Toxicon 93 (2015) 37–40

Contents lists available at ScienceDirect

Toxicon

journal homepage: www.elsevier.com/locate/toxicon

Short communication

Anti-loxoscelic horse serum produced against a recombinant dermonecrotic protein of Brazilian *Loxosceles intermedia* spider neutralize lethal effects of *Loxosceles laeta* venom from Peru

C.G. Duarte [b], C. Bonilla [a], G. Guimarães [b], R.A. Machado de Avila [d], T.M. Mendes [b], W. Silva [a], B. Tintaya [a], A. Yarleque [c], C. Chávez-Olórtegui [b, *]

[a] *Instituto Nacional de Salud, Lima, Peru*
[b] *Departamento de Bioquímica-Imunologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte 31270-901, Minas Gerais, Brazil*
[c] *Universidad Nacional Mayor de San Marcos, Lima, Peru*
[d] *Universidade do Extremo Sul Catarinense – UNESC, Criciúma, Santa Catarina, Brazil*

ARTICLE INFO

Article history:
Received 8 August 2014
Received in revised form
20 October 2014
Accepted 29 October 2014
Available online 30 October 2014

Keywords:
Antivenom
Loxosceles
Recombinant protein
Immunization
Neutralization

ABSTRACT

In this work, an anti-loxoscelic serum was produced by immunizing horses with a recombinant dermonecrotic protein from *Loxosceles intermedia* (rLiD1). Anti-rLiD1 antibodies were able to recognize different species of *Loxosceles* venoms by Western Blot and ELISA. The efficacy of anti-rLiD1 serum against the toxic effects of *Loxosceles laeta* (Peru) venom was tested, showing that anti-rLiD1 serum can neutralize those effects. This study confirms that recombinant proteins can be good candidates to replace crude venoms for antivenom production.

Loxosceles spiders are distributed worldwide, found in several continents. These spiders represent a group of medical importance in North America, Latin America, Europe, Middle East and other parts of Asia, Africa and Australia (Futrell, 1992; da Silva et al., 2004). *Loxosceles* venoms are responsible for a syndrome called loxoscelism, which presents itself in two forms: the cutaneous form, which causes pain and erythema that can develop into a necrotic ulcer; and the systemic loxoscelism, which is characterized by intravascular hemolysis and occasional renal failure (da Silva et al., 2004; Ministério da Saúde, 2011).

Loxosceles venoms present a complex mixture of toxic protein components such as hyaluronidases, metalloproteases, peptides and sphingomyelinases D. The sphingomyelinase D family members are also called dermonecrotic toxins and are the main responsible for toxic effects and pathogenesis of *Loxosceles* envenomation (Tambourgi et al., 1998).

The Public Health Organizations recommend the application of commercial antivenoms produced by hyperimmunization of horses with *Loxosceles* venoms as treatment for spiders bites (Pauli et al., 2009). The production of anti-loxoscelic antivenom is limited, due to the difficulty in obtaining the necessary amounts of venom, since these spiders have small venom glands with restricted production. As an alternative for the utilization of crude venom, recombinant dermonecrotic proteins or their epitopes has been used as immunogens for therapeutic serum development or vaccination approaches (Olvera et al., 2006; de Almeida et al., 2008; Dias-Lopes et al., 2010; de Moura et al., 2011; Mendes et al., 2013; Figueiredo et al., 2014).

In the present study, we compared the neutralization efficacy of an anti-loxoscelic serum produced by immunization of horses with a recombinant dermonecrotic protein (rLiD1) from Brazilian *Loxosceles intermedia* spider venom, produced previously (Kalapothakis et al., 2002; Felicori et al., 2006) and standard antivenom used for loxoscelism therapy in Peru, developed against the toxic effects of venom from *Loxosceles laeta* (Peru) spiders.

The venoms used throughout this study were collected from dissected venom glands of mature spiders, according to da Silveira

* Corresponding author. Departmento de Bioquímica e Imunologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, CP: 486. CEP: 30.161-970, Belo Horizonte, Brazil.
E-mail address: olortegi@icb.ufmg.br (C. Chávez-Olórtegui).

http://dx.doi.org/10.1016/j.toxicon.2014.10.023
0041-0101/

et al. (2002). *L. laeta* (Peru) were collected in the region of Cañete (Lima, Peru) and maintained in the herpetarium INS in Lima, Peru. Brazilian *L. intermedia, L. gaucho* and *L. laeta* (Brazil) 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 venom protein concentration was determined by the method of Bradford (1976). The minimum necrotizing dose (MND) (lowest venom concentration that induces a necrotic area of 1 cm^2) used throughout this study was 6 μg.

rLiD1 was obtained as described by Felicori et al. (2006) and used to immunize adult male horses. After collection of pre-immune serum, the animals received an initial subcutaneous injection of 200 μg of rLiD1 in complete Freund's adjuvant (day 1). Six booster injections were made subcutaneously 30, 60, 90, 120, 150 and 180 days later, with the same dose in incomplete Freund's adjuvant. Blood samples were withdrawn one week after the last injection.

Indirect ELISA and immunobloting assays were performed for immunological characterization of anti-rLiD1 horse sera (Fig. 1), according to Guimarães et al., 2013. For ELISA, plates were coated with 100 μl of a 10 μg/ml solution of either rLiD1, *L. intermedia, L. gaucho, L. laeta* (Brazil) and *L. laeta* (Peru). Sera from pre-immune and immune horse were added in serial dilutions. Triplicate readings were taken for all samples and means were calculated. Anti-rLiD1 serum showed high reactivity against rLiD1, showing that the immunization protocol was efficient. An important cross-

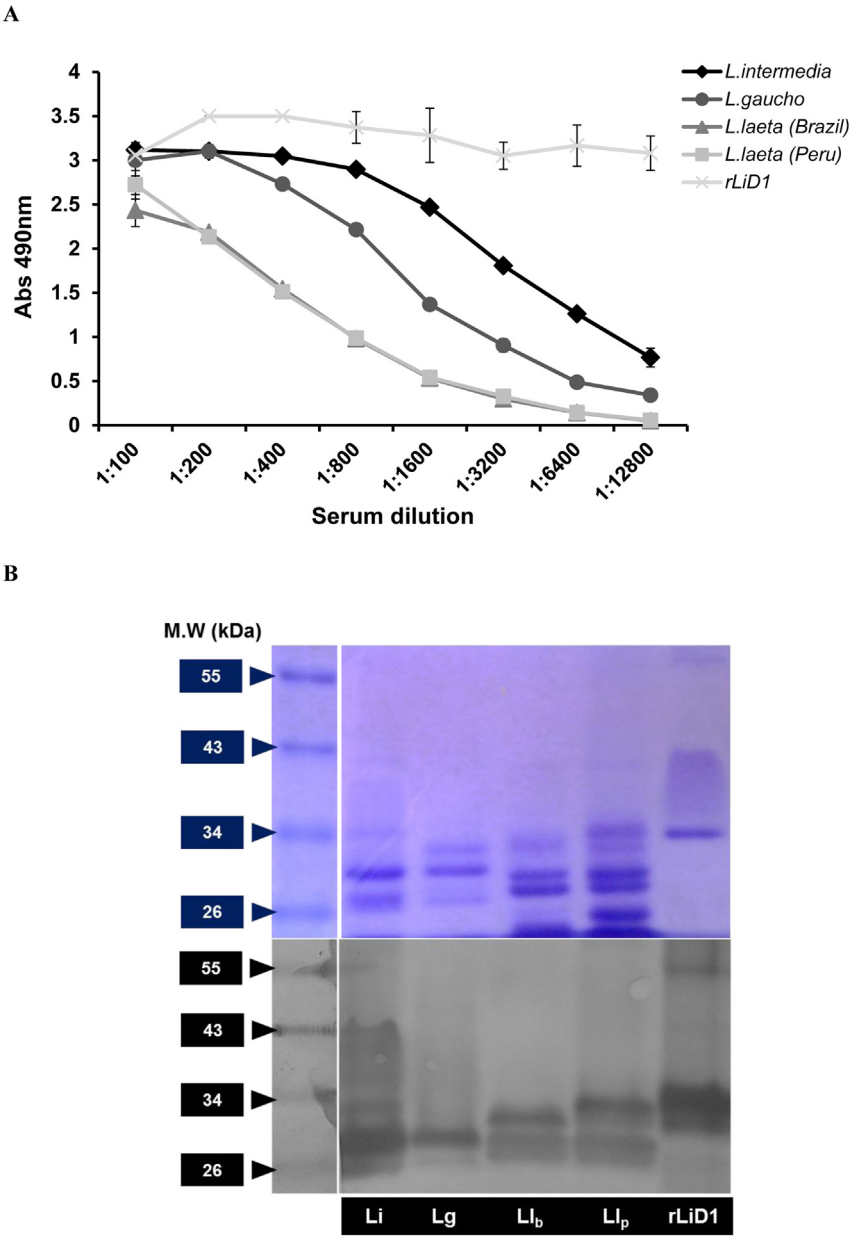

Fig. 1. Immunoreactivity characterization of anti-rLiD1 horse serum. (A) ELISA reactivity against rLiD1, *L. intermedia, L. gaucho, L. laeta* (Brazil) and *L. laeta* (Peru) spider venoms. Plates were coated with 1 μg of recombinant protein or venoms per well and reactivity tested against anti-rLiD1 serum using serial dilution from 1:100 to 1:12,800. A considerable cross-reactivity was observed for all venoms. (B) SDS-PAGE 12.5% of Loxosceles venoms and western blotting with anti-rLiD1 horse serum. Twenty μg of each *Loxosceles* venom *L. intermedia* (Li), *L. gaucho* (Lg), *L. laeta* Brazil (LI_b) and *L. laeta* Peru (LI_p) and 10 μg of rLiD1 were analyzed. After analysis by SDS-PAGE, the gel was blotted in nitrocellulose membrane and tested against anti-rLiD1 horse serum (1:500). An intense reaction with bands in the range of 34–26 kDa was observed. This molecular mass corresponds to proteins from the sphingomyelinase D family, responsible for the toxic effects of Loxosceles venoms.

reactivity against different species of *Loxosceles* venoms was also detected, indicating that these antibodies could have venom neutralization potential (Fig. 1A).

To access whether anti-rLiD1 antibodies could bind to venom's sphingomyelinases D, immunoblotting was performed. *L. intermedia. L. gaucho, L. laeta* (Brazil), *L. laeta* (Peru) venoms (20 μg) and rLiD1 (10 μg) were ran on 12.5% SDS-PAGE (Laemmli, 1970). Gels were blotted onto nitrocellulose membranes (Towbin et al., 1979). After transfer, the membrane was incubated with anti-rLiD1 horse serum (1:500) for 1 h. Anti-rLiD1 serum was able to recognize the analyzed venoms and rLiD1 (Fig. 1B). As expected, there was a strong reactivity with components ranging from 26 to 34 kDa, which is the typical molecular mass of the sphingomyelinases D family (Silvestre et al., 2005), the major component from *Loxosceles* venoms and responsible from the main effects of these spiders venom (da Silva et al., 2004). Machado et al. (2005), reported several isoforms of dermonecrotic toxins in the venoms of *L. laeta*, *L. gaucho* and *L. intermedia* Brazilian spiders, and Guimarães et al. (2013) also shown that remarkable differences were detected within this molecular weight range between Brazilian and Peruvian populations of *L. laeta* venoms, explaining why different bands were recognized in Western Blotting.

Since antibodies capable of binding to sphingomyelinases D were detected in the anti-rLiD1 serum, neutralization assays were performed. To evaluate the dermonecrotic, hemorrhagic and oedematogenic neutralization, adult female New Zealand rabbits (2–2.5 kg) were maintained at the Centro de Bioterismo, ICB-UFMG (Belo Horizonte, MG, Brazil). For the *in vitro* neutralization test, 100 μl of pre-immune serum, anti-rLiD1 serum or commercial Peruvian anti-venom, produced at INS by hyperimmunization of horses with *L. laeta* (Peru) venom, were incubated with 1 MND of *L. laeta* (Peru), for 1 h, at 37 °C and then injected intradermically in rabbits. The animals were evaluated 24 h, 48 h and 72 h after the challenge. The measurements of the dermonecrotic lesions were determined as described by Furlanetto (1962). The diameters of hemorrhagic and oedematogenic lesions were measured with a scale meter and pachymeter, respectively. The venom incubated with pre-immune serum was capable of producing a dermonecrotic lesion of 0.2 cm^2, a hemorrhagic area of 8.05 cm^2, and a 0.6 mm thick edema. The commercial antivenom completely neutralized the dermonecrotic and hemorrhagic activities of *L. laeta* (Peru) and produced edema of 0.067 mm. Anti-rLiD1 serum was also capable of completely neutralizing the dermonecrotic and hemorrhagic activities of *L. laeta* (Peru) venom, but induced edema of 0.5 mm. The oedematogenic activity of *Loxosceles* venoms has already shown to be difficult to neutralize (Mendes et al., 2013; Guimarães et al., 2013). Other venom components may be responsible for edema formation, as described by (Barbaro et al., 2010). However, the pathophysiology of edema formation in loxoscelism is still unknown.

To perform lethal neutralization assays, the median lethal dose (LD_{50}) of *L. intermedia* and *L. laeta* (Peru) venoms were established in male CF1 mice (18–22 g). The animals were maintained in the Centro de Bioterismo of the Centro Nacional de Producción de Biológicos of Instituto Nacional de Salud (INS), in Lima, Peru. The experimental protocols followed the Guide for the Care and Use of Laboratory Animals, (NIH Publication No. 85-23, revised 1996). Groups of four mice were injected with different doses of each venom, via intra-peritoneal (i.p.) route, dissolved in 0.5 ml of PBS-BSA 0.1%. Forty-eight hours later, deaths were recorded and LD_{50} was calculated by Probit analysis (Finney, 1971).

Neutralization of venoms lethal activity by horse anti-rLiD1 antivenom or commercial antivenom were done by i.p. injection of 2.5 LD_{50} of each crude venom, incubated previously (1 h, 37 °C) with 100 μl of either PBS, pre-immune serum, anti-rLiD1 serum or commercial antivenom, as previously described (de Roodt et al., 2002). Deaths were counted after 48 h of venom injection. The neutralization tests, traditionally used for determination of neutralizing potency of anti-loxoscelic antivenom in Peru (Ministério de Salúd, Peru, 2004), showed that the lethal activity of *L. intermedia* was completely neutralized by horse sera raised against rLiD1 and this serum achieved a 75% protection against *L. laeta* (Peru) venom (Table 1). Protection against *L. laeta* (Peru) venom may have not been complete because anti-rLiD1 serum is directed against only one recombinant isoform of dermonecrotic protein. Others isoforms within Loxosceles venoms may share extensive similarity, but are not identical (Kalapothakis et al., 2007). Some of *L. laeta* (Peru) isoforms could not have been completely neutralized by anti-LiD1r antibodies which can be responsible for the residual lethality observed. It is not excluded either that other venom components, such as metalloproteases and hyaluronidases, may also contribute to venom's lethality (Ferrer et al., 2013).

Table 1
Neutralization of lethal activity. Groups of four mice received a subcutaneous doses of 2.5 LD_{50} of *L. intermedia* or *L. laeta* (Peru) venoms (10 and 12.5 μg/20 g of mice, respectively), pre-incubated with 100 μl of anti-rLiD1, anti-loxoscelic or pre-immune sera. The venom was incubated only with PBS as a control. Forty-eight hours after the injections, the deaths were recorded and the number of survivors counted.

	Surviving/Challenged (%)	
	L. intermedia	*L. laeta* (Peru)
PBS	1/4 (25%)	0/4 (0%)
Pre-immune	1/4 (25%)	1/4 (25%)
Anti-rLiD1	4/4 (100%)	3/4 (75%)
Anti-*L. laeta* (Peru)	4/4 (100%)	4/4 (100%)

These experiments show the crossed neutralization of Peruvian *L. laeta* venom by anti-rLiD1 antibodies. The use of recombinant proteins combined with crude venoms has proven to be capable of generating antivenoms with satisfactory neutralization results using reduced amounts of crude venom (Figueiredo et al., 2014). The present results provide the scientific basis to support the use of rLiD1 in the production of Peruvian anti-loxoscelic antivenom. The best protocol for immunization for production of anti-loxoscelic antivenom using recombinant proteins is still under studies, but seems to be a promising approach.

Acknowledgments

This research was supported by Instituto Nacional de Salud (INS), Lima, Peru (R.D. N°1099-2009-DG-OGITT-OPE/INS) and executed by specific agreement with the Universidad Nacional Mayor de San Marcos, Lima, Peru. The authors gratefully acknowledge the financial support of Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Brazil – CAPES (TOXINOLOGIA N° 23038000825/2011-63), Fundação de Amparo a Pesquisa do Estado de Minas Gerais, Brazil (FAPEMIG) and by funds of the INCTTOX PROGRAM of Conselho Nacional de Desenvolvimento Científico e Tecnológico, Brazil (CNPq).

Conflict of interest statement

The authors declare that there are no conflicts of interest.

References

Barbaro, K.C., Lira, M.S., Araújo, C.A., Pareja-Santos, A., Távora, B.C.L.F., Prezotto-Neto, J.C., Kimura, L.F., Lima, C., Lopes-Ferreira, M., Santoro, M.L., 2010.

Inflammatory mediators generated at the site of inoculation of *Loxosceles gaucho* spider venom. Toxicon 56 (6), 972–979.
Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.
da Silva, P.H., da Silveira, R.B., Appel, M.H., Mangili, O.C., Gremski, W., Veiga, S.S., 2004. Brown spiders and loxoscelism. Toxicon 44 (7), 693–709. http://dx.doi.org/10.1016/j.toxicon.2004.07.012.
da Silveira, R.B., dos Santos Filho, J.F., Mangili, O.C., Veiga, S.S., Gremski, W., Nader, H.B., Von Dietrich, C.P., 2002. Identification of proteases in the extract of venom glands from brown spiders. Toxicon 40 (6), 815–822. http://dx.doi.org/10.1016/S0041-0101(02)00078-8.
de Almeida, D.M., Fernandes-Pedrosa, M.F., de Andrade, R.M., Marcelino, J.R., Gondo-Higashi, H., de Azevedo, I.L., Ho, P.L., van den Berg, C., Tambourgi, D.V., 2008. A new anti-loxoscelic serum produced against recombinant sphingomyelinase D: results of preclinical trials. Am. J. Trop. Med. Hyg. 79 (3), 463–470.
de Moura, J., Felicori, L., Moreau, V., Guimaraes, G., Dias-Lopes, C., Molina, L., Alvarenga, L.M., Fernandes, P., Frezard, F., Ribeiro, R.R., Fleury, C., Nguyen, C., Molina, F., Granier, C., Chavez-Olortegui, C., 2011. Protection against the toxic effects of *Loxosceles intermedia* spider venom elicited by mimotope peptides. Vaccine 29 (45), 7992–8001. http://dx.doi.org/10.1016/j.vaccine.2011.08.065.
de Roodt, A.R., Salomon, O.D., Lloveras, S.C., Orduna, T.A., 2002. Poisoning by spiders of *Loxosceles* genus. Medicina (B Aires) 62 (1), 83–94.
Dias-Lopes, C., Guimaraes, G., Felicori, L., Fernandes, P., Emery, L., Kalapothakis, E., Nguyen, C., Molina, F., Granier, C., Chavez-Olortegui, C., 2010. A protective immune response against lethal, dermonecrotic and hemorrhagic effects of Loxosceles intermedia venom elicited by a 27-residue peptide. Toxicon 55 (2–3), 481–487. http://dx.doi.org/10.1016/j.toxicon.2009.09.019.
Felicori, L., Araujo, S.C., de Avila, R.A., Sanchez, E.F., Granier, C., Kalapothakis, E., Chavez-Olortegui, C., 2006. Functional characterization and epitope analysis of a recombinant dermonecrotic protein from *Loxosceles intermedia spider*. Toxicon 48 (5), 509–519. http://dx.doi.org/10.1016/j.toxicon.2006.06.019.
Ferrer, V.P., de Mari, T.L., Gremski, L.H., Silva, D.T., da Silveira, R.B., Gremski, W., Chaim, O.M., Senff-Ribeiro, A., Nader, H.B., Veiga, S.S., 2013. A novel hyaluronidase from brown spider (*Loxosceles intermedia*) venom (Dietrich's hyaluronidase): from cloning to functional characterization. PLoS Negl. Trop. Dis. 7 (5), e2206.
Figueiredo, F.M., Dias-lopes, C., Alvarenga, L.M., Mccormack, J., Mendes, T.M., Machado-de- Avila, R.A., Minozzo, C., Kalapothakis, E., Chavez-Olortegui, C., 2014. Innovative immunization protocols using chimeric recombinant protein for the production of polyspecific loxoscelic antivenom in horses. Toxicon 86, 59–67.
Finney, D.J., 1971. Probit Analysis. Cambridge Univ. Press, Cambridge, p. 333.
Furlanetto, R.S.R.R.R., 1962. Efeito neutralizante do soro anti-loxoscélico administrado em tempos variáveis após a inoculação do veneno. Ciênc. Cult. 14, 253.
Futrell, J.M., 1992. Loxoscelism. Am. J. Med. Sci. 304 (4), 261–267.
Guimarães, G., Dias-Lopes, C., Duarte, C.G., Felicori, L., Machado de Avila, R.A., Figueiredo, L.F., de Moura, J., Faleiro, B.T., Barro, J., Flores, K., Silva, W., Tintaya, B., Yarleque, A., Bonilla, C., Kalapothakis, E., Salas, C.E., Chávez-Olortegui, C., 2013. Biochemical and immunological characteristics of Peruvian *Loxosceles laeta* spider venom: neutralization of its toxic effects by anti-loxoscelic antivenoms. Toxicon 70, 90–97. http://dx.doi.org/10.1016/j.toxicon.2013.04.018.
Kalapothakis, E., Araujo, S.C., de Castro, C.S., Mendes, T.M., Gomez, M.V., Mangili, O.C., Gubert, I.C., Chavez-Olortegui, C., 2002. Molecular cloning, expression and immunological properties of LiD1, a protein from the dermonecrotic family of *Loxosceles intermedia* spider venom. Toxicon 40 (12), 1691–1699. http://dx.doi.org/10.1016/S0041-0101(02)00201-5.
Kalapothakis, E., Chatzaki, M., Gonçalves-Dornelas, H., de Castro, C.S., Silvestre, F.G., Laborne, F.V., de Moura, J.F., Veiga, S.S., Chávez-Olórtegui, C., Granier, C., Barbaro, K.C., 2007. The loxtox protein family in loxosceles intermedia (Mello-Leitao) venom. Toxicon 50 (7), 938–946.
Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227 (5259), 680–685.
Machado, L.F., Laugesen, S., Botelho, E.D., Ricart, C.A., Fontes, W., Barbaro, K.C., Roepstorff, P., Sousa, M.V., 2005 May. Proteome analysis of brown spider venom: identification of loxnecrogin isoforms in Loxosceles gaucho venom. Proteomics . 5 (8), 2167–2176. http://dx.doi.org/10.1002/pmic.200401096 2167.
Mendes, T.M., Oliveira, D., Figueiredo, L.F., Machado-de-Avila, R.A., Duarte, C.G., Dias-Lopes, C., Guimarães, G., Felicori, L., Minozzo, J.C., Chávez-Olortegui, C., 2013. Generation and characterization of a recombinant chimeric protein (rCpLi) consisting of B-cell epitopes of a dermonecrotic protein from Loxosceles intermedia spider venom. Vaccine 31 (25), 2749–2755. http://dx.doi.org/10.1016/j.vaccine.2013.03.048, 7.
Ministério de Salúd, Peru, 2004. Norma Técnica sobre Prevencion y Tratamiento de Acccidentes por Animales Ponzoñosos.
Ministério da Saúde, Brazil, 2011. Sistema nacional de notificação de agravos. Ministério da Saúde, Brasília, Distrito Federal.
Olvera, A., Ramos-Cerrillo, B., Estevez, J., Clement, H., de Roodt, A., Paniagua-Solis, J., Vazquez, H., Zavaleta, A., Arruz, M.S., Stock, R.P., Alagon, A., 2006. North and South American *Loxosceles* spiders: development of a polyvalent antivenom with recombinant sphingomyelinases D as antigens. Toxicon 48 (1), 64–74. http://dx.doi.org/10.1016/j.toxicon.2006.04.010.
Pauli, I., Minozzo, J.C., da Silva, P.H., Chaim, O.M., Veiga, S.S., 2009. Analysis of therapeutic benefits of antivenin at different time intervals after experimental envenomation in rabbits by venom of the brown spider (*Loxosceles intermedia*). Toxicon 53 (6), 660–671. http://dx.doi.org/10.1016/j.toxicon.2009.01.033.
Silvestre, F.G., de Castro, C.S., de Moura, J.F., Giusta, M.S., de Maria, M., Alvares, E.S., Lobato, F.C., Assis, R.A., Goncalves, L.A., Gubert, I.C., Chavez-Olortegui, C., Kalapothakis, E., 2005. Characterization of the venom from the brazilian brown spider *Loxosceles similis* Moenkhaus, 1898 (Araneae, Sicariidae). Toxicon 46 (8), 927–936. http://dx.doi.org/10.1016/j.toxicon.2005.09.002.
Tambourgi, D.V., Magnoli, F.C., van den Berg, C.W., Morgan, B.P., de Araujo, P.S., Alves, E.W., Da Silva, W.D., 1998. Sphingomyelinases in the venom of the spider *Loxosceles intermedia* are responsible for both dermonecrosis and complement-dependent hemolysis. Biochem. Biophys. Res. Commun. 251 (1), 366–373. http://dx.doi.org/10.1006/bbrc.1998.9474.
Towbin, H., Staehelin, T., Gordon, J., 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. U. S. A. 76, 4350–4354.