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Biochemical and immunological characteristics of Peruvian *Loxosceles laeta* spider venom: Neutralization of its toxic effects by anti-loxoscelic antivenoms

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ABSTRACT

This manuscript describes the general biochemical properties and immunological characteristics of Peruvian spider *Loxosceles laeta* venom (P*Llv*), which is responsible for the largest number of accidents involving venomous animals in Peru. In this work, we observed that the venom of this spider is more lethal to mice when compared with *L. laeta* venom from Brazil (B*Llv*). The LD_{50} of P*Llv* was 1.213 mg/kg when the venom was intradermally injected. The venom displayed sphingomyelinase activity and produced dermonecrotic, hemorrhagic and edema effects in rabbits. 2-D SDS-PAGE separation of the soluble venoms resulted in a protein profile ranging from 20 to 205 kDa. Anti-P*Llv* and anti-B*Llv* sera produced in rabbits and assayed by ELISA showed that rabbit antibodies cross-reacted with P*Llv* and B*Llv* and also with other Brazilian *Loxosceles* venoms. Western blotting analysis showed that bands corresponding to 25–35 kDa are the proteins best recognized in every *Loxosceles* spp venoms analyzed. The immunized rabbits displayed protective effect after challenge with P*Llv* and B*Llv*. *In vitro* assays with horse anti-loxoscelic antivenoms produced in Brazil and Peru demonstrated that these commercial antivenoms were efficient to inhibit the sphingomyelinase activity of P*Llv* and B*Llv*.

1. Introduction

Loxoscelism is the most important clinical syndrome resulting from *Loxosceles* spp spider bite and follows two well-defined clinical variants: the cutaneous form which manifests as erythema and edema that may develop into necrotic ulcer, whilst systemic loxoscelism is characterized by intravascular hemolysis and occasional renal failure (da Silva et al., 2004; Ministério da Saúde, 2011).

Loxosceles laeta (Nicolet, 1849) (Araneae, Sicariidae), known as “brown spider”, “corner spider” and “spider violin”, is an endemic species of South America, which has been introduced into the East of this continent and also into both North and Central America (Gerstch, 1967). *L. laeta*

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species is found throughout Argentina (de Roodt et al., 2002), frequently reported in the South region of Brazil (Malaque et al., 2002), widely distributed in Chile (Manriquez and Silva, 2009) and also found throughout the Peruvian territory, where it is also named "killer spider", due to the association of this spider with many fatal cases of loxoscelism (Maguiña-Vargas et al., 2004). Loxoscelism is a serious public health problem in Peru, the number of human accidents caused by spiders of *Loxosceles* genus attains 2500 per year (Panaftosa, 2007). *L. Laeta* and in a lesser extent *Loxosceles rufipes* are the most medically relevant species in Peru (Sanabria and Zavaleta, 1997). The highest incidence of envenomations is recorded in cities along the Peruvian Coast (Sanabria and Zavaleta, 1997).

In Peru, although early work on loxoscelism dates from 1953 (Yzu, 1953), hardly anything is known about the structural and functional characteristics of this Peruvian spider venom, while significant progress has been made in other regions where *Loxosceles* spp spider live. *Loxosceles* venoms contains several protein toxins including alkaline phosphatases, hyaluronidases, metalloproteases, sphingomyelinases, and insecticidal peptides (da Silva et al., 2004). Among venom toxins, sphingomyelinases, also called dermonecrotic toxins, are the major toxic components and play an essential role on the pathogenesis of loxoscelism (Tambourgi et al., 2010). By using molecular biology tools, dermonecrotic toxins have been identified, the crystal structure determined, the cDNAs encoding toxins isolated, characterized and the recombinant proteins expressed, providing new insight for this group of toxins (Kalapothakis et al., 2002; Murakami et al., 2006; de Santi Ferrara et al., 2009; Catalán et al., 2011; da Silveira et al., 2006). Immunization strategies using crude *Loxosceles* venoms, recombinant toxins or synthetic epitopes derived from these toxins support the notion of using these immunogens as therapeutics via anti-sera development or vaccine strategy (Olvera et al., 2006; de Almeida et al., 2008; Dias-Lopes et al., 2010; de Moura et al., 2011). Antivenoms prepared from horse sera immunized with crude *Loxosceles* venoms are an important tool for treatment of human envenomation by spider and its use recommended by the Public Health Organizations (Pauli et al., 2009).

In view of the absence of information about the properties of P*Llv* toxins, the main goal of this work is to report some biochemical, immunological and toxic properties of this venom. In this paper, the sphingomyelinase, dermonecrotic, hemorrhagic, edematogenic and lethal activities of crude venom were investigated. This manuscript also describes the separation of soluble venoms proteins by 2-D SDS-PAGE, highlighting the differences between P*Llv* and B*Llv* protein pattern. In addition, this study shows the capacity of rabbit polyclonal anti-P*Llv*, anti-B*Llv* and, also horse anti-loxoscelic sera to neutralize Brazilian and Peruvian *Loxosceles laeta* venoms toxic effects.

2. Materials and methods

2.1. Animals, venoms and antivenoms

Adult male Swiss mice (weighing 18–22 g) were maintained at the Centro de Bioterismo of the Instituto de Ciências Biológicas of the Universidade Federal de Minas Gerais (UFMG), Belo Horizonte, MG, Brazil. All animals received water and food *ad libitum*. The experimental protocols conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) (A5452-01). Eight- to nine-week-old New Zealand rabbits were used to produce the sera anti-P*Llv* and anti-B*Llv*. Animals were maintained and handled as described above.

L. laeta (Peru) mature spiders were collected in the region of Cañete (Lima, Peru) and maintained in the herpetarium of the Centro Nacional de Producción de Biologicos of Instituto Nacional de Salud (INS), in Lima, Peru. Spiders were maintained in plastic boxes with water *ad libitum* and were fed weekly with cockroaches. The venom from mature spiders was obtained from dissected venom glands of ten spiders. Venom was collected according to da Silveira et al. (2002), pooled and stored at −20 °C until use. Protein concentration was determined by Bradford method (Bradford, 1976). *L. laeta, Loxosceles intermedia and Loxosceles gaucho* Brazilian mature spiders were collected in the region of Curitiba, PR, Brazil and maintained at the Centro de Produção e Pesquisa de Imunobiológicos (CPPI) of the State of Paraná, Brazil. The venoms from mature spiders were obtained as described before. *Phoneutria nigriventer* spiders and *Tityus serrulatus* scorpions were collected in the region of Belo Horizonte and maintained at the "Seção de Animais Peçonhentos" of Ezequiel Dias Foundation (FUNED) of Belo Horizonte, Brazil. The crude venoms were obtained by electric stimulation, lyophilized and stored at −20 °C in the dark until use.

Two commercial antivenoms were used for the neutralization assay, the antivenom produced in CPPI, Brazil (Lot. S02100) against B*Llv*, *L. intermedia* and *L. gaucho* venoms and an antivenom produced by Instituto Nacional de Salud del Perú (INS) (Lot. 0300069), containing antibodies against P*Llv*.

2.2. Toxic activities of L. laeta venom

2.2.1. Determination of median lethal dose (LD_{50})

The lethality was assessed via intradermal (i.d.) route. Groups of four mice were injected with different doses of venoms (0.4, 0.56, 0.784, 1.098, 1.537, 2.152 mg per kg of body weight) dissolved in 0.1 mL of PBS-BSA 0.5%. Seventy-two hours later deaths were recorded and the LD_{50} was then calculated by Probit analysis (Finney, 1971).

2.2.2. Determination of dermonecrotic, hemorrhagic and edematogenic activities

The dermonecrotic, hemorrhagic and edematogenic activities of P*Llv* and B*Llv* were determined by intradermal injection of 10 μg of crude venom in 100 μL of PBS pH 7.2 into the shaved back of five rabbits for each venom, as described by Furlanetto (1962). Injection of PBS alone was used as negative control. The diameters of dermonecrotic, hemorrhagic and edematogenic lesions were measured in the skin areas with a scale meter and caliper rule, 72 h after injection. Three measures of each lesion were made and their arithmetic mean was considered the mean diameter of the lesion.

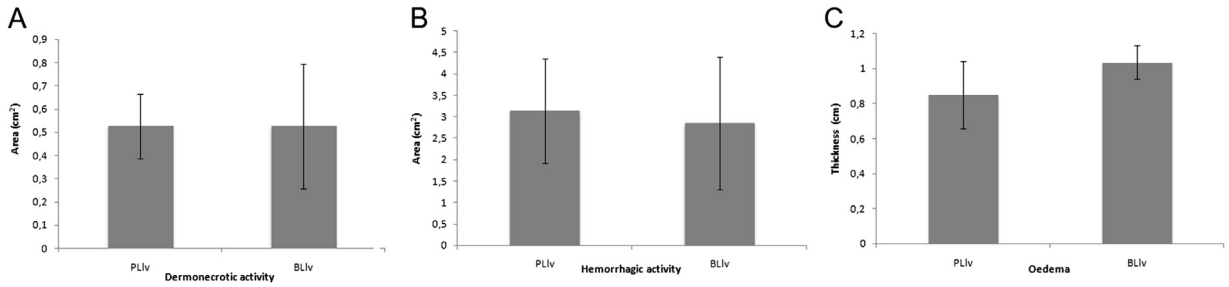

Fig. 1. Biological activities of P*LIv* and B*LIv*. (A) Dermonecrotic activity, (B) hemorrhagic activity and (C) edema of P*LIv* and B*LIv* were measured (area) 72 h after intradermal injection of 10 μg of each venom..

2.2.3. Determination of sphingomyelinase activity

The sphingomyelinase (SMase) activity was measured using the Amplex Red Sphingomyelinase Assay Kit (Invitrogen) as previously described (Gatt et al., 1978; Binford et al., 2009). Briefly, different amounts of the venoms (0.125, 0.25, 0.5, 1.0 μg) were assayed in triplicates. Varion Cary eclipse fluorescence spectrophotometer was used to measure the fluorescence emission from the reactions.

2.3. Electrophoretic determinations

Protein profile of P*LIv* and B*LIv* was analyzed by two-dimensional electrophoresis using the IPG-SDS-PAGE system (Gorg, 1993). The venom was solubilized in lysis buffer containing 8 M urea, 2 M thiourea, 4% w/v CHAPS, 65 mM dithioerythritol, 40 mM Tris, 0.002% bromophenol blue, protease inhibitor and 1% of IPG buffer. Nonlinear immobilized pH 3–10 gradient IPG strips were rehydrated with 100 μg of the venom for 4 h (no electric field) and then for 12 h at 30 V. Isoelectric focusing was carried out using a gradient mode to a total of 50 kVh. Strip equilibration was done with 65 mM DTT and then 135 mM iodoacetamide. For the second dimension the proteins were separated in 18 cm 12% SDS-PAGE gel at 200 V. The proteins were stained with silver nitrate. To analyze and compare P*LIv* and B*LIv* profile the software Progenesis SameSpot was used.

2.4. Immunological studies

2.4.1. Immunization protocols

Adult New Zealand female rabbits were used for the production of anti-P*LIv* and anti-B*LIv* antibodies (3 rabbits for each venom). After collection of pre-immune sera, the animals received an initial subcutaneous injection of 20 μg of crude venom absorbed in aluminum hydroxide adjuvant (day 1). Three booster injections were made subcutaneously 14, 28 and 52 days later with a same dose (20 μg). The animals were bled one week after the last injection.

2.4.2. Indirect ELISA and immunoblotting assays

Falcon flexible microtitration plates (BD Biosciences, USA) were coated overnight at 5 °C with 100 μl of a 5 μg/ml solution of P*LIv*, B*LIv*, *L. intermedia*, *L. gaucho*, *Phoneutria nigriventer*, or *Tityus serrulatus* whole venoms in carbonate buffer (0.02 M, pH 9.6). The assay was performed as previously described (Chavez-Olortegui et al., 1998). Absorbance values were determined at 492 nm using an ELISA plate reader (BIO-RAD, 680 models). All the samples were done in triplicate.

For immunoblotting assays, SDS-PAGE gels of B*LIv*, P*LIv*, *L. intermedia* and *L. gaucho* venoms (20 μg of each) were used. The venoms were solubilized in non reducing sample buffer and electrophoresed on 12.5% SDS-PAGE gel, according to Laemmli (1970), and then transferred onto Hybond-P PVDF membranes (Amersham Life science). The membrane 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 anti-P*LIv* and anti-B*LIv* rabbit sera (1:250) for 1 h. The membrane was washed (PBS-Tween 0.05%) three times and immunoreactive proteins were detected using anti-rabbit 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.

2.5. In vivo and in vitro *neutralization assays*

For the *in vivo* neutralization assays, immunized rabbits were challenged with 10 μg of P*LIv* and B*LIv* 10 days after the last immunization. The diameters of dermonecrotic, hemorrhagic and edematogenic lesions were measured 72 h after the injection as described before. Non-immunized rabbits were used as positive control.

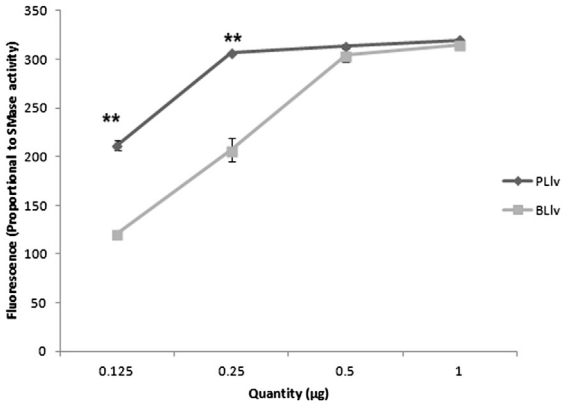

Fig. 2. Sphingomyelinase activity of P*LIv* and B*LIv*. The SMase activities using different amounts (0.125, 0.25, 0.5, 1.0 μg) of P*LIv* (A) and B*LIv* (B) were assessed by Amplex Red Sphingomyelinase assay kit (Invitrogen) (**$p < 0.01$ P*LIv* vs. B*LIv*; values shown represent mean plus standard errors).

The neutralization of sphingomyelinase activity of P*LI*v and B*LI*v was assessed by pre-incubation of 0.125 μg of P*LI*v or 0.25 μg of B*LI*v (values previously established as the same amount of sphingomyelinase activity for these venoms) with different dilutions (1:100, 1:500, 1:2500 and 1:12,500) of the commercial horse anti-loxoscelic antivenom produced in Brazil (CPPI) and commercial horse anti-P*LI*v antivenom produced in Peru (INS), for 1 h at 37 °C. The venom alone was used as positive control, established as 100% of sphingomyelinase activity. After pre-incubation, the sphingomyelinase activity was assessed as described before.

3. Results and discussion

3.1. General venom characterization

The lethal activity of P*LI*v and B*LI*v was compared in mice subjected to intradermal toxin injection. We observed that both venoms are lethal to mice, but P*LI*v was more efficacious than B*LI*v (LD_{50} = 1.21 mg/kg and 2.18 mg/kg, respectively).

In previous similar studies, with whole venom of five Brazilian *Loxosceles* species, it was shown that the LD_{50} of *Loxosceles similis* was the most lethal in mice (LD_{50} = 0.32 mg/kg (Silvestre et al., 2005)); followed by LD_{50} for *L. intermedia* (0.48 mg/kg (Barbaro et al., 1996) and 0.5 mg/kg (Braz et al., 1999), respectively). Different LD_{50} values were found for *L. gaucho* venom (0.74 mg/kg (Barbaro et al., 1996) and 0.574 mg/kg (Pretel et al., 2005), respectively); in *L. laeta* the venom LD_{50} was 1.45 mg/kg (Barbaro et al., 1996) and for *Loxosceles adelaida* venom 0.696 mg/kg (Pretel et al., 2005). The LD_{50} for B*LI*v obtained here is 1.5-fold higher than that obtained by Barbaro et al. (1996). This divergence can be explained because in our experiments venom was collected by extraction after gland dissection as described by da Silveira et al. (2002),whilst in their study the venom was obtained by electrical stimulation. In addition, interspecies variations in *Loxosceles* venom composition have been reported (de Oliveira et al., 2005). The standard murine lethal assay (LD_{50} of venom and ED_{50} for antivenom), is viewed as yardstick to determine the neutralizing potency of antivenoms for therapeutic use, and is currently the most accepted method in various countries (Theakston and Reid, 1983). In Peru, this is the pre-clinical test for assessing the antivenom potency of anti-loxoscelic antivenom.

Since the main effect of *Loxosceles* envenomation is the development of skin lesions on experimental or fortuitous inoculation (da Silva et al., 2004), we studied the ability of P*LI*v to induce dermonecrosis, hemorrhage and edema in rabbits using 10 μg of crude venom. The rationale for this

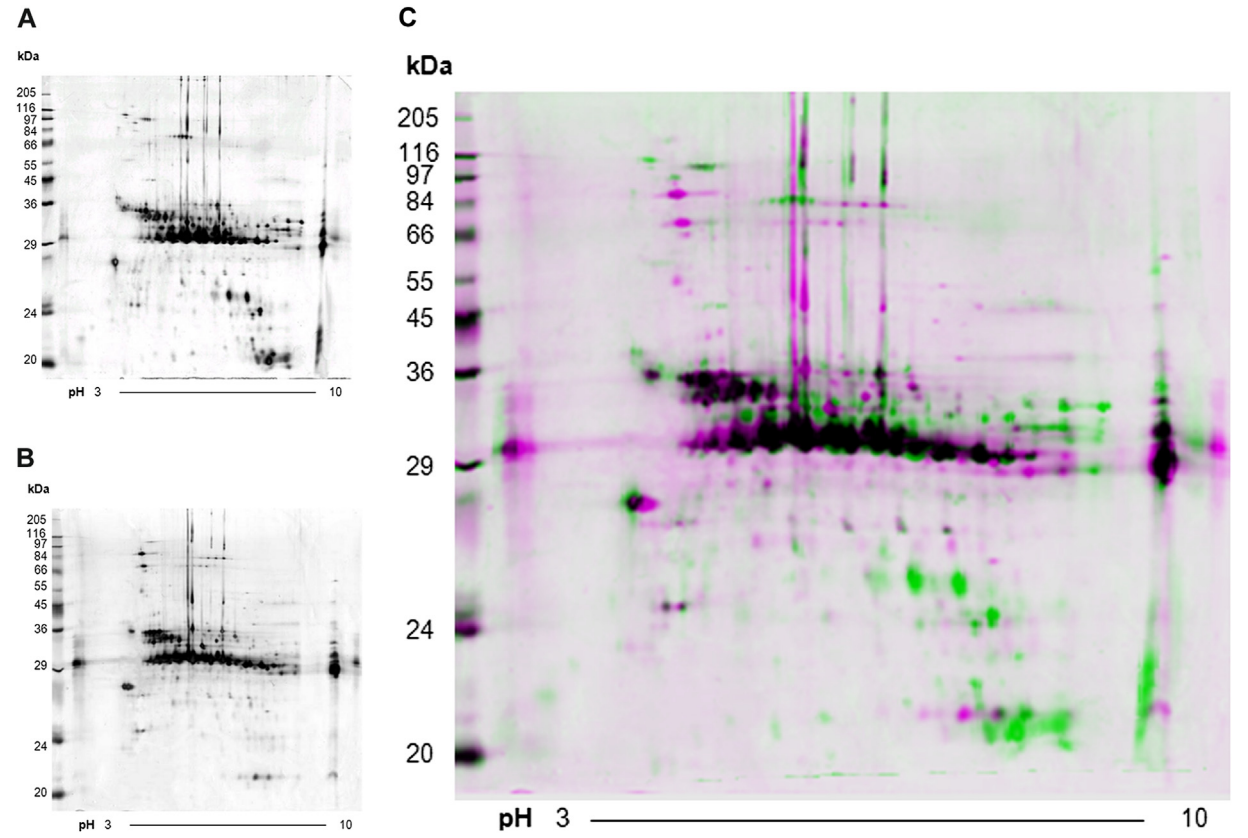

Fig. 3. *Loxosceles laeta* venoms 2D protein profile. PLIv (A) and BLIv (B) were separated by two dimensional electrophoresis of 100 μg of each venom using IPG strip 3–10 non linear, 18 cm. The proteins were stained with silver. (C) Alignment of both two-dimensional SDS-PAGE gels was done using the software Progenesis SameSpot. The green spots are from PLIv gel, the pink spots from BLIv gel, and the dark ones represent the overlapping spots.

dose of *Loxosceles* venom is that we determined that this value represents a Minimum Necrotizing Dose (MND)/kg in rabbits when *L. intermedia* venom (considered as reference venom) is injected (Felicori et al., 2006). The results (Fig. 1) showed that P*Llv* was capable to produce, 72 h after injection, dermonecrosis, hemorrhage and edema effects with typical pattern development of loxoscelic lesions. Comparative analysis of P*Llv* and B*Llv* showed that both Peruvian and Brazilian venoms exhibited same dermonecrotic activities (P*Llv* and B*Llv* = 0.53 cm^2, approximately). Rabbits injected with P*Llv* and B*Llv* showed hemorrhagic area of 3.12 cm^2 and 2.85 cm^2, respectively. Concerning the edematogenic activity, the rabbits injected with P*Llv* showed an edematogenic area smaller than the rabbits injected with B*Llv* (P*Llv* = 0.845 cm^2 and B*Llv* = 1.04 cm^2).

To determine if differences in lethal dose and skin lesions effects by P*Llv* and B*Llv* correlate with changes in sphingomyelinase activity, both venoms were analyzed for their ability to cleave sphingomyelin. Fig. 2 confirms that both venoms were able to hydrolyze sphingomyelin, but P*Llv* exhibited higher sphingomyelinase activity than B*Llv*, and this difference was statistically significant. These data confirm previous observations suggesting that lethal and skin effects of *Loxosceles* venoms are correlated to their sphingomyelinase activity (de Oliveira et al., 2005). The higher lethal and sphingomyelinase activity observed in P*Llv*, may explain the higher frequency of systemic loxoscelism reported in Peru: 25–32% of cases in this country are reported as viscerocutaneous loxoscelism (Sanabria and Zavaleta, 1997; Instituto Nacional de Salud, 2006), compared to 13–16% of cases reported with *Loxosceles* spp in Brazil (Isbister and Fan, 2011).

The components of P*Llv* (Fig. 3A) and B*Llv* (Fig. 3B) were separated by two-dimensional gel electrophoresis and the gels were stained with silver nitrate. Differences in the number and intensity of spots were found between the venoms. A large portion of proteins from P*Llv* and B*Llv* venoms (52 of 105 and 52 of 134 for, respectively) had molecular mass between 29 and 36 kDa. Fig. 3C shows the alignment between P*Llv* and B*Llv* profiles, using the software Progenesis SameSpot. The green spots belong to P*Llv*, the pink spots to B*Llv* and the dark signals are overlapping spots. The alignment revealed 40.4% of difference in the protein pattern between both venoms, within the 29–36 kDa region, particularly in the zone with basic isoelectric point (p*I*), where several P*Llv* proteins are located (green spots). This region corresponds to proteins with dermonecrotic and/or sphingomyelinase activity previously isolated from the venom gland of *Loxosceles* spiders (Kalapothakis et al., 2007). In addition, P*Llv* presents several other proteins, between 20 and 29 kDa, with basic p*I*. This region probably corresponds to proteins with metalloprotease (astacin-like) activity, described as a protein family in venoms of *L. intermedia*, *L. gaucho* and B*Llv* (Trevisan-Silva et al., 2010). Machado et al. (2005), reported several isoforms of dermonecrotic toxins in the venoms of *L. laeta, L. gaucho* and *L. intermedia* Brazilian spiders, thus, corroborating our results showing intraspecific differences in the protein profile. Dermonecrotic toxins, sphingomyelinases D (SMases D), phospholipase D family or Loxtox protein family (Tambourgi et al., 1995; Chaim et al., 2006; Kalapothakis et al., 2007), are the main toxic venom components, responsible for local and systemic effects induced by whole venom from *Loxosceles* spiders. These proteins constitute a family of homologs with 190 non-redundant sequences described in 21 species of the Sicariidae family (Binford et al., 2009). SMase D (EC number 3.1.4.41) catalyzes the hydrolysis of sphingomyelin resulting in formation of ceramide 1-phosphate (C1P) and choline or the hydrolysis of lysophosphatidyl choline, generating the lipid mediator lysophosphatidic acid (LPA) (van Meeteren et al., 2004). C1P is implicated in the stimulation of cell proliferation via a pathway that involves inhibition of acid sphingomyelinase and the simultaneous blocking of ceramide synthesis (Gomez-Munoz et al., 2004). LPA is known to induce various biological and pathological responses such as platelet aggregation, endothelial hyperpermeability, and pro-inflammatory responses by signaling through three G-protein-coupled receptors (Anliker and Chun, 2004; Moolenaar et al., 2004).

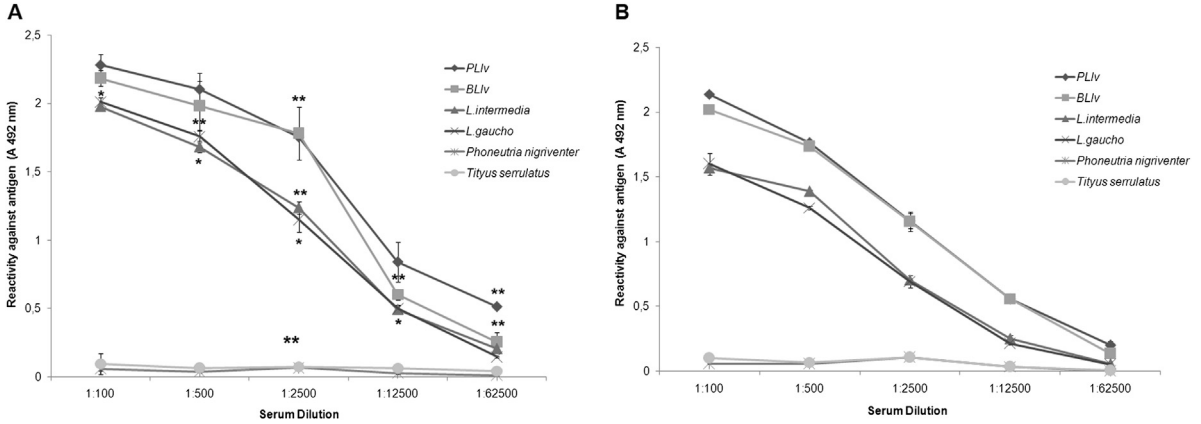

Fig. 4. Immunoreativity of P*Llv* and B*Llv* by ELISA. ELISA showing the reaction of anti-P*Llv* (A) and anti-B*Llv* (B) rabbits sera against arachnidic venoms (P*Llv*, B*Llv*, *L. intermedia*, *L. gaucho*, *Phoneutria nigriventer* and *Tityus serrulatus*). ELISA plates were coated with 5 μg/mL solution of each venom. Antivenom rabbit sera were tested at 5 different dilutions (1:100; 1:500; 1:2500; 1:12,500 and 1:62,500). The absorbance of the samples was determined at 492 nm ($^*p < 0.05$ P*Llv* vs. B*Llv*; $^{**}p < 0.01$ P*Llv* vs. B*Llv*; values shown represent mean plus standard errors).

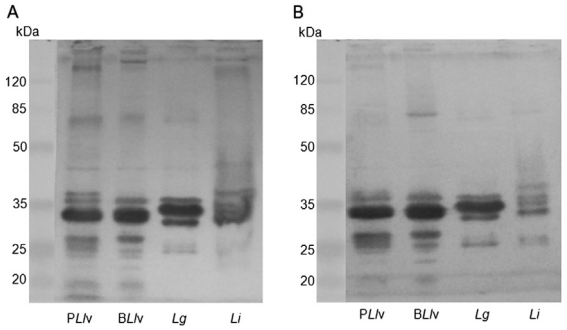

Fig. 5. Immunoreativity of P*Llv* and B*Llv* by western blotting. Four *Loxosceles* species (P*Llv*, B*Llv*), *Loxosceles gaucho* (*Lg*) and *Loxosceles intermedia* (*Li*) were separated by 12.5% SDS-PAGE gel (20 μg), and then transferred to Hybon-P PVDF membrane (Amersham Life Science). The cross-reactivity was assayed by incubating anti-P*Llv* (A) and anti-B*Llv* rabbits (B) sera (1:250 dilution) with the membrane.

3.2. Immunological studies

In this study, we defined the antigenic/immunogenic potential of P*Llv* and B*Llv* by ELISA and immunoblotting. Immune sera anti-P*Llv* and anti-B*Llv* were produced in rabbits and their cross-reactivity against *L. gaucho, L. intermedia, Phoneutria nigriventer* venoms and *Tityus serrulatus* scorpion venom was evaluated. Fig. 4A and B show the ELISA reactivity (A_{492} nm) using different serum dilutions (1:100 to 1:62,500). As expected, each serum reacted strongly against its own venom antigens, and also with venoms from *L. intermedia* and *L. gaucho*. Notably, P*Llv* (Fig. 4A) is moderately more immunogenic than B*Llv* (Fig. 4B). None of the antivenoms reacted with *P. nigriventer* spider or *T. serrulatus* scorpion venoms. These observations suggest the presence of similar antigenic identities or common epitopes across the four *Loxosceles* spiders venoms studied. The antigen–antibody reactivity was also examined using western blotting and the cross-reactivity between *Loxosceles* venoms and anti-P*Llv* and anti-B*Llv* antivenom sera were confirmed (Fig. 5A and B). A strong cross-reactivity with components ranging from 25 to 35 kDa was evident. Proteins with molecular masses between 25 and 35 kDa have been found to be the most immunogenic components of *Loxosceles* venoms (Barbaro et al., 1996). Antibodies against dermonecrotic toxins can be responsible for the strong cross-reactivity in the ELISA assay of the four spider venoms analyzed in this study (Barbaro et al., 1994; Guilherme et al., 2001).

The *in vivo* neutralizing activity in rabbits immunized with whole P*Llv* or B*Llv* venoms was studied by assaying protection against dermonecrosis, hemorrhage and edema. Ten days after the last immunization, rabbits were challenged by intradermal injection of 10 μg whole venoms (P*Llv* or B*Llv*), an amount equivalent to 1 MND/kg (Felicori et al., 2006). Rabbits immunized with P*Llv* and challenged showed full protection against dermonecrosis and 80–90% protection against the hemorrhagic activity induced by both venoms (Fig. 6A). Concerning the edematogenic activity, immunized rabbits afforded about 50% protection to B*Llv*, but lower protection against P*Llv* (Fig. 6A). On the other hand, rabbits immunized with B*Llv* (Fig. 6B) showed similar pattern of neutralization for dermonecrosis and edema, but close to 50% protection against the hemorrhagic-inducing activities by B*Llv*. These results suggest that an efficient neutralization of the dermonecrotic and hemorrhagic effects are obtained by immunizing animals with P*Llv* and B*Llv*, and demonstrate the ability of each specific antibody to neutralize the non-cognate venom, confirming the cross neutralization capability, previously described by Gomez et al. (2001). These results support the notion that toxins venoms share similar epitopes for dermonecrotic toxins (Guilherme et al., 2001). In these assays, the neutralization of edema-inducing activity by P*Llv* afforded lower protection in immunized rabbits.

Finally, we investigated the neutralization of sphingomyelinase activity by commercial sera produced in Brazil and Peru. An *in vitro* neutralization assay was performed by pre-incubating P*Llv* and B*Llv* with different antivenom dilutions from CPPI and INS. The applied doses were 0.125 μg of P*Llv* and 0.250 μg of B*Llv*, once these values showed similar sphingomyelinase activity. Both antivenoms neutralized about 100% of both venoms activities in the dilution 1:100, and more than 80% in the dilution 1:500 (Fig. 6A and B). On the other hand, with the 1:2500 dilution,

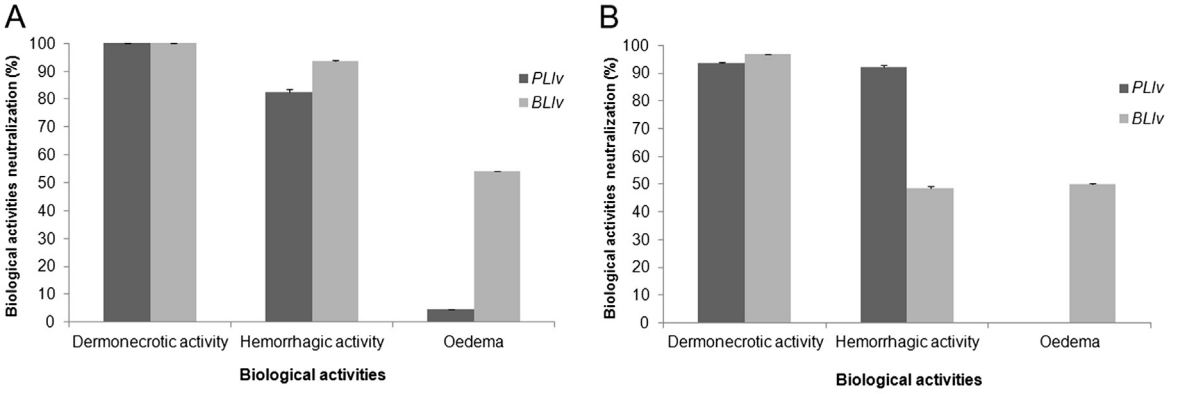

Fig. 6. Neutralization of *in vivo* activities. Rabbits immunized with P*Llv* (A) and B*Llv* (B) were intradermally challenged with 10 μg of both venoms in PBS, and the neutralization of the dermonecrotic and hemorrhagic activity and edema were measured (area) 72 h after the injection. Non immunized animals were used as positive control, and their lesions were considered 100% (**$p < 0.01$ P*Llv* vs. B*Llv*; ***$p < 0.001$ P*Llv* vs. B*Llv*; values shown represent mean plus standard errors).

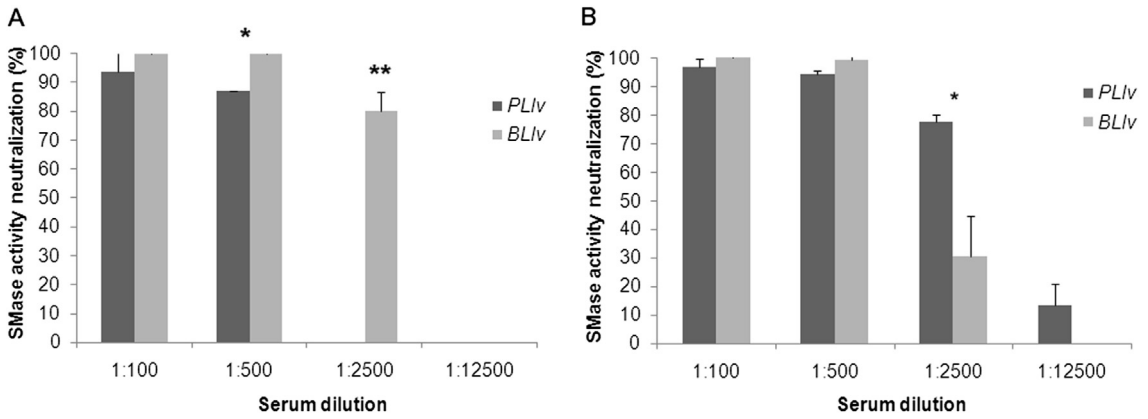

Fig. 7. Neutralization of *in vitro* activity. Both venoms were pre-incubated with different dilutions (1:100, 1:500, 1:2500 and 1:12,500) of anti-P*LIv* serum (INS)(A) Or anti-loxoscelic serum (CPPI) (B) For 1 h at 37 °C and the SMase activity was tested using the Amplex Red sphingomyelinase assay kit (Invitrogen) ($^{*}p < 0.05$ P*LIv* vs. B*LIv*; $^{**}p < 0.01$ P*LIv* vs. B*LIv*; values shown represent mean plus standard errors).

only the CPPI serum partially neutralize both venom (30% for B*LIv* and 80% for P*LIv*, respectively). Previously, Olvera et al. (2006), had suggested designing a polyvalent antivenom and our results confirm that two different and interspecific commercial antivenoms are able to cross neutralize venoms from different species, supporting the idea of developing a "pan-American" or global loxoscelic antivenom (Barbaro et al., 2005; Olvera et al., 2006). Fig. 7

In conclusion, our data suggest, based on the *in vivo* lethal effect and *in vitro* sphingomyelinase activity, that venom of *Loxosceles laeta* from Peru is more toxic than B*LIv* and that antivenom antibodies raised in immunized rabbits or commercial sera produced in Brazil and in Peru are efficient in neutralizing the toxic activity of both venoms.

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Conflict of interest

None declared.

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