), located in Belo Horizonte, Brazil. All venoms were manually extracted from a pool of individuals, lyophilized and kept at −20 °C until use. Five milligrams of each venom pool were diluted in 1 mL of deionized water and its protein content was determined with Bio Rad DC™ Protein Assay. Peruvian anti-bothropic polyvalent anti-venom (PAV), produced from horse plasma previously immunized with a defined pool of venoms [*B. atrox* (50%), *B. pictus*, *B. barnetti*, *B. brazili* and

Bothrocophias hyoprora (12.5%each)] was provided by INS. Brazilian anti-bothropic polyvalent anti-venom (BAV) produced from horse plasma previously immunized with a defined pool of venoms [*B. jararaca* (50%), *Bothrops jararacussu*, *Bothrops alternatus*, *Bothrops neuwiedi* and *Bothrops moojeni* (12.5%each)], was provided by Instituto Butantan, São Paulo, Brazil. Antivenoms were kept at 4 °C as indicated on their prescription.

2.2. Enzymatic activities

2.2.1. Hyaluronidase

For quantifying hyaluronidase activity, dilution curves from 120 to 0.625 μg of each Peruvian species venom were incubated in acetate buffer (0.2M sodium acetate–acetate acid and 0.15 M NaCl, pH 6.0) with 12.5 μg of hyaluronic acid (HA) in 96-well microtiter plates (Horta et al., 2014; with modifications). A control curve was made with 5 points of known HA concentrations, corresponding to 0%, 25%, 50%, 75% and 100% of hyaluronidase activity. The plate was incubated at 37 °C for 15 min. After the incubation, 200 μL of a solution containing 2.5% (w/v) cetyltrimethylammoniumbromide (CTAB) dissolved in 2% (w/v) NaOH were added to each well and produced turbidity was measured at 405 nm in microplate reader Biorad Model 680. All assays were performed in duplicates. Results from four independent experiments were plotted as means in a dose–response curve and values for 50% activity were determined.

2.2.2. Phospholipase A_2

To analyze phospholipase A_2 activity, the EnzChek® Phospholipase A_2 Assay Kit (Life Technologies) was used. The experiment was made following EnzCheck's protocol, using 2 μg of each *Bothrops* spp. venoms. A solution of purified PLA_2 from bee venom (10 units/ml) in 1X PLA_2 reaction buffer was used as positive control and the same buffer without PLA_2 was considered the negative control. All assays were performed in duplicates. Means of the results from four independent experiments were calculated and plotted as percentage of activity, relating to the positive control.

2.2.3. Proteolytic activities (SVMP and SVSP)

To measure snake venom metalloprotease activity (SVMP), a FRET peptide (Abz-LVEALYQ-EDDnp) containing the specific cleavage site for these enzymes, previously produced by our group (Schneider et al., 2014) was used. Twenty microliters of the FRET peptide diluted in 60 μL of Tris–HCl 100 nM/NaCl 50 nM buffer was incubated with 1 μg of each Peruvian *Bothrops* venoms. After 30 min of incubation, the plate was read in fluorometer (Synergy, BioTek) at 340 nm for excitation and 440 nm for emission. Results of means of three independent experiments were plotted as arbitrary units of fluorescence.

To measure the snake venom serine protease (SVSP) activity, venoms were pre-incubated with EDTA 0.2 μM for 1 h minutes and then followed the same protocol described above, using a FRET peptide with the specific active site for serine proteases (Abz-FLPRSFRQ-EDDnp). All assays were performed in duplicates. Results of means three independent experiments were plotted as arbitrary units of fluorescence.

2.2.4. L-amino acid oxidase (LAAO)

For L-amino acid oxidase (LAAO) activity quantification, 2 μg of each *Bothrops* sp. venom were incubated with a solution containing L-leucine as substrate, 2 mM OPD (o-phenylenediamine dihydrochloride) and peroxidase (5U/mL) in 100 mM Tris- HCl buffer (pH 8.5), in 96 wells microtitre plates, at 37 °C. After one hour of incubation, the reaction was stopped by adding 50 μL of H_2SO_4 2M. The reaction was measured by the absorbance intensity at 490 nm, in microplate reader Biorad Model 680. Four independent assays

performed in duplicates were made (Bregge-Silva et al., 2012; with slight modifications) and one unit of LAAO activity was considered as the amount of venom that produced 1 μmol of H_2O_2 per minute, under these specific conditions.

2.3. Cytotoxicity

MGSO-3 cell line, derived from human breast cancer tissue was used (Correa et al., 2009). These cells were characterized, immortalized and provided by Correa group. VERO (from normal epithelial monkey kidney) and HeLa (from human cervical adenocarcinoma) cells were also used, since they are already the standardized cell lines for viability assays. All cell lines were cultured in Dulbecco's Modified Eagle's (DMEM, Sigma–Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Thermo Scientific-HyClone), 0.2% gentamicin (Gilbco by Life Technologies) and kept in controlled atmosphere (10% CO_2 incubator at 37 °C).

For venom cytotoxicity evaluation (Damico et al., 2007; with modifications), Alamar Blue® assay was conducted. Previously to the assay, 10^4 cells/well of either MGSO3, VERO or HeLa were plated in 96-wells plates for 24 h at 37 °C and 10% CO_2. Then, the cells were incubated under the same conditions with different amounts of Peruvian Bothrops venoms for 24 h. A dilution curve of the venoms was made from 80 μg/mL to 1.25 μg/mL. After incubation period, Alamar Blue® was diluted in DMEM with a concentration of 10% v/v and added to each well. The plate was incubated for another 3 h and then the values were measured in fluorometer Synergy 2 (Bio-tek) at 540 nm for excitation and 590 nm for emission. The software GraphPad Prism 5 was used to calculate the CD_{50} (amount of venom able to reduce in 50% cell viability) for each venom, using results of three independent experiments, performed in duplicates.

2.4. ELISA

Microtitre plates were coated with 100 μl of a solution containing 1 μg/well in carbonate buffer, pH 9.8 of Peruvian Bothrops venoms and incubated overnight at 4 °C. The plate was washed and then blocked with 100 μL/well of a solution of 3% skimmed milk in PBS and incubated at 37 °C for one hour. After washing, either Peruvian anti-bothropic polyvalent anti-venom (PAV), Brazilian anti-bothropic polyvalent anti-venom (BAV) or pre-immune horse serum (PI) were diluted from 1/500 to 1/508,000 in a final volume of 100 μL, in 0.01% milk-PBS and again the plate was incubated at 37 °C for an hour. The plate was washed and the secondary antibody (anti-horse peroxidase, diluted to 1/6000 with incubation buffer), was added in a final volume of 100 μL and incubated at 37 °C for one hour. SigmaFast OPD tablets detected recognition, according to manufacturer's instructions. The plate was incubated in the dark for 20 min. After that, 20 μL of H_2SO_4 2M was added to each well to stop the reaction. Absorbance at 492 nm was measured in microplate reader Biorad Model 680.

2.5. SDS-PAGE and Western Blot

Twenty micrograms of Bothrops spp. venoms were diluted in sample buffer and separated in 12.5% SDS-PAGE gel, according to Laemmli, 1970. The gel ran for 30 min at 50 V and then at 150 V until the end. The gel was stained with silver or transferred to nitrocellulose membranes for immunoblotting (Towbin et al., 1979). The nitrocellulose membrane containing the transferred proteins was blocked with PBS-Tween 0.3% for 1 h. After washing three times for 5 min with PBS-Tween 0.05%, the membrane was incubated with either pre-immune serum, Peruvian anti-bothropic polyvalent anti-venom (PAV) or Brazilian anti-bothropic polyvalent anti-venom (BAV), diluted 1/5000 for 1 h. The membrane was washed (PBS-Tween 0.05%) three times and immunoreactive proteins were detected using anti-horse IgG conjugated to peroxidase for 1 h at room temperature. After washing three times for 5 min with PBS-Tween 0.05%, blots were developed using DAB/chloronaphthol according to manufacturer's instructions (Sigma–Aldrich).

3. Results

3.1. Enzymatic activities

Enzymatic activities of the eleven studied species of Bothrops venoms are summarized in Fig. 2. B. jararaca venom was used as a reference for all the performed experiments. Fig. 2A shows the results for the hyaluronidase activity test, expressed as the amount of venom needed to degrade 50% of the hyaluronic acid present in the solution. The results show that Bothriopsis oligolepis venom requires the smallest dose to achieve 50% activity. B. microphthalmus venom has the lowest hyaluronidase activity, needing more than 100 μg to degrade 50% of the hyaluronic acid available. It was not possible to detect any hyaluronidase activity in B. andianus venom, even at very high concentrations (>200 μg/well), in the conditions used in this assay.

As shown on Fig. 2B, B. atrox had the highest PLA_2 activity among the tested species, followed closely by B. taeniata and B. pictus. Three of the twelve species tested had a result close to zero (Bothrops castelnaudi, Bothriopsis chloromelas and B. oligolepis), indicating very low PLA_2 activity within these conditions.

Analyzing specific metalloprotease activity over FRET substrate (Fig. 2C), B. atrox presented the highest activity among the studied Peruvian venoms. B. castelnaudi was the least active venom, with a result close to zero, but B. chloromelas, B. oligolepis and B. taeniata also presented very low results.

For the serine protease assay (Fig. 2D), B. andianus had the highest result, with B. chloromelas, B. oligolepis, B. peruviana and B. taeniata presenting similar values, which were considerably higher than B. jararaca controls. On the other hand, B. barnetti presented the lowest activity.

In LAAO quantification assay (Fig. 2E), the species B. chloromelas showed the highest reactivity, presenting 14 units per mg per minute of the active form of the enzymes L-amino acid oxidase. The species that presented the lowest LAAO activity was B. barnetti, with less than two units of the enzyme L-amino acid oxidase.

3.2. Cytotoxicity

Concerning cytotoxicity (Fig. 3), three different cell lineages (MGSO3, VERO and HeLa) were tested against a serial dilution (80 μg/ml to 1.25 μg/ml) of the Peruvian venoms. Although there were some differences in the CD_{50} dose for each lineage (Table 1), all venoms were capable of reducing cell viability of all lineages. In addition, each venom presented a specific pattern of toxicity, with dose–response curves presenting similar shape for all tested lineages.

3.3. ELISA

In ELISA assays (Fig. 4), both Peruvian (PAV) and Brazilian (BAV) anti-bothropic polyvalent anti-venoms strongly recognized the different venoms coated to the plates. PAV presented a slightly higher recognition then BAV, even with venoms that do not belong to the antigenic pool [B. atrox (50%), B. pictus, B. barnetti, B. brazili and Bothrocophias hyoprora 12.5%, each] used by INS to produce the Peruvian antivenom. Pre-immune serum did not present significant recognition.
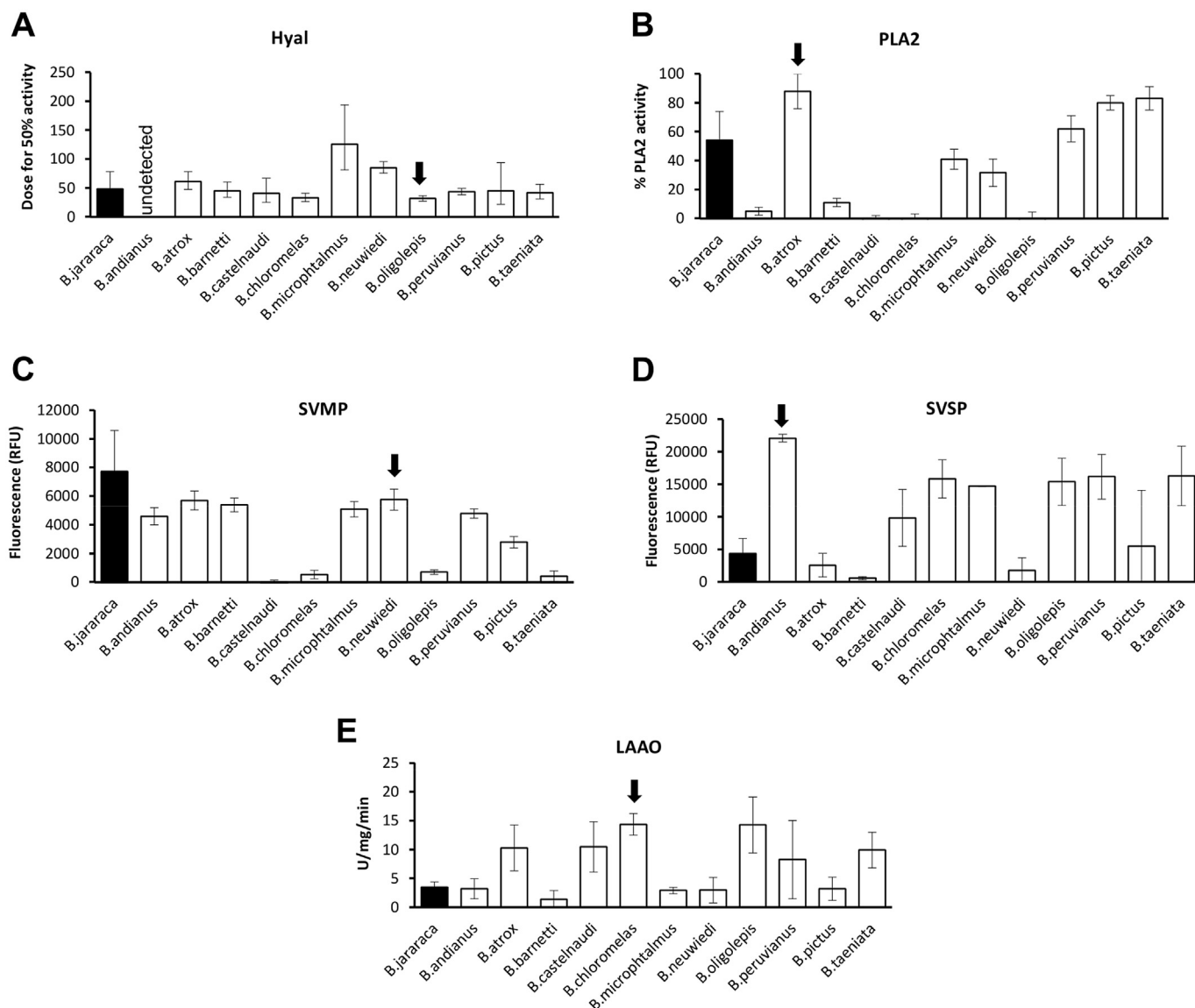
Fig. 2. Enzymatic activities of Peruvian Bothrops venoms. (A) Hyaluronidase activity results, measured in turbidimetric assay. Data are represented as the amount of venom needed for degradation of 50% of hyaluronic acid, according to standard curve (means ± SEM; n = 4). (B) PLA2 activity results, measured with EnzChek® Phospholipase A$_2$ Assay Kit. Data are represented as percentage of activity in relation to positive control of purified PLA2 (means ± SEM; n = 4). (C) Metalloprotease from snake venom activity results, measured by fluorescence (λex = 340 nm and λem = 440 nm) produced by FRET substrate hydrolysis. Data are represented as arbitrary units of produced fluorescence (means ± SEM; n = 3). (D) Serine protease from snake venom activity results, measured by fluorescence (λex = 340 nm and λem = 440 nm) produced by FRET substrate hydrolysis. Data are represented as arbitrary units of produced fluorescence (means ± SEM; n = 3). (E) ʟ-amino acid oxidase activity results, measured in colorimetric assay at 490 nm. Data are represented as the amount of venom that produced 1 μmol of H$_2$O$_2$ per minute (means ± SEM; n = 4). Black arrows indicate the species who presented the highest activity for the tested enzymatic activity.

3.4. SDS-PAGE and Western Blot

From SDS-PAGE (Fig. 5), it was possible to note that there are some similarities between the venoms tested in this article. *B. chloromelas* and *B. oligolepis* seem to have the same protein content in their venoms. *B. peruviana*, which has been described as a synonym of *B. oligolepis*, presented a very different band pattern. *B. castelnaudi*, described elsewhere as a possible *B. taeniata* synonym, also presented different bands.

In Western Blotting assay (Fig. 6), both Peruvian anti-bothropic polyvalent anti-venom (PAV) and Brazilian anti-bothropic polyvalent anti-venom (BAV) had a considerable reaction with the Peruvian Bothrops species tested, showing full interaction with the venoms. Reactivity intensities were comparable and both sera recognized the majority of the bands. No reactivity was detected when the membrane was probed with pre-immune serum (data not shown).

4. Discussion

Peru is a country with remarkable topographic diversity, and such diversity allows the formation of a great number of different ecosystems and microclimates, resulting in rich biodiversity. According to Espiñoza and Icochéa (1995), there are 33 different venomous snakes inhabiting Peruvian territory, being 9 endemic species and another 24 shared with other countries. This makes
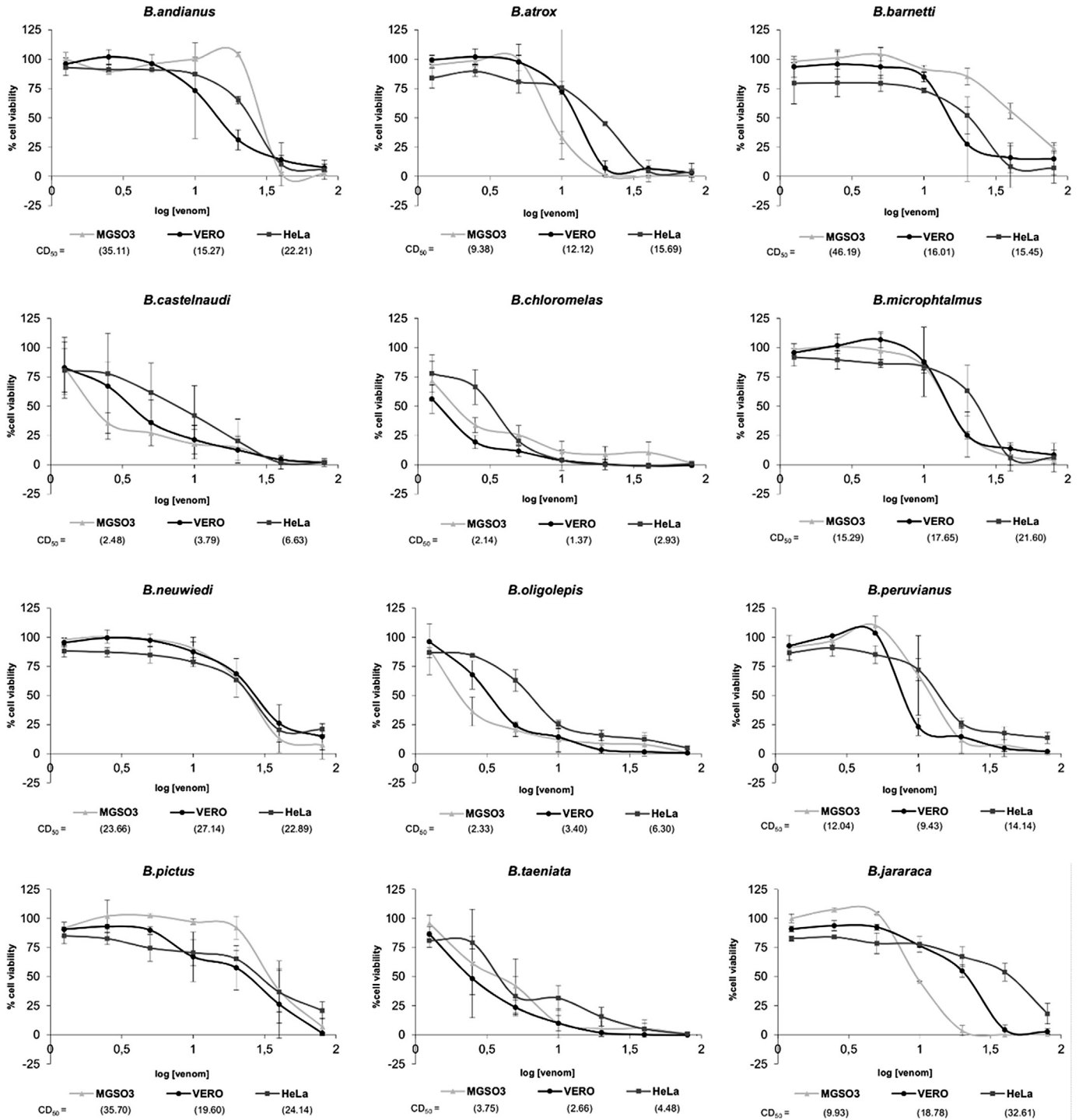
Fig. 3. Cell viability of MGSO-3, VERO and HeLa cells (1×10^4), following 24 h treatment with different concentrations of *Bothrops spp.* venoms (1.25−80 μg/mL). Cell viability was analyzed by Alamar Blue® assay. Data are represented as means ± SEM (n = 4).

Peru the second Latin American country in snake diversity.

The Bothrops complex possesses many representatives in Peruvian fauna. The complex presents great morphological and ecological niches, portraying the diversity of habitats in this country. The precise taxonomic identification of species pertaining to Bothrops complex (*Bothrops*, *Bothriopsis*, *Bothrocophias*, *Rhinocerophis*) is still controversial (Carrasco et al., 2012), but it is accurate to say that 97% of reported accidents with venomous snakes in Peru

are caused by members of this group (Ministerio de Salud del Peru, 2003). The study of different Bothrops snakes venom composition may help understanding the variability within these taxa as well as the development of improved medication protocols in case of accidents, since venom toxin content can be related to clinical features of envenomation (Queiroz et al., 2008).

The initial in vitro characterization of enzymatic activities (HYAL, SVMP, SVSP, PLA$_2$ and LAAO) of Peruvian bothropic venoms

Table 1
Summary of Peruvian Bothrops venom enzymatic and cytotoxic properties. Venom activities were assayed as described in Material and Methods and are expressed as means of independent experiments. Hyal (hyaluronidase activity); SVMP (snake venom metalloproteinase); SVSP (snake venom serin protease); PLA$_2$ (phospholipase activity); LAAO (L-amino acid oxidase); CD$_{50}$ (median cytotoxic dose). The gray scale represents the intensity of the detected activities.

	Hyal (µg/50%act)	PLA 2 (% activity)	SVMP (RFU)	SVSP (RFU)	LAAO (U/mg/min)	Citotox (CD50)		
						MGSO3	VERO	HeLa
B.jararaca (C+)	48.39	54.19	7739.88	4350.17	3.48	9.93	18.78	26.45
B.andianus	undetected	5.09	4597.50	22106.5	3.22	35.11	15.27	22.21
B.atrox	61.03	87.93	5698.75	2571.5	10.31	9.38	12.12	13.9
B.barnetti	45.04	11.02	5391.92	578.17	1.39	46.19	16.01	12.08
B.castelnaudi	40.74	-8.73	-109.67	9841.83	10.48	2.48	3.79	8.13
B.chloromelas	33	-5.44	525.75	15839.33	14.38	2.14	1.37	2.94
B.microphtalmus	125.5	40.93	5089.17	10122.33	2.93	15.29	17.75	22.69
B.neuwiedi	84.98	31.63	5763.00	1774.50	2.99	23.66	27.14	22.16
B.oligolepis	31.67	-2.99	700.67	15397.00	14.29	2.33	3.4	6.81
B.peruvianus	43.5	62.03	4796.92	16190.83	8.27	12.04	9.43	12.52
B.pictus	45.01	80.03	2797.42	5501.67	3.22	35.70	19.6	16.24
B.taeniata	48.39	83.19	413.92	16316.67	9.93	3.75	2.66	3.91

was performed and showed diverse results for each enzyme tested among the samples. It is important to stress that, although these enzymes were chosen because they are relatively easily detected and play key roles in the onset of bothropic envenoming, other molecules, such as catalytically inactive Lys49 phospholipase A$_2$ (Villalobos et al., 2007), disintegrins and C-type lectins/thrombin inhibitors (Öhler et al., 2010), also contribute to venom toxicity.

The compilation of the present results made possible the classification of the eleven venoms into four distinct venom profiles, which may help to better characterize this group of snakes (Fig. 7). Important differences in venom composition between members of the Bothrops complex were already demonstrated for some Brazilian species, confirming that there is a high venom content variability within this group (Leme et al., 2009; Queiroz et al., 2008).

The first identified profile formed group 1 (G1), comprising *B. barnetti* and *B. neuwiedi*, is characterized by high SVMP and low SVSP and LAAO activities. SVMP are often the most abundant toxins in Viperidae venoms and are the main responsible for venom's hemorrhagic activity (reviewed by Fox and Serrano, 2009; Markland and Swenson, 2013).

B. barnetti (Parker, 1938, known as Macanche, Sancarranca, Doble X, Cascabel) is found along the Pacific coast of northern Peru (Fig. 9), at low elevations in arid, tropical scrub. This species venom compose the antigenic pool used to produce Peruvian bothropic antivenom but, in spite of this, there are not many studies concerning its venom characterization (Orejuela et al., 1991; Vivas-Ruiz et al., 2013). Its high SVMP activity justifies further studies on this species, considering it presents a high risk of causing severe accidents.

The other member of G1, *B. neuwiedi*, has been referred as a taxonomic complex, comprising 12 subspecies (Silva and Rodrigues, 2008), with wide geographical distribution in South America. The subspecies found in Peru was classified as *B. neuwiedi mattogrossenssis*. It has been reported (Ministerio de Salud del Peru, 2003) in forest regions of eastern Andes (Fig. 9). This subspecies venom induces lethal, myotoxic, edema-forming and defibrinogenating effects, in addition of being strongly hemorrhagic (Minimum Hemorrhagic Dose of 0.90 µg) (Fernández et al., 2010), which is in accordance with the high SVMP activity detected in the present analysis.

The second profile (Group 2) shows high SVMP and SVSP

activities and almost undetectable HYAL activity. It is known for long that hyaluronidase act as a venom spreading factor (Hechter, 1947). It has been demonstrated that this enzyme is crucial to enhance venom toxicity and it is possible to say that the lack of this enzyme may diminish mortality due to systemic complications, confining the local effects to the extent close to the snakebite (Bordon et al., 2012; Girish et al., 2004; Mahadeswaraswamy et al., 2011; Wahby et al., 2012). However, SVMPs can also degrade the extracellular matrix in some extent (Gutiérrez et al., 2009). In this group, proteinases can be responsible for venom spreading, since it presents high activity of this class, compensating the lack of HYAL activity. HYAL properties and its role in the envenoming process have not been extensively studied (Fox, 2013), especially within the Bothrops complex. Only the description of a single cDNA sequence from a hyaluronidase-like protein from *Bothrops pauloensis* has been achieved so far (Castanheira et al., 2014).

G2 is composed by *B. andianus* and *B. microphthalmus*. *Bothrops andianus* (Amaral, 1923, known as Andean Lancehead, Jergona, Amantita or Marianito), is found in the southern mountains of Peru in the departments of Cuzco and Puno, at elevations of 1800–3300 m. The geographical distribution of *B. andianus* overlaps Machu Picchu's area (Peruvian Historical Sanctuary and a UNESCO World Heritage Site in 1983) (Fig. 9), which receives many tourists, putting themselves in potential risk of accidents. Our group published an initial characterization of this venom (Schneider et al., 2012). It was possible to detect hemorrhagic, hemolytic and proteolytic activities, as well as cross-reactivity of *B. andianus* venom with Peruvian antibothropic antivenom by ELISA and Western Blotting, consistent with the results shown here. The antivenom was also capable of neutralizing the aforementioned activities. Recently, the first serine protease with thrombin-like activity, called TLBan, from *B. andianus* venom was purified and well characterized by Valeriano-Zapana et al., 2012. The confirmed presence of this enzyme is in accordance with the high SVSP activity found in *B. andianus* venom.

In addition to be highly present in G2, SVSP activity is also notable in G4 and in *B. peruviana*, from G3. This class of enzymes causes various disturbances in the hemostatic system (platelet aggregation, coagulation, fibrinolysis, etc.) (Sajevic et al., 2011), a characteristic sign of bothropic envenoming. SVSP with thrombin-like activity has caught the attention of pharmaceutical industry for
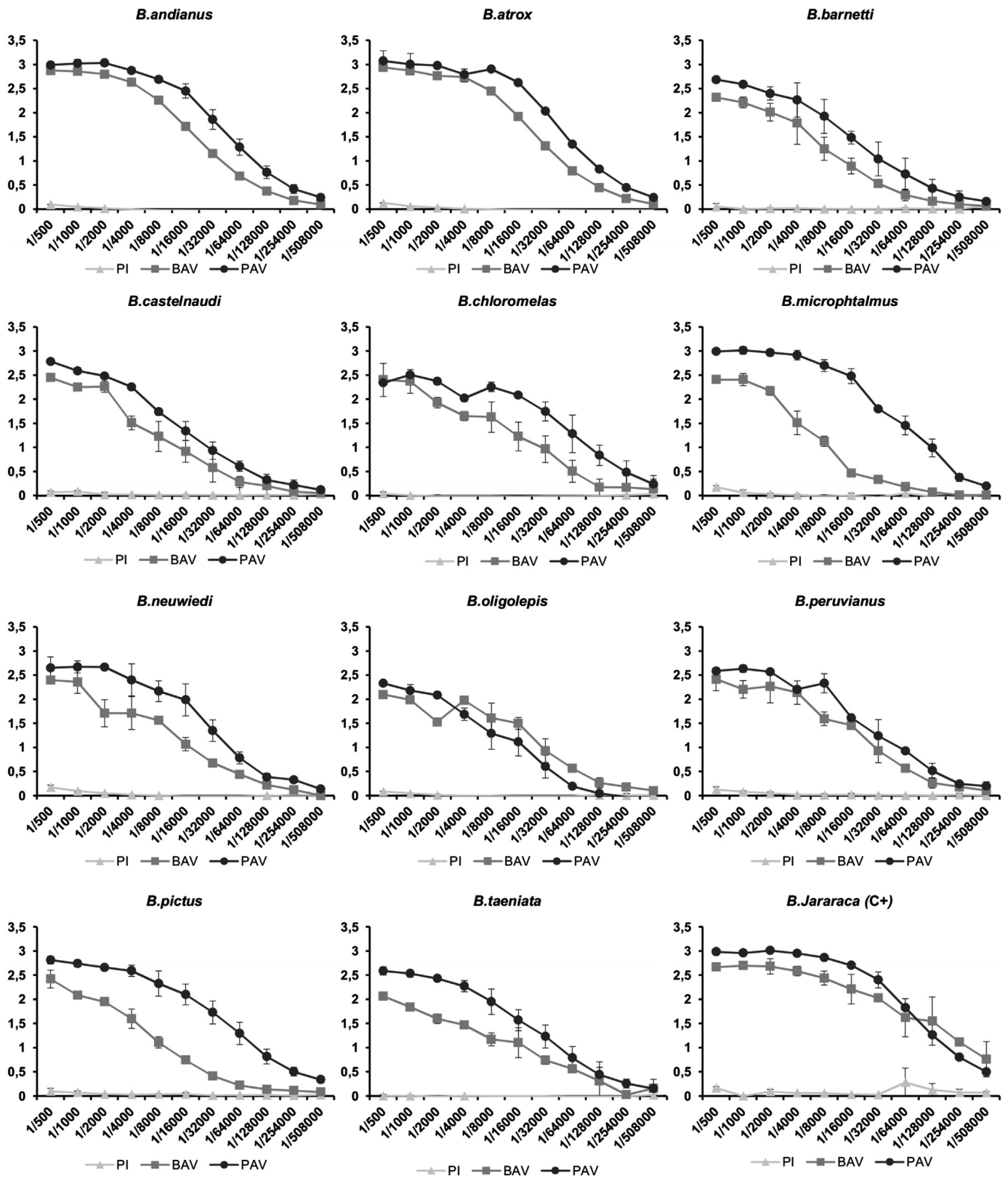
Fig. 4. ELISA of venoms tested against PAV (Peruvian anti-bothropic polyvalent anti-venom), BAV (Brazilian anti-bothropic polyvalent anti-venom) and PI (pre-immune sera). All Peruvian venoms were recognized by both antivenoms, but PAV's reaction was slightly higher, as expected. PI did not recognize any of the venoms. *B. jararaca* venom was used as a control for BAV recognition, but PAV showed higher values for this venom as well.
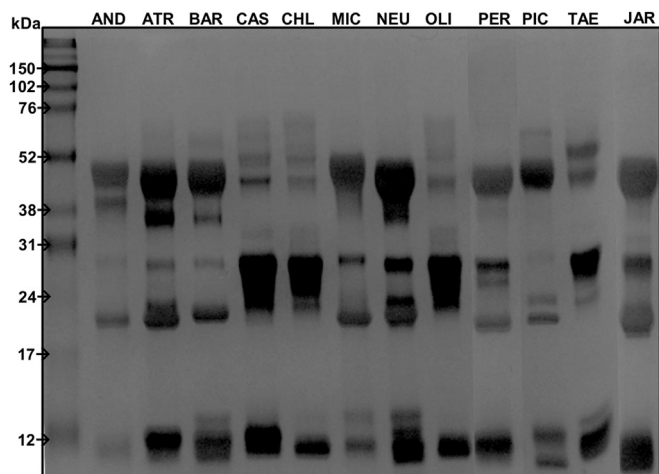
Fig. 5. SDS-PAGE of Peruvian Bothrops venoms. Samples (20 μg) of *B. andianus* (AND), *B. atrox* (ATR), *B. barnetti* (BAR), *B. castelnaudi* (CAS), *B. chloromelas* (CHL), *B. microphthalmus* (MIC), *B. neuwiedi* (Peru) (NEU), *B. oligolepis* (OLI), *B. peruviana* (PER), *B. pictus* (PIC)and *B. taeniata* (TAE), and Brazilian *B. jararaca* (JAR) as a reference were analyzed in 15% polyacrylamide gel and stained with silver.

catalytically active PLA$_2$, which is responsible for venom's myotoxicity, inhibition of platelet aggregation (Ferreira et al., 2013; Teixeira et al., 2011), among other deleterious effects.

G3 venoms, although not apparently related phylogenetically (Pyron et al., 2013) nor found in neighbor regions, share a similar eletrophoretic profile (Fig. 6). *B. atrox* (Linnaeus, 1758, known as Jergón, Jergona, Catari, Achujergón) is one of the better known Peruvian species, the most toxic species from Bothrops complex and one of the most widespread as well, covering all the Amazonian region, in altitudes below 600 m (Furtado et al., 1991). In addition, this species co-exists well with humans (Kohlhoff et al., 2012), increasing its relevance as a health problem. Due to these features, this snake has aroused a greater interest from researchers (Calvete et al., 2011; López-Lozano et al., 2002; Moreira et al., 2012; Sousa et al., 2013). *B. atrox* venom pattern defined here present high SVMP and PLA$_2$ activities, maybe justifying its increased venom toxicity, since these two enzymes are responsible for many deleterious effects and, in addition, can act synergistically (Bustillo et al., 2012). A previous proteomic characterization performed by Kohlhoff et al. (2012) also found high content of these two enzymes within Peruvian *B. atrox* venom.

B. pictus (Tschudi, 1845, known as Macanche, Sancarranca, Víbora, Jergón de la Costa, Jergona) is found along the Pacific coast of Peru and the occidental Andean slope, at altitudes from sea level to 1800 m. This region comprises the metropolitan area of Lima, where this species is the main responsible for bothropic accidents (Maguiña et al., 1998). This venom also integrates the antigenic pool for Peruvian bothropic anti-venom. Taxonomically, the species *B. pictus* is considered as a monophyletic group and a sister taxon to the clade containing all other subdivisions of the Bothrops complex (Rhinocerophis, Bothropoides, Bothriopsis, and Bothrops), according to Pyron et al., 2013. More data are needed to better characterize *B. pictus* venom, since it has not been the main subject of many studies (Orejuela et al., 1991; Rojas et al., 2005). The work of Kohlhoff et al. (2012) also described a high content of PLA$_2$ and SVMP in the proteomic analysis of *B. pictus* venom. However, unlike our findings, they did not detect the presence of LAAO. This can be accounted on technical sensitivity differences, since this enzyme is found in minor amounts within bothropic venoms. Another hypothesis is that the previous work was conducted with venom
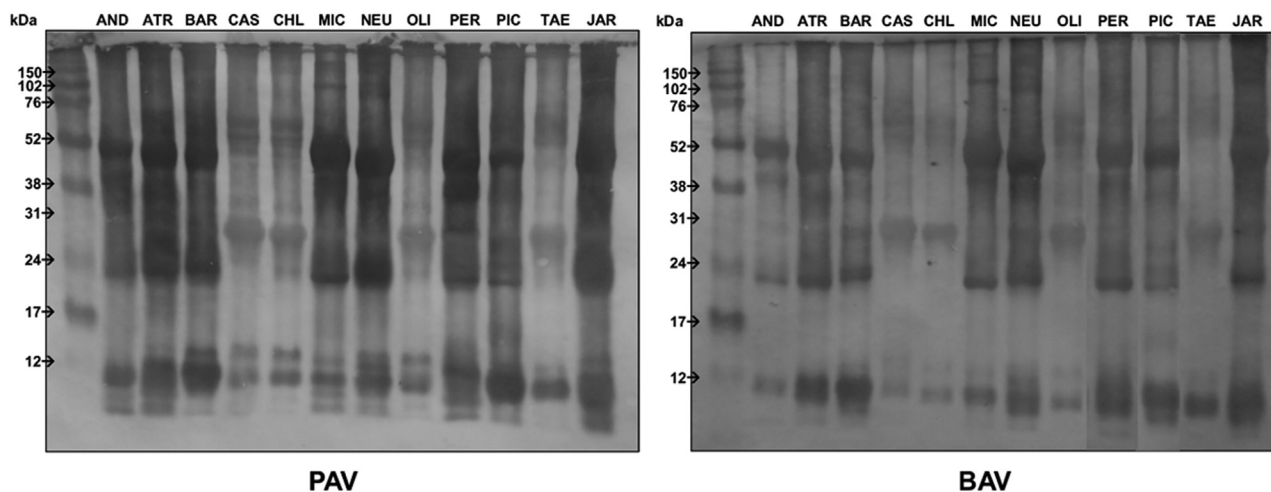
its potential use for patients with thrombosis and vascular disorders (reviewed by Serrano, 2013).

The other member of G2, *Bothrocophias microphthalmus* (Amaral, 1935, known as Jergón pudridora), is found within forests in Colombia, Ecuador, Peru and Bolivia (Carrasco et al., 2012). This species is also very poorly studied, but it seems to be commonly found in Peruvian Amazonia. It occurs in sympatry with *B. atrox*, and it is not clear the actual contribution of *B. microphthalmus* to the accidents occurring in this area (Kuch and Freire, 1995).

B. andianus presents some features of the Bothrocophias genus, such as small eyes and dorsal scales pattern, and its re-classification as belonging to this genus was proposed (Carrasco et al., 2012). The species tested in this work supposedly pertaining to *Bothrocophias* genus were put in the same enzymatic activity-defined group (Fig. 7), corroborating the phylogeny proposed by Carrasco.

Species pertaining to Group 3 (*B. atrox*, *B. peruviana*, *B. pictus*) show the highest PLA$_2$ activity. In this work, we detected only the

PAV

BAV

Fig. 6. Western Blotting analysis of Peruvian Bothrops venoms against Peruvian anti-bothropic polyvalent antivenom (PAV) and Brazilian anti-bothropic polyvalent antivenom (BAV). Samples (20 μg) of *B. andianus* (AND), *B. atrox* (ATR), *B. barnetti* (BAR), *B. castelnaudi* (CAS), *B. chloromelas* (CHL), *B. microphthalmus* (MIC), *B. neuwiedi* (Peru) (NEU), *B. oligolepis* (OLI), *B. peruviana* (PER), *B. pictus* (PIC) and *B. taeniata* (TAE) were analyzed in 12.5% poliacrylamide gel, transferred to nitrocellulose membranes and probed against either PAV, BAV or PI (1/1000) and incubated with anti-horse IgG conjugated to peroxidase. Reactivity was visualized using DAB/chloronaphthol according to manufacturer's instructions as substrate.
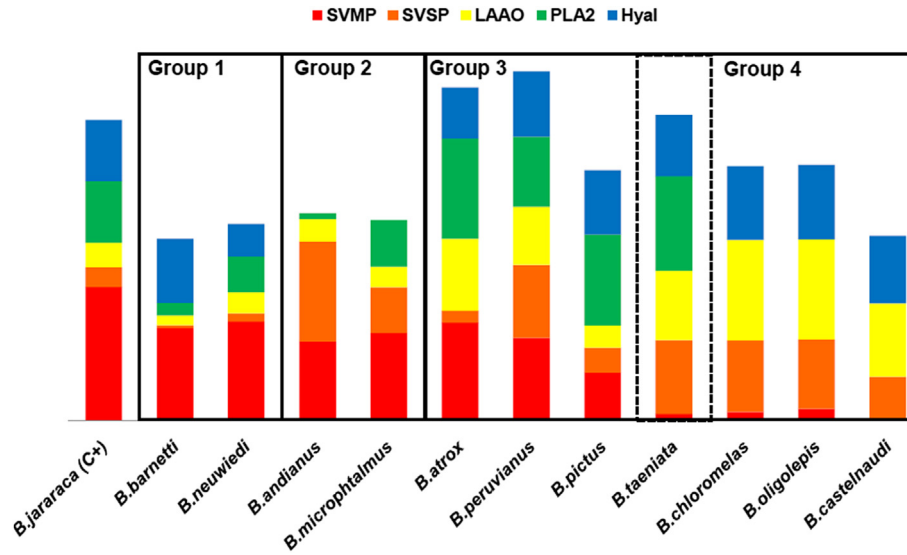
Fig. 7. Peruvian Bothrops species venom profile, according to measured *in vitro* activities. The relative proportion of each venom activities was plotted in a piled graphic. Species were grouped according to the similarity of its profile into four groups. *B. jararaca* was used as a parameter.
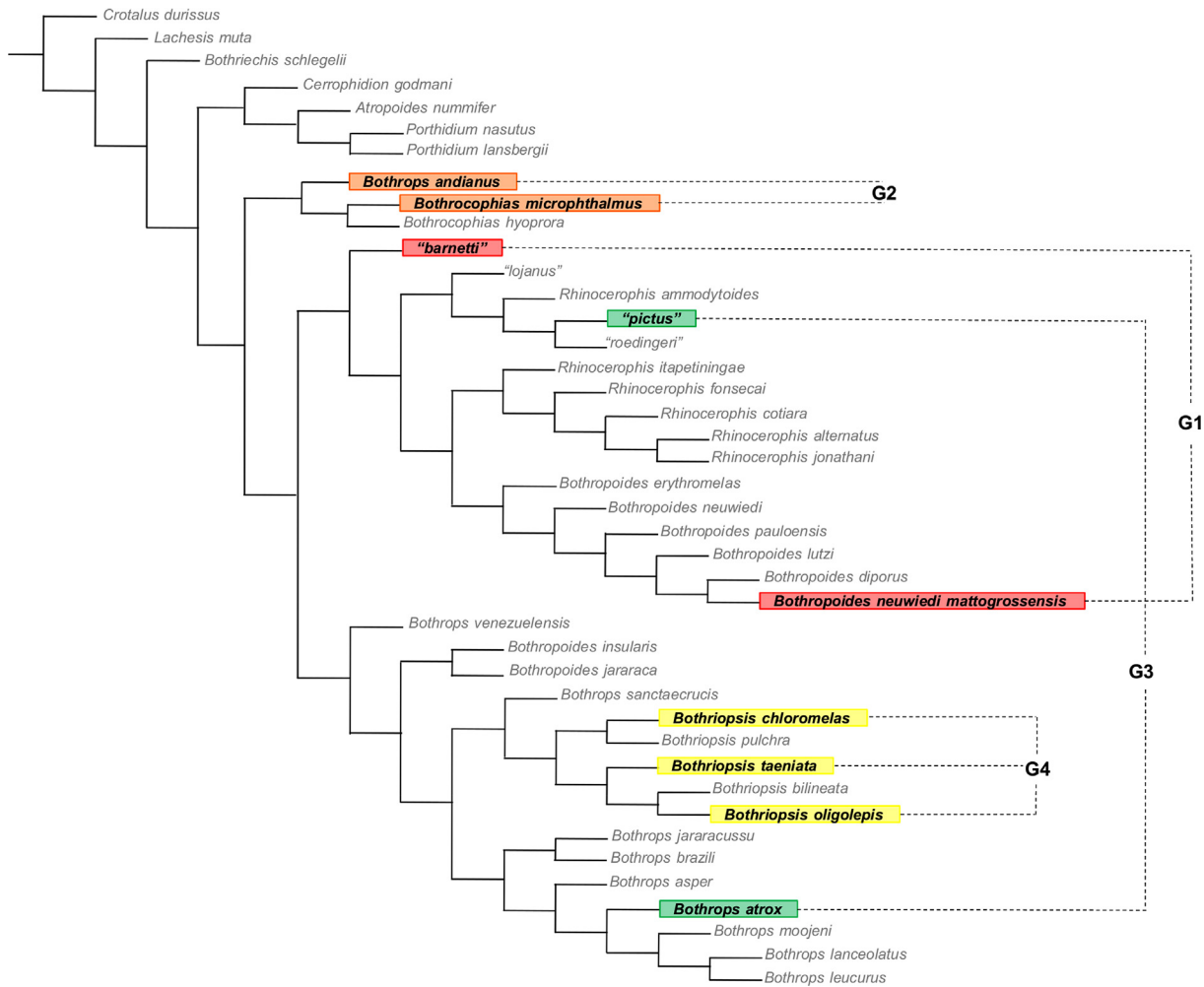

Fig. 8. Cladogram of South American Bothropoides pitvipers, obtained in the analysis of total evidence (adapted from Carrasco et al., 2012). The species whose venom was characterized in the present work are highlighted. The colors correspond to the group based on the established profile (Red = Group 1; Orange = Group 2; Green = Group 3; Yellow = Group 4). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
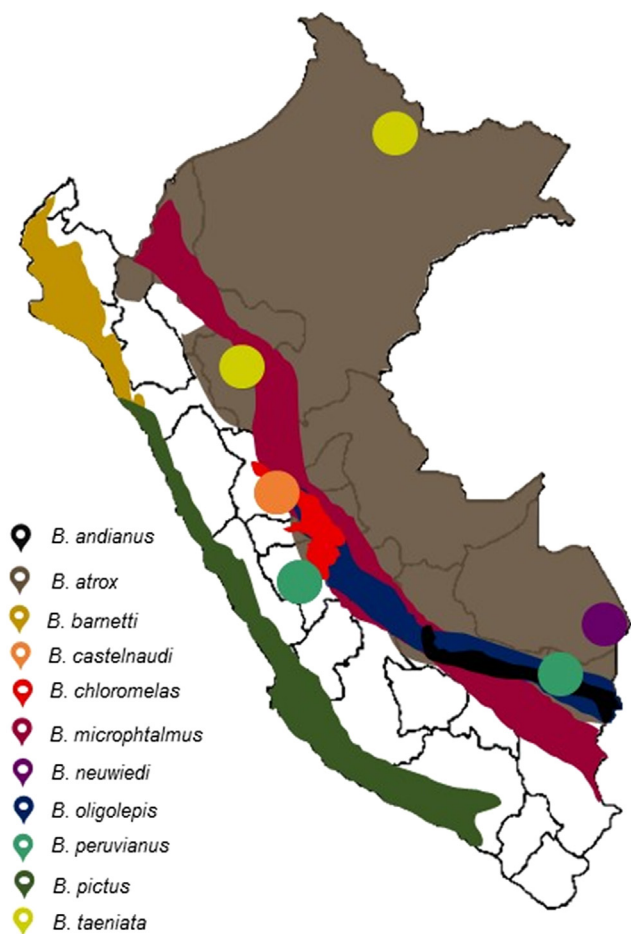
Fig. 9. Main distribution of Peruvian Bothroxps species. Colored areas mark the regions where the reported species are most commonly found. Round spots correspond to punctual reports (Zamora et al., 2010; http://www.bothrops-theultimatepitviper.com; access 01 may 2015).

extracted from juvenile snakes, which can present significant differences from adult venom content (Calvete et al., 2011).

Bothriopsis peruviana (known as Jergón negro, Sachavaca machaco) is found in southeastern Peru. Campbell and Lamar (2004) includes this name in the synonymy of *B. oligolepis* (Werner, 1901), but the lather variant is reported to be found on the eastern slopes of the Andes in Peru and Bolivia (Quijadas-Mascareñas and Wüster, 2010). Analyzing these venoms' characterization in this work, they presented a diverse profile, consistent with a classification of two different species. Further information and characterization of this species are still lacking.

Groups 4 shows very low values for SVMP activity and the highest LAAO activity. This enzyme is present in smaller amounts within bothropic venoms, compared to others tested here, and its exact role in the envenoming pathophysiology remains not fully understood (Fox, 2013). LAAO seems to be related to platelet aggregation, edema formation and can exert cytotoxic effects in bacteria, *Leishmania*, tumoral and normal mammalian cell lineages (Izidoro et al., 2006; Naumann et al., 2011; Rodrigues et al., 2009; Stábeli et al., 2007), possibly through apoptosis (Alves et al., 2008).

G4 is composed by representatives of the arboreal and semi-arboreal *Bothriopsis* sub-group, including *B. chloromelas*, *B. oligolepis*, *B. taeniata*, and *B. castelnaudi*. Unlike the members of this group, *B. taeniata* also presented high PLA$_2$ activity, but since other features, such as cytotoxicity pattern, were more similar to

the members of this group, it was placed in G4.

Bothriopsis chloromelas (Boulenger, 1912, known as Lamon or Achujergón) is endemic to the Central slopes of Peru, generally at altitudes above 1000 m and has been reported as a synonym of *B. oligolepis* (Schatti et al., 1990). In spite of being recognized as possibly belonging to the same species, venoms from these snakes were classified differently by the National Institute of Health from Peru (INS) and analyzed separately in this work. However, *B. chloromelas* and *B. oligolepis* presented very similar eletrophoretic profile, enzymatic activities and cytotoxicity, corroborating the synonymy. It cannot be ruled out that previous erroneous taxononomic classification, which is still confusing, could have happened and be responsible for the equivalence detected here for these two venoms.

Bothriopsis taeniata (Wagler, 1824) is widespread in the equatorial forests of South America (Ecuador, Colombia, Venezuela, Guyana, Suriname, French Guyan, Brazil, Peru and Bolivia). *B. taeniata* accident was reported as a mild bothropic poisoning without noticeable coagulopathy, but more case reports are necessary to better characterize clinical conditions caused by this snake (Torrez et al., 2009). This is a rather pacific snake that prefers hiding then attacking. However, considering its adult size and its ability of injecting great amounts of venom deep in the tissues, due to their long fangs, it can cause a potential severe harm. The low SVMP activity detected for this venom is in accordance with the low coagulopathy observed, since this group of enzymes is responsible for this type of effect.

B. castelnaudi (Dumeril, 1854) has been classified as a synonym of *B. taeniata*. The presented results show that, indeed, their venoms share some similarities, but significant differences were also observed, such as the high PLA$_2$ activity of *B. taeniata*, which was undetectable in *B. castelnaudi* venom. The present observations hence are not sufficient to state the synonymy between the two species.

B. castelnaudi showed no in vitro coagulant activity in the work published by Sanchez et al., 1992. It can induce weak or no fibrinogen clotting activity in vitro but, *in vivo*, it shows a high hemorrhagic activity. Kamigutti et al., 1985, also noted a very weak proteolytic activity on casein and on azocoll for this venom. In the conditions tested in this work, we have not found detectable SVMP activity and only low SVSP activity for this venom, confirming these previous data.

Bothrops is a very diverse group and its systematic analysis is very complex. To the moment, no consensus has been reached in establishing a definitive phylogeny for this group. Carrasco et al., 2012 proposed a combined analysis, using morphological, molecular and ecologic features to classify South-American pitvipers (Fig. 8). Comparing the enzymatic venom profile traced in this work and the phylogeny proposed by Carrasco, it is possible to observe that species classified in groups 2 and 4 are in accordance with the taxonomy and similarities detected in venom's activities may reflect evolutionary proximity in these groups.

On the other hand, members of groups 1 and 3 are distant from each other in the cladogram. It is important to stress, however, that *B. barnetti* (group 1) and *B. pictus* (group 3) are still considered as '*incertae sedis*', meaning that their presented classification is not well defined. According to Wüster et al., 2008, phylogenetic studies are important to venom research and improvement of antivenom production. In addition to this, we propose that venom composition determination may also help phylogenetic studies, constituting an additional feature to be considered, together with molecular, morphological and ecology characteristics, helping to understand evolution of the Bothrops complex. Using venom composition as additional criteria may help defining *B. barnetti* and *B. pictus* positioning in the evolutionary tree. Also, the venoms from majority of
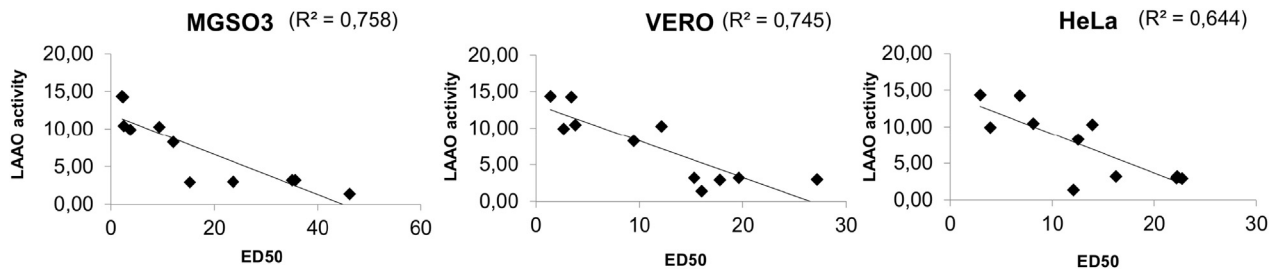
Fig. 10. Correlation between cytotoxicity and LAAO activity. Graphs represent the results for LAAO activity of each venom plotted against the CD_{50} values found for each cell lineage (MGSO3, VERO and HeLa). The established correlation indicates that LAAO activity may be responsible for the observed cell toxicity.

the South American bothropoides classified by Carrasco have not been characterized. Performing this kind of analysis with these other venoms may clarify and improve the initial profiling proposed in the present work. It is also noteworthy that *B. castelnaudi* (group 4) and *B. peruvianus* (group 3) were not included in the phylogenetic analysis.

In cell viability assays, all venoms were considerably toxic. Cytotoxicity has been featured as a property of bothropic venoms in many studies, using normal and cancer cell lineages (Jorge et al., 2011; Mello et al., 2014; Novak-Zobiole et al., 2015; Nunes et al., 2012). Both normal (VERO cells) and tumoral cells (MGSO-3, a breast cancer lineage characterized within our institute; and HeLa, cervix cancer lineage) were used in the present study, to confirm these previous findings.

In spite of using different cell lineages, the pattern of the dose–response curve seems to be species-specific. Venoms from G1 were the least cytotoxic, whereas venoms from G4 were the most toxic ones. It has been stated that venom cytotoxicity is related to LAAO activity (De Vieira Santos et al., 2008). In this present work, we found that indeed G1, in addition to being less cytotoxic, also presented the lowest LAAO activities; and Group 4, the most cytotoxic, also had the highest LAAO activity values. When these features were plotted against each other (Fig. 10), they showed to be correlated ($R^2 > 0.64$). Such correlation was not found for other groups of activities tested in this work ($R^2 < 0.5$).

Immunological studies of cross reactivity between the tested venoms and different antivenoms (Peruvian — PAV, and Brazilian — BAV) showed that in spite of the diversity of venom activities, all venoms were recognized by the antidotes. This is a preliminary sign that the tested antivenoms might be efficient for treatment of accidents involving all of these eleven species.

5. Final remarks

Peru has a remarkable biodiversity and therefore many native species of medically relevant snakes remain poorly studied. To assure proper care in case of accidents with less common snakes and to expand the current knowledge on snake venoms, this work attempted to perform an initial in vitro characterization of eleven Peruvian snake venoms pertaining to the Bothrops complex. The obtained results reflect the diversity found in nature, since different enzymatic and antigenic profiles were found within the venoms tested. Much work is still needed to properly characterize these venoms, but the preliminary information provided here might be useful to guide further research.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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