Toxicon 130 (2017) 63–72

Contents lists available at ScienceDirect

Toxicon

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

Toxicity of the venom of *Latrodectus* (Araneae: Theridiidae) spiders from different regions of Argentina and neutralization by therapeutic antivenoms

Adolfo Rafael de Roodt [a, b, c, *], Laura Cecilia Lanari [a], Rodrigo Daniel Laskowicz [a], Vanessa Costa de Oliveira [b, c], Lucia Elvira Irazu [d], Alda González [e], Luis Giambelluca [e], Néstor Nicolai [f], Javier Hugo Barragán [g], Leticia Ramallo [f], Raúl Alfredo López [h], Jorge Lopardo [i], Oscar Jensen [i], Edmundo Larrieu [j], Arnoldo Calabró [k], Miriam Guadalupe Vurcharchuc [l], Néstor Rubén Lago [c], Susana Isabel García [m], Ernesto Horacio de Titto [m], Carlos Fabián Damín [b]

[a] *Área Investigación y Desarrollo-Venenos, Instituto Nacional de Producción de Biológicos, Administración Nacional de Laboratorios e Institutos de Salud "Dr. Carlos G. Malbrán", Ministerio de Salud de la Nación, Argentina*
[b] *Primera Cátedra de Toxicología, Facultad de Medicina, Universidad de Buenos Aires, Argentina*
[c] *Laboratorio de Toxinopatología, Centro de Patología Experimental y Aplicada, Facultad de Medicina, Universidad de Buenos Aires, Argentina*
[d] *Departamento de Parasitología, Instituto Nacional de Enfermedades infecciosas, Administración Nacional de Laboratorios e Institutos de Salud "Dr. Carlos G. Malbrán", Ministerio de Salud de la Nación, Argentina*
[e] *Centro de Estudios Parasitológicos y de Vectores (CEPAVE)/CONICET, Universidad Nacional de La Plata, Argentina*
[f] *Laboratorio Central de Salud Pública, Ministerio de Salud de la Provincia de Buenos Aires, Argentina*
[g] *Cátedra de Inmunología II, Microbiología Clínica e Industrial, Facultad de Ciencias Veterinarias, Universidad Nacional de La Plata, Argentina*
[h] *Departamento de Zoonosis, Ministerio de Salud de la Provincia de Catamarca, Argentina*
[i] *Departamento de Investigación, Secretaría Salud de la Provincia de Chubut, Argentina*
[j] *Escuela de Veterinaria, Universidad Nacional de Río Negro, Provincia de Rio Negro, Argentina*
[k] *Dirección de Salud Ambiental, Ministerio de Salud de la Provincia de Rio Negro, Argentina*
[l] *Instituto de Animales Venenosos "Jorge Washington Ábalos", Ministerio de Salud de la Provincia de Santiago del Estero, Argentina*
[m] *Dirección Nacional de Determinantes de la Salud e Investigación, Ministerio de Salud de la Nación, Argentina*

ARTICLE INFO

Article history:
Received 5 November 2016
Received in revised form
23 February 2017
Accepted 24 February 2017
Available online 27 February 2017

Keywords:
Antivenom
Envenomation
Black widow
Latrodectus
Toxicity
Venom

ABSTRACT

"Black widow" spiders belong to the genus *Latrodectus* and are one of the few spiders in the world whose bite can cause severe envenomation in humans and domestic animals. In Argentina, these spiders are distributed throughout the country and are responsible for the highest number of bites by spiders of toxicological sanitary interest. Here, we studied the toxicity and some biochemical and immunochemical characteristics of eighteen venom samples from *Latrodectus* spiders from eight different provinces of Argentina, and the neutralization of some of these samples by two therapeutic antivenoms used in the country for the treatment of envenomation and by a anti-*Latrodectus* antivenom prepared against the venom of *Latrodectus mactans* from Mexico. We observed important toxicity in all the samples studied and a variation in the toxicity of samples, even in those from the same region and province and even in the same *Latrodectus* species from the same region. The therapeutic antivenoms efficiently neutralized all the venoms studied.

1. Introduction

Spiders of the genus *Latrodectus* ("widow spiders" or "black widows") are distributed worldwide (Garb et al., 2004). The toxicity of their bites is known from the beginning of the history of medicine (Maretic and Stanic, 1954). Their bites causes severe pain at the

* Corresponding author. Área Investigación y Desarrollo, INPB-ANLIS "Dr. Carlos G. Malbán", Av. Velez Sarsfield 563, CP 1281, CABA, Argentina.
E-mail address: aderoodt@gmail.com (A.R. de Roodt).

http://dx.doi.org/10.1016/j.toxicon.2017.02.029
0041-0101/

site of inoculation of the venom, which irradiates, causing muscle cramps, spasms, motor unrest, salivation, sweating, precordial oppression, hypertension, board-like abdomen, oliguria, anxiety, mental excitation, and agonizing pain; in addition, these symptoms are responsible both for the "*pavor mortis*" experienced by some patients after the bite and for the extended convalescence (Maretic, 1971; Martino et al., 1979; Isbister and White, 2004; Isbister and Fan, 2011; Ministerio de Salud, 2012). Death is not common, but if it occurs, it is generally due to pulmonary edema and cardiac failure (Ministerio de Salud, 2012).

Envenomation is caused by the presence in their venoms of a group of neurotoxins called latrotoxins, which bind proteins of the pre-synaptic membranes (latrophilin and neurexin), triggering the massive release of neurotransmitters (Volynski et al., 2000; Van Renterghem et al., 2000; Bittner, 2000; Ushkaryov et al., 2008). There are different latrotoxins but only α-latrotoxin is toxic to humans and domestic animals. This toxin binds to tissues of mammals, whereas the other latrotoxins are specific to other zoological groups (Grishin, 1998).

The classification of *Latrodectus* spiders is controversial. Although around 31 species have been described (World Spider Catalog, 2017), the taxonomy of the genus has experienced a chaotic history and has not been revised on a worldwide scale (Garb et al., 2004).

In Argentina, "black widows" are represented by two groups of *Latrodectus*: the *mactans* group, with the species *L. mirabilis*, *L. corallinus*, *L. diaguita* and *L. quartus*, and the *curacaviensis* group, with the species *L. antheratus* and *L. variegatus* (Ábalos, 1980; González, 1985). The approximate distribution of these species is shown in Fig. 1. The other representative of the genus *Latrodectus* in Argentina is *L. geometricus* ("brown widow"), which does not represent a toxicological risk in the country. The total content of four venomous glands of *L. geometricus* spiders cannot kill or cause neurotoxic signs in 20-g mice (de Roodt, unpublished).

In Argentina, almost all accidents are related to job activities (González, 1985; Martino et al., 1979; Ministerio de Salud, 2012). Because of this epidemiological feature and because most of the accidents were formerly on populations of agricultural workers, other common names used in Argentina are "araña del lino" ("flax spider"), "araña del trigo" ("wheat spider") or "araña de los rastrojos" or "araña rastrojera" ("stubble spider") (González, 1985). At present, due to the mechanization of the agricultural work, although almost all accidents are rural, the principal groups affected are workers of the harvest of olives and grapes, principally in the north-west of the country, those of the harvest of potatoes and onions in the south of the province of Buenos Aires, and those in oil and gas extraction plants and harvest of berries in the Patagonia, in the south of the country.

In Argentina in the period 2001–2011 there were registered 16.165 accidents and 24 deaths by spiders (Casas et al., 2013; de Roodt et al., 2013). *Latrodectus* bites would represent the most important number of notifications by spiderbite envenoming being around 75% of the total (de Roodt et al., 2002b; Ministerio de Salud, 2012).

Despite the medical and sanitary importance of these spiders in Argentina, up to date, little is known about the toxicity of the venom of *Latrodectus* spiders from this country or its neutralization by antivenoms (de Roodt et al., 2006, 2012). Thus, in the present study, we analyzed the lethal potencies of the venoms of *Latrodectus* spiders from different regions of the country, in order to determine their toxicity and variability, as well as their immunological reactivity against two specific therapeutic antivenoms used for the treatment of *Latrodectus* envenomation in Argentina and a anti-*Latrodectus mactans* antivenom from Mexico. In addition, we analyzed some biochemical and toxicological data of the venom and morphometric data useful from the toxicological point of view.

2. Material and methods

2.1. Spiders and venomous apparatuses

Black widows from different provinces (n = 4,593 females) were used for the preparation of the distinct pools. Spiders from the provinces of Santa Cruz (localities of El Calafate and Esperanza), Chubut (localities of Península Valdés, Puerto Madryn, Rawson, Comodoro Rivadavia and Sarmiento), Río Negro (localities of Las Grutas and San Antonio Oeste), Neuquén (locality of Aguada Pichana) and La Rioja (locality of Aimogasta) were captured by members of the Area of Research and Development of the National Institute for Production of Biologics of the National Administration of Laboratories and Institutes of Health "Dr. Carlos G. Malbrán" of the National Ministry of Health (henceforth INPB). Spiders from Catamarca (localities of San Fernando del Valle de Catamarca and El Portezuelo), were obtained by personnel of the INPB and/or members of the Zoonosis Department of the Ministry of Health of the province of Catamarca. Spiders from Santiago del Estero (locality of the province of Santiago del Estero) were obtained by members of the Institute of Venomous Animals "J. W. Abalos", from the Ministry of Health of Santiago del Estero. Spiders from the province de Buenos Aires (locality of Sierra de la Ventana) were obtained by members of the Central Laboratory of Public Health "Tomás Perón" of the province of Buenos Aires (henceforth LCSP). The different localities where the spiders were collected are shown in Fig. 2. With the exception of one pool from Catamarca constituted by *L. anterathus* (*curacaviensis* group), all the rest of spiders were from the *mactans* group. The characteristics of the pools and the spiders can be seen in Tables 1 and 2.

In all the cases, the methodology used to capture the spiders and obtain their venomous apparatuses was the same.

2.2. Venom

The venomous apparatuses and venom were obtained from the homogenates of the venomous glands as previously described (de Roodt et al., 2001, 2002a,b, 2007; Ozkan et al., 2006, 2007). The supernatants of the centrifuged macerated tissues were diluted in 0.15 M NaCl, aliquoted and stored at −20 °C until use. Only the venomous apparatuses containing both venomous glands were considered for the study of the different pools of venoms.

2.3. Lethal potency

The lethal potency of the venom was determined by injecting CF-1 mice through the intraperitoneal (i.p.) route, following the recommendations to determine the lethal potency of venom from venomous animals (World Health Organization, 1981, 2010; Theakston and Reid, 1983). Briefly, different doses of venom diluted in 0.15 M NaCl in a final volume of 0.5 ml were injected in series of five mice per dose level. After 48 h the plot of the log of the venom dose versus the percentage of deaths was analyzed by non-linear regression (Casasola et al., 2009) and the median lethal dose was estimated as the dose of venom expressed as micrograms of protein or as the theoretical proportion of venomous apparatus content that kills 50% of the challenged mice. This estimation comes from the relation: lethal dose expressed in microliters/volume of solution from homogenate corresponding to one venomous apparatus. In example, if in a pool of 10 venomous apparatuses/one milliliter of solution, the LD_{50} obtained was 50 μl/mouse, the potency expressed in this way would be 0.5 venomous apparatus/mouse.

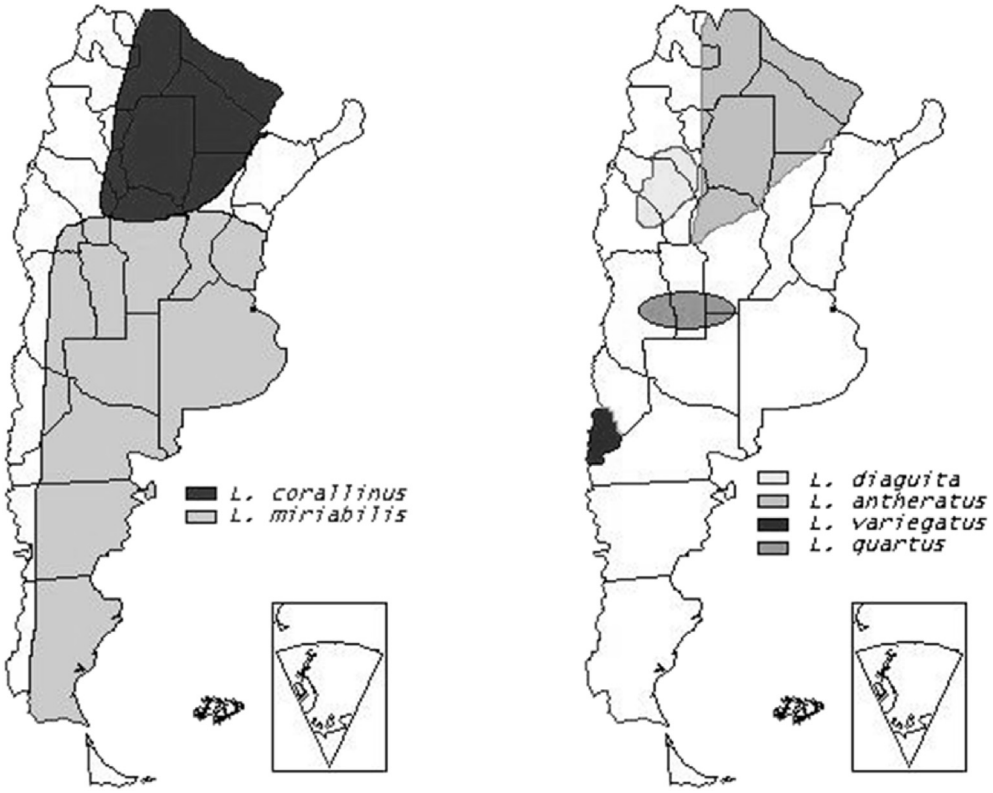

Fig. 1. Distribution of the different species of *Latrodectus* in Argentina. The maps show the distribution of the different species of black widow in Argentina. Note the superposition of several species in some regions of the country, and the wide distribution of the *mactans* group (*L. corallinus, L. mirabilis, L. quartus* and *L. diaguita*). *Latrodectus* species can also be found in other regions not shown in the maps, but the maps show only the distribution of spiders in the regions from which the spiders used to obtain the venom were captured.

2.4. Antivenoms

The antivenoms used in this study are routinely used for the treatment of *Latrodectus* envenomation in Argentina and Mexico. These antivenoms are prepared from hyperimmunized horses. These were: 1) Suero Anti-*Latrodectus* produced by the INPB, Batch 836 bis, expiration date January 31st, 2013, protein content 48.7 mg/ml (henceforth AL-1). This antivenom is raised against the venom of *Latrodectus* from different regions of Argentina and obtained by treating the plasma by ammonium sulfate precipitation and pepsin digestion. Presentation: 2 ml vial of liquid equine $F(ab')_2$ fragments. 2) Suero Anti-*Latrodectus* produced by the LCSP, Batch 012, expiration date September 2013, protein content 47.3 mg/ml (henceforth AL-2). This antivenom is raised against the venom of *L. mirabilis* from the province of Buenos Aires and obtained by treating the plasma with caprylic acid. Presentation: 2-ml ampoule of liquid equine whole IgG. 3) Aracmyn-Plus, produced by Instituto Bioclón, Mexico DF, Mexico, Batch B-7L-06, expiration date February 2010, protein content after re-dissolved 2.6 mg/ml (henceforth AL-3). This antivenom is raised against the venom of the North American *L. mactans*. Presentation: vial of lyophilized $F(ab')_2$ fragments of equine immunoglobulins to be re-dissolved in 5 ml. The antivenom was re-dissolved immediately before use. All the antivenoms were used during their period of validity. The protein content of the antivenoms used in the study was determined by the Biuret method, using the commercial Proti II kit (Wiener, Rosario Argentina).

2.5. Mice

The mice used in this study were CF-1 of 18–22 g of body weight. The animals were used following the ethical statements of the INPB, which are in accordance with the suggestions of the National Research Council (National Research Council, 2002).

2.6. Determination of the body size and weight of the spiders

The cephalothorax-abdomen length was measured with a caliper from the chelicerae to the silk-producing glands in the pool of spiders from Santa Cruz, Río Negro, Chubut (pool of the province and individual pools from two localities), Catamarca, Neuquén and La Rioja (n = 920), and expressed in mm. Additionally, the weight of these spiders (n = 921) was recorded by individually weighting the spiders in an analytical scale (Ohaus) and expressed in milligrams. The data obtained for each region were analyzed by Kruskal-Wallis and by Mann Whitney tests, and the relationships between these two variables were analyzed by lineal regression.

2.7. Determination of the weight of the venomous apparatuses

The dried venomous apparatuses of spiders from Rio Negro (n = 50), La Rioja (n = 99), Neuquén (n = 59) and Santiago del Estero (n = 50) and a pool from La Rioja and Rio Negro (n = 26) were individually weighed in an analytical scale (Ohaus). The differences between the distinct regional samples were statistically analyzed by Mann Whitney test.

2.8. Determination of the protein content of the venomous apparatuses

To determine the individual variation of each of the venomous apparatuses, individual dried venomous apparatuses (n = 26), from

Fig. 2. Provinces and points where the specimens of black widows were collected. 1- Santiago del Estero province, Santiago del Estero city. 2- Catamarca province, from east to west: San Fernando del Valle de Catamarca and El Portezuelo. 3- La Rioja province, locality of Aimogasta. 4- Buenos Aires province, Sierra de la Ventana: 5- Neuquén province, Aguada Pichana. 6- Río Negro province, from east to west: San Antonio Oeste and Las Grutas. 7- Chubut province, from east to west: Península Valdés (Caleta Valdés), Puerto Madryn, Rawson, Comodoro Rivadavia and Sarmiento. 8- Santa Cruz province, from west to east: El Calafate and Esperanza.

Chubut and La Rioja were homogenized in a same volume for each venom apparatus was centrifuged and the supernatant was read in a spectrophotometer at wavelengths of 214 and 280 nm. In addition the protein content was determined by the Bradford method (1976), using the commercial Protein-Assay Kit (Bio Rad). The values obtained from the individual measurements of protein content and weight, were analyzed by linear regression.

To estimate the protein by venomous apparatuses of the pools of the venoms from different regions, it was used the method of Bradford. The protein by venomous apparatus was estimated as:

Table 1
Characteristics of the spiders. Some physical characteristics of the spiders and their venomous apparatuses. The body length (from chelicerae to silk glands), body weight and the protein content of the venomous glands of the spiders studied as well the dry weight from some groups of the spiders used to obtain the venom in this study are showed. Values indicate the median with the minimal and maximal values found in each sample into brackets. ND: not determined. The protein content column indicates the protein by venomous apparatus in each pool (total protein of the pool/number of venomous apparatuses) and the standard deviation (±) of the determinations. The regression column indicates the regression values between body weight and body length for each region. The n values are indicated in the correspond column and in all the cases the p values of the regression were <0.0001.

Provinces	Venomous Apparatuses			Body			
	Number of samples	Dry Weight (μg)	Protein content (μg)	Number of samples	Body Weight (mg)	Body Length (mm)	Regression (weight-length)
Río Negro	50	450 (100–900)	79 ± 3	182	152 (12–583)	9.7 (4.2–13.0)	0.82
Neuquén	59	200 (100–700)	59 ± 6	106	63 (10–190)	7.4 (4–9.6)	0.76
La Rioja	99	200 (50–600)	58 ± 6	285	56 (7–350)	7.4 (4.2–13.0)	0.59
Santiago del Estero	50	200 (100–300)	35 ± 3	ND	ND	ND	ND
Chubut (P. de Valdés)	200	ND	53 ± 10	115	54 (8–348)	6.8 (3.6–11.9)	0.59
Chubut (C. Rivadavia)	103	ND	67 ± 7	102	123 (11–729)	8.8 (4.2–13.7)	0.77
Santa Cruz	186	ND	48 ± 9	108	83 (16–221)	8.0 (5.3–10.9)	0.76
Catamarca (El Portezuelo)	23	ND	46 ± 15	23	84 (7–190)	6.9 (3.3–9.1)	0.87
Catamarca (S.F. del Valle de Catamarca)	70	ND	51 ± 6	ND	ND	ND	ND

Table 2
Toxicity of the samples. Composition of the different samples of venom studied. The source of material for the preparation of the different pools of venoms studied as well as the number of animals used and the species are presented. The lethal potencies of the different samples are expressed as the amount of protein required to kill half of challenged 18–22-g mouse (LD_{50} in μg/mouse) expressing the value and 95% confidence intervals, or as the LD_{50} contained in one venomous apparatus of the spiders (LD_{50}/venomous apparatus). (*) Pool composed of venom from Península Valdés, Comodoro Rivadavia, Sarmiento, Rawson and Puerto Madryn, all localities of Chubut). (**) Pool composed of venom from spiders of Santiago del Estero, La Rioja, Catamarca, (*L. diaguita*) and Santa Cruz (*L. mirabilis*).

Province	Regions	Number of spiders	Species	LD50 (μg/mouse)	LD50/Venom. Apparatus
Chubut	Península Valdés	200	*L. mirabilis*	7.9 (6.4–9.8)	6.7
Chubut	Comodoro Rivadavia	103	*L. mirabilis*	6.2 (5.2–7.1)	14.7
Chubut	Pool Chubut (*)	130	*L. mirabilis*	12.2 (8.7–17.1)	6.7
Río Negro	San Antonio Oeste - Las Grutas	50	*L. mirabilis*	8.7 (8.3–9.0)	15.2
Río Negro	San Antonio Oeste - Las Grutas	100	*L. mirabilis*	9.3 (8.8–9.9)	9.9
Santa Cruz	Esperanza - El Calafate	136	*L. mirabilis*	3.9 (2.9–5.2)	10.3
Santa Cruz	Esperanza - El Calafate	50	*L. mirabilis*	3.1 (2.7–3.5)	21.3
Neuquén	Aguada Pichana	59	*L. mirabilis*	22.5 (19.4–26.0)	3.2
S. del Estero	Santiago del Estero	50	*L. corallinus*	11.1 (9.7–13.5)	2.6
La Rioja	Aimogasta	99	*L. diaguita*	6.2 (4.4–8.9)	9.3
La Rioja	Aimogasta	943	*L. diaguita*	12.7 (5.4–19.7)	2.7
S.F.V. de Catamarca- El Portezuelo	S.F. del Valle de Catamarca	70	*L. diaguita*	6.7 (5.6–8.3)	9.1
S.F.V. de Catamarca- El Portezuelo	S.F. del Valle de Catamarca - El Portezuelo	167	*L. diaguita*	21.6 (18.8–24.9)	2.5
S.F.V. de Catamarca- El Portezuelo	El Portezuelo	23	*L. antheratus*	8.0 (4.1–15.6)	5.7
Buenos Aires	Sierra de la Ventana	58	*L. mirabilis*	15 (9.5–24.0)	4.2
Buenos Aires	Sierra de la Ventana	153	*L. mirabilis*	19.5 (16.8–22.6)	4.6
Buenos Aires	Sierra de la Ventana	402	*L. mirabilis*	8.7 (4.2–19.2)	9.1
Pool	Pool (**)	1800	*L. mirabilis* -*L. diaguita*	7.7 (6.2–9.7)	3.2

total protein/number of venomous apparatuses.

2.9. SDS-PAGE

SDS-PAGE (12.5% polyacrylamide concentration) was carried out under non-reducing conditions, as described by Laemmli (1970), running 5 μg of each sample of venom and molecular mass markers (Bio Rad Broad Range). The protein bands were either stained with Coomassie Brilliant Blue G-250 or blotted for 1 h onto nitrocellulose membranes (Bio Rad), according to Towbin et al. (1979). To determine the proportion of the different bands, the gels were analyzed with the graphic software Gel Pro Analyzer (Media Cybernetics, Rockville, MD).

2.10. Immunochemical studies

2.10.1. Double immunodiffusion (Ouchterlony method)

The double immunodiffusion test was carried out by conventional methods (Margni, 1990). Briefly, 10 μl of each antivenom or its dilutions were confronted against 10 μl of a venomous apparatus homogenate of the samples from Buenos Aires (Sierra de la Ventana), Neuquén, Río Negro, Chubut (pool), Chubut (Comodoro Rivadavia), Santa Cruz, Catamarca, Santiago del Estero, La Rioja (all those venoms from *mactans* group), and Catamarca (*antheratus* group) and from the pool of all the venoms. The venom concentrations ranged from 0.5 to 1.0 mg/ml in 0.15 M NaCl. The gels were incubated for 48 h at room temperature, washed in 0.15 M NaCl for 48 h, dried and stained with Amido Schwartz.

2.10.2. Immunoblot

Protein profiles of the different venoms were generated by SDS-PAGE by using 5 μg of each venom, which were electrophoretically resolved using the same conditions as described above. After the run, the protein was transferred to nitrocellulose membranes and probed with AL-1 and AL-3. After transfer, nitrocellulose membranes were blocked overnight with 3% defatted milk in Tris-buffered saline pH 7.4 and incubated with AL-1 or AL-3 for 1 h at room temperature. Anti-horse immunoglobulin peroxidase conjugate (The Binding Site) was used as the secondary antibody and the protein bands were developed by the addition of diaminobencidine (Sigma) plus hydrogen peroxide (Riedel) as substrate. As negative control, a western blot made in the same conditions of blotting, was treated with horse Anti-*Bothrops* antivenom (Bivalent antivenom, INPB, batch 262) as primary antibody. As positive control of the binding of the conjugate, another gel in the same conditions, including lanes containing 5 μg of equine IgG and $F(ab')_2$ fragments was used.

Between each step in all the cases the membranes were washed four times by 5 min each using pH 7.4 tris-saline buffer solution with 0.3% defatted milk.

2.11. Neutralization of lethal activity

The neutralizing capacity of the three antivenoms was studied on the venoms of *L. mirabilis* from the provinces of Chubut and Buenos Aires. Series of five CF-1 mice (18–22 g) per dose of antivenom level were injected with a mixture of 3 LD_{50} of venom-different doses of antivenom, preincubated at 37 °C for 30 min.

The number of deaths 48 h after injection was recorded and the median effective dose (ED_{50}) was estimated as the antivenom dose that protected 50% of mice. In addition the dose that protected 100% of challenge mice (ED_{100}) was recorded. Positive controls were injected with 3 LD_{50} of venom in 0.5 ml of 0.15 M NaCl. The plot between the log of the dose of antivenom and the percentage of survival was studied by non-linear regression (Casasola et al., 2009). Both doses were expressed in microliters and milligrams of antivenom. In addition, the potency of the antivenoms was expressed as the LD_{50} neutralized by 1 ml of antivenom.

2.12. Statistics

The Kolmogorov-Smirnov test was used to study the distribution of data. For the comparison of groups, the non-parametric Kruskal-Wallis and Mann-Whitney tests were used, whereas for the comparison of the relation between some variables we used linear regression analysis. To determine the lethal potencies of the venoms and the neutralizing potency of the antivenoms, the non-linear regression analysis of variable slope was used. For the application of the Kruskal-Wallis test, the method described by Conover (1999) was followed using the software Excel (Excel: mac 2011®, Microsoft®, CA). For all the rest of calculations, we used the software Prism 5.0 (Graph Pad, La Jolla, CA).

3. Results

3.1. Morphometric data

The relationships between the length of the cephalothorax and the body weight showed a direct relation in all the cases studied (r^2 around 0.6 to over 0.8, $n = 7$, $p < 0.0001$) (see Table 1 and Appendix 3 in Supplementary data). The length of the cephalothorax of all the black widows measured ($n = 920$, females) ranged between 3.3 and 13.7 mm long, with a median of 7.7, and the body weight ($n = 921$ females) ranged between 7 and 729 mg, with a median of 72.9 mg, (Table 1 and Appendices 1 to 3 in Supplementary data). The highest body weights were those of the samples from Río Negro and Comodoro Rivadavia (Chubut) ($p < 0.05$), followed by the samples from Catamarca, Santa Cruz and Neuquén ($p < 0.05$), being the samples from La Rioja and Península Valdés (Chubut) those with the lowest body weight ($p < 0.05$) (Table 1, Appendix 1 and 4). The length of the cephalothorax seemed to be a better variable to make measurements since it was not affected by factors like pregnancy or feeding status. The highest values of length and weight were those of the spiders from Río Negro and Comodoro Rivadavia (Chubut) ($p < 0.05$). The following cephalothorax length was that of spiders from Santa Cruz, followed by those from La Rioja and Neuquén, and by those from Catamarca and Península Valdés ($p < 0.05$). (Table 1, Appendices 2 and 5).

A direct relationship between the protein content and the dry weight of the individual venomous apparatuses was observed, with $r^2 > 0.6$ ($n = 26$) when the A_{280}nm ($r^2 = 0.63$, $p < 0.0001$) or A_{214}nm ($r^2 = 0.71$, $p < 0.0001$) were measured or when the protein content was determined by Bradford method ($r^2 = 0.70$, $p = 0.0012$).

Regarding the dry weight and protein content of the venomous apparatuses of the pool of venoms, the values ranged from 50 μg to 900 μg of dry weight and from 34 to 79 μg of protein per venomous apparatus (Table 1) and showed a good relationship although with no statistical significance ($r^2 = 0.63$, $n = 4$, $p = 0.20$) possibly due the low number of data. Nevertheless the venomous apparatuses of spiders from Rio Negro were the higher in weight ($p < 0.0001$) and protein content ($p < 0.05$) while samples with lower weights possessed the lower protein content ($p < 0.05$), which could suggest a relation similar to that observed with the individual venomous apparatuses (See Table 1 and Supplementary data, Appendix 6).

We found a strong relation between the protein content of the venomous apparatuses and the length of the cephalothorax (r^2 0.73, $n = 7$, p 0.0147).

3.2. SDS-PAGE

The electrophoretic profile in all the cases showed bands in the order of 120–130 kDa, representing 3–10% of the homogenate mass. The molecular masses were compatible with the molecular mass of latrotoxins. In addition, an important band in the range of 60–70 kDa in concordance with the molecular weight of hemocyanin (which was expected because the samples were from homogenates) was observed in all the samples (Fig. 3).

3.3. Lethal potency

The median lethal dose was 10 μg, with a range from 3.1 to 22.5 μg. Expressed as the LD_{50}s contained by venomous apparatus, the range was 2.5–21.3 (Table 2). The relationship between the lethal potencies expressed as mass of venom or as the LD_{50}/venomous apparatus was low (r^2 0.43, n = 18, p = 0.003).

The most potent lethal doses were those of spiders from Santa Cruz, being one of this samples (LD_{50} 3.1 μg/mouse) the most potent of the tested venoms ($p < 0.05$) whereas the least potent ones were those from one sample from Catamarca and the sample from Neuquén. Values of LD_{50}s and 95% confidence intervals are seen in Table 2 and Appendix 7 in Supplementary data. Important differences were observed between samples from different regions, even in a same species of spider.

3.4. Double immunodiffusion

All the antivenoms showed strong reactivity against the different venoms in almost all the cases. AL-1 and AL-2 cross-reacted with the venoms of all the regions, with AL-1 showing more strongly stained bands. AL-3 showed strong reactivity only against the venoms from spiders of Buenos Aires, Neuquén, Santa Cruz and Catamarca, being the reactivity against the other venoms either absent or very weak; nevertheless, the reactivity against *L. antheratus* venom gland homogenates was important (Fig. 4).

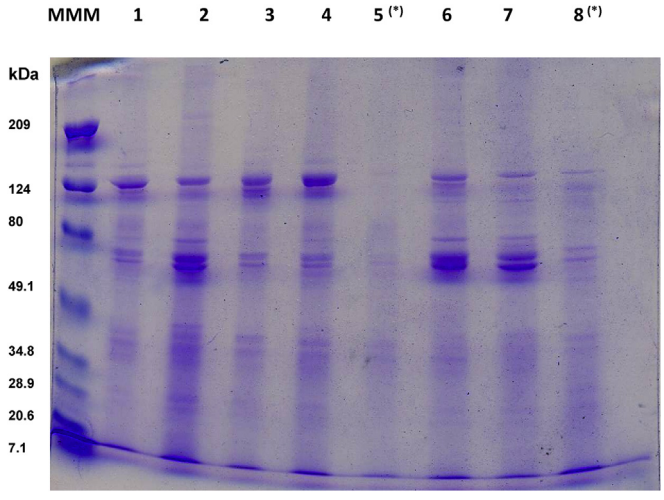

Fig. 3. SDS-PAGE. SDS-PAGE in non-reducing conditions in a 12.5% gel in a tris-glycine system. Five micrograms of different samples were run with the molecular mass markers (Bio Rad Broad Range prestained). 1- Molecular mass markers; 2- Santa Cruz; 3- Río Negro; 4- Chubut (Península Valdés); 5- Chubut (Comodoro Rivadavia [*]); 6- Neuquén; 7-La Rioja; 8-Catamarca [*]. The mark [*] means that in these lanes 1 μg of venom was run.

3.5. Immunoblot

The two antivenoms tested by western blot recognized all the venoms transferred (Fig. 5), but this recognition was different. AL-1 showed higher immunochemical reactivity because all the components of the venoms were recognized in a 1/100 dilution, whereas AL-3 recognized the components of the venoms only when using a 1/2 dilution.

3.6. Seroneutralization assays

The three antivenoms showed neutralizing capacity against the two venoms studied. AL-1and AL-2 were more efficient to neutralize the venom from Buenos Aires and AL-1 showed the better neutralization than AL-3 on the venom from Chubut ($p < 0.05$) (See Table 3). Nevertheless the neutralization by AL-3 although lower, would be acceptable since using this experimental model provided 50–75% of the protection conferred by the specific antivenoms. However, the clinical health status of the surviving mice treated with AL-3 was rather worse than that of those treated with other antivenoms. Indeed, although AL-3 showed an important para-specific neutralization considering the technique used (the one required for the different pharmacopeias), the surviving animals, 48 h after injection, showed severe contractures of the skeletal muscle, slow motion and respiratory difficulty and other signs not observed in the surviving mice treated with AL-1 or AL-2.

4. Discussion

Despite the very wide distribution of black widows in Argentina and the different climate and geographical characteristics of the regions where the specimens studied came from, the size of the spiders was very close. Nevertheless slight differences in the body size were observed, even between spiders from a same province (Table 1). The largest specimens were those from Río Negro ($p < 0.05$) and Comodoro Rivadavia (Chubut province) ($p < 0.05$), whereas one of the smallest ones were those from Península Valdés (Chubut province). This shows that differences are not a characteristic of the province but of the different populations. See Table 1 and Supplementary data.

We observed a direct relation in the size of individual venomous apparatuses and the protein content ($r^2 > 0.63$ to 0.71, n = 26, $p < 0.0001$) as well as in the size and protein content of the pool of venomous apparatuses, where the biggest venomous apparatuses showed higher protein content ($p < 0.05$). In addition larger spiders seems to have the larger venomous apparatuses when the protein content is considered ($r^2 > 0.73$, n = 7, $p < 0.001$). These observations could indicate that large spiders contain more venom in their glands than smaller spiders.

The electrophoretic pattern of the different samples of venom gland homogenates showed in all the cases up to 10% of components in the order of the molecular mass of latrotoxins, i.e. around 110–130 kDa (Grishin, 1998) (Fig. 3), which can also be observed in the western blot (Fig. 5). This indicates that the latrotoxins are an important percentage in the mass of the venom since we analyzed the homogenates of the glands, which contains a big amount of other proteins in addition of venom components.

The lethal potencies were variable, ranging from 7 to 10 fold from the maximal to the minimal potency, depending on whether they were expressed as mass of venom or LD_{50} contained in a venomous apparatus respectively (Table 2). The variation of toxicity was important even in spiders from the same species and in the same province and region, as can be seen in the case of *L. mirabilis*, a species widely distributed in Argentina (Figs. 1 and 2, Table 2).

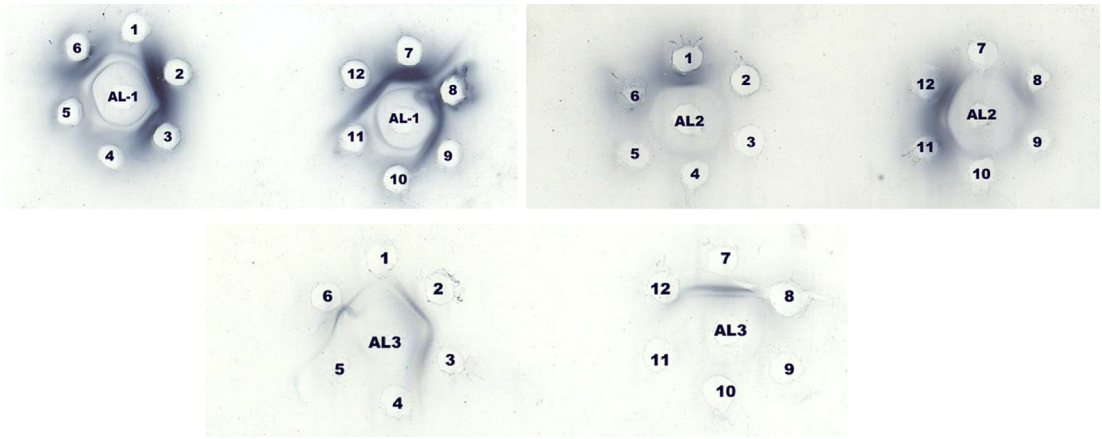

Fig. 4. Double immunodiffusion. The venoms from the different regions (10 μl) were confronted with 10 μl of the three antivenoms studied. AL-1: Suero Anti-*Latrodectus*, INPB (dilution 1/3, upper left), Argentina; AL-2: Suero Anti-*Latrodectus*, LCSP, Argentina (dilution 1/3, upper right); AL-3: Aracmyn Plus: Instituto Bioclón, Mexico (no dilution, down, in the center). The venoms were 1: Buenos Aires; 2: Neuquén; 3: Río Negro; 4- Chubut (Pool); 5 Chubut (Comodoro Rivadavia); 6: Santa Cruz; 7: Catamarca (Pool); 8: Catamarca (*L. anterathus*); 9: Santiago del Estero; 10: La Rioja; 11: Pool of all the venoms (1–10); 12: Pool from Catamarca, La Rioja, Santiago del Estero and Santa Cruz. Venom concentrations used for testing the South American antivenom was 1 mg/ml and for the North American antivenom, 0.5 mg/ml. Staining with Amido black.

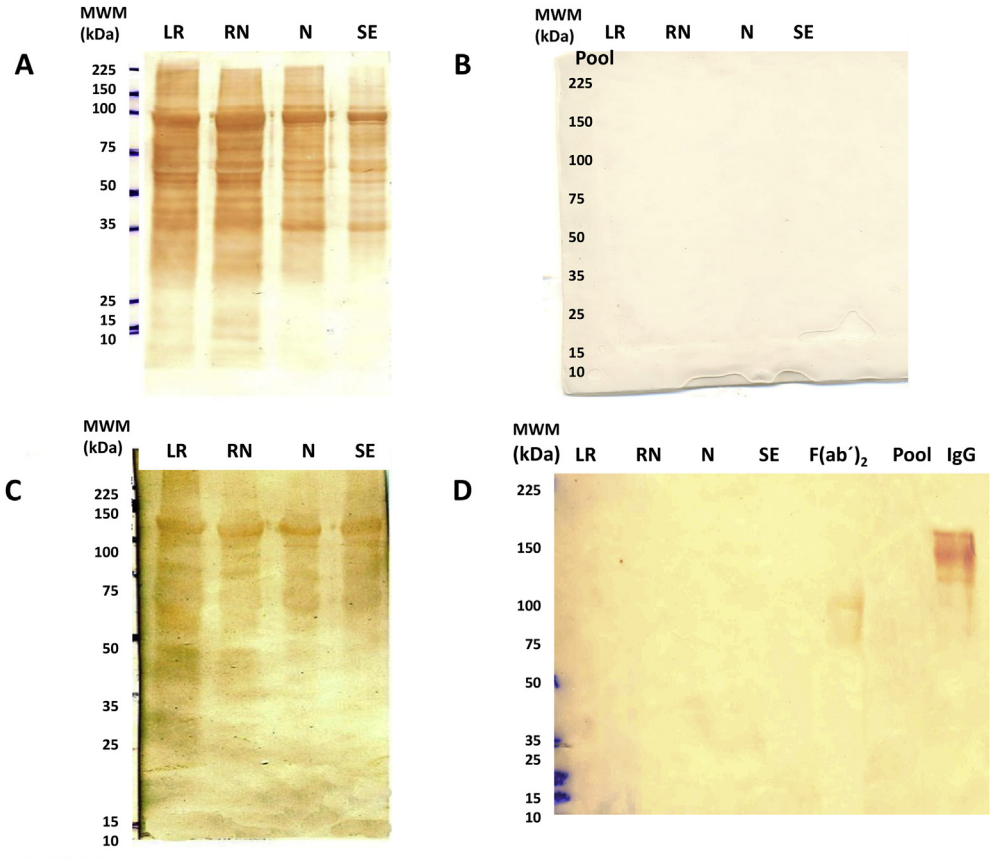

Fig. 5. Westernblot of two antivenoms against different venoms. Venoms from La Rioja (LR), Río Negro (RN), Neuquén (N) and Santiago del Estero (SE) were transferred after the SDS-PAGE to a nitrocelullose membrane and incubated with the antivenoms. A) reactivity of AL-1 antivenom (dilution 1/100). B) negative control using antibothropic antivenom 1/100 as primary antibody. C) reactivity of AL-3 (dilution 1/2) antivenom. D) negative control and control of conjugate: blot of a gel run in the same conditions but with the addition of lanes containing 5 μg of equine IgG or F(ab')$_2$. As primary antibody antibothropic antivenom in ½ dilution was used. Note that both antivenoms recognize the components, especially those with molecular masses related to latrotoxins. Controls show the absence of unspecific binding even using ½ dilution of the control antivenom and the specific reactivity of the anti-horse IgG conjugate.

Considering the variations in LD_{50} as the mass of protein, no differences were observed between the samples from the north-west of the country and those from Patagonia ($p > 0.05$). In this case, the variations in the LD_{50}s (minimal to maximal) were wide, ranging from 3.5 fold in the samples from the north-west of the country to 7.3 fold in the samples from Patagonia.

Table 3
Seroneutralization assays. Neutralizing potency of the three antivenoms studied against the venom of spiders *L. mirabilis*. from Buenos Aires and Chubut provinces. The median effective doses (ED_{50}s) and the ED_{100} are expressed as μl or μg of antivenom required to neutralize 3 LD_{50} of venom. In addition the neutralizing potency is expressed as the LD_{50} neutralized by 1 ml of antivenom (ED_{50}/ml). Note that it was necessary 400% the dose (Buenos Aires) or 25–67% more dose (Chubut) of AL-3 to protect 100% of mice regarding the protection provided by the South American antivenoms. AL-1 and AL-2 were better to neutralize the venom of spiders from Buenos Aires, and AL-1 was better in neutralizing the venom of spiders from Chubut.

Antivenoms	Samples of venoms									
	Buenos Aires					Chubut				
	ED50		ED100		LD50/ml	ED50		ED100		DL50/ml
	μl	μg	μl	μg		μl	μg	μl	μg	
AL-1	3.5	170.5	5	244	857	2.0 (1.5–2.5)	97.4 (10.1–16.8)	3	146	1500
AL-2	3.5	165.6	5	237	857	2.9 (1.9–3.7)	137.2 (21.6–42.0)	4	189	1035
AL-3	5.9 (5.2–6.7)	15.3 (13.5–17.4)	20	52	509	3.9 (3.3–4.6)	10.4 (5.6–10.4)	5	13	769

Regarding the expression for the lethal potency of the venom, some considerations should be made. Since we observed different sizes of the spiders and venomous apparatuses and despite the differences in the protein content (possibly related to the filling status of the glands), it is clear that a larger venomous apparatus will possibly have a larger amount of venom than a smaller one. In fact, we observed that samples composed of larger spiders possessed higher protein content in their venomous apparatuses ($r^2 > 0.73$, $n = 7$, $p = 0.0147$). In addition, we observed that the relation between the size of the individual glands and the protein content of their homogenate was important ($r^2 > 0.6$, $n = 26$, $p < 0.0001$). By this reasons, at a similar toxicity of the venom, pools obtained from larger spiders could seem more toxic than those obtained from smaller spiders and consequently from smaller venomous apparatuses if we expressed their toxicity as the LD_{50}s contained in the venomous apparatuses. Then, to compare the lethal potencies of the venoms, those expressed as mass of protein give better information than those expressed as LD_{50} contained in a venomous apparatus, a tool commonly used to estimate the potencies of the pool of venomous apparatuses from arthropods. Although a relation may be expected in the lethal potencies expressed in these two ways, in the present case, it was rather low (r^2 0.43, $n = 18$, p 0.003). The expression of the toxic potency of a venom as LD_{50}/venomous apparatus could be useful if it is not possible to know its protein content. The expression of toxicity by venomous apparatuses helps to compare the "dangerousness" of a population of spiders (larger spiders with large venomous apparatuses will have more venom to inject and, at a similar toxicity, their bite may be more dangerous). Nevertheless, the comparison of the toxicity from different venoms for toxicological or pharmaceutical studies, should be made by analyzing the protein required to obtain the desired toxic effect.

The immunochemical reactivity was very important. The Ouchterlony method showed that both antivenoms used for the treatment of black widow envenomation in Argentina recognized well the different venoms studied. AL-1 showed more highly stained bands in the double immunoprecipitation, possibly related to the fact that this antivenom is raised with venom from *Latrodectus* from different regions of the country. This can be the same explanation for the more strongly stained bands in the case of AL-2 and the venom from spiders from Sierra de la Ventana, which is the venom used to prepare this antivenom. Although AL-3 recognized the venoms from South American *Latrodectus,* it showed lower reactivity (Fig. 4). In the case of the westernblot, the reactivity of AL-3 on the venoms assayed was observed only when using a dilution of ½. Nevertheless, the recognition of the bands in concordance with latrotoxins was clear (Fig. 5). This fact was very possibly was related to the low protein content of AL-3 regarding the other two antivenoms (around 18 fold), since 20 fold more antivenom was necessary to visualize the bands.

The neutralization assays showed that all the antivenoms were able to neutralize the venoms studied, showing AL-1 and AL-2 highest neutralizing potency against Buenos Aires venom ($p < 0.05$) and AL-1 higher neutralizing potency on Chubut venom regarding the AL-3 ($p < 0.05$). No differences were observed in the neutralizing potency of AL-1 and AL-2, which is very interesting because although both venoms were from *L. mirabilis*, they were from two distant populations and with different lethal potencies. Although AL-2 antivenom is produced using only the venom of *L. mirabilis* from Buenos Aires province, it neutralized very efficiently the venom of spiders from Chubut, whereas although AL-1 does not contain in its immunogens venom from spiders of Buenos Aires province, it neutralized this venom efficiently. These data are important for the antivenom production. Surprisingly, although AL-3 is produced using the venom of *L. mactans* as immunogen and showed very low immunochemical reactivity, it showed neutralizing ability against *L. mirabilis* venom.

Independently of the differences that may exist in the venom of these spiders from different hemispheres, when interpreting the immunochemical results, we must consider some other aspects. Regarding the immunogens used to produce the antivenoms, AL-3 is produced by immunizing horses with milked venom, whereas AL-1 and AL-2 are produced by immunizing horses with homogenates of venomous apparatuses. Thus, again, a greater immunochemical reactivity of AL-1 and AL-2 is logical because these antivenoms recognize not only the venom components but also the other components of the venomous apparatuses, which was the material used for the studies (Fig. 5). Another factor is that the South American antivenoms, as mentioned, possessed a higher amount of protein by vial than AL-3.

The lower neutralizing capacity and immunochemical reactivity of AL-3 in this context does not necessarily imply that the latrotoxins of *L. mactans* used as immunogen do not provide neutralizing antibodies of quality against latrotoxins of other *Latrodectus*; it only implies that, in this pharmaceutical presentation, AL-3 has lower reactivity and neutralizing potency, possibly related to the lower amount of proteins and consequently of antibodies (Dias da Silva and Tambourgi, 2011; de Roodt et al., 2014). These neutralization results are similar to those observed in previous studies, in which, when using different batches of antivenoms, we observed a good neutralization by AL-1 against the venom of *Latrodectus* from Neuquén, Santiago del Estero, La Rioja and Río Negro, and a rather lower, but present, neutralization against these venoms using AL-3 when the neutralization was volumetrically considered (de Roodt

et al., 2006). These results suggest that the antivenoms against *Latrodectus* in America possess a cross neutralizing capacity of a degree enough to be used in heterologous envenomation, in the case of AL-3 on South American black widows at higher doses and possibly the anti-*Latrodectus* from South America could be useful in the neutralization of the venom from *L. mactans*.

However, regarding the neutralization observed, as described before, important considerations must be made. Regardless of survival after 48 h, the health status of the surviving mice was worse in those treated with AL-3 than in those treated with the other antivenoms. Although the observation of the health of the surviving animals after the neutralization experiments is not a requirement for these experiments and it is not done as a routine or required by any pharmacopeia to determine the neutralizing potency of antivenoms, should be considered at the moment to evaluate the real usefulness of a product to be used as a therapeutic tool. This point, not only regarding this punctual case, deserves to be deeply investigated.

The cross neutralization provided by different anti-*Latrodectus* antivenoms has been previously described. Some examples are those of the cross neutralization between antivenoms against *L. halsetti* from Australia on *L. mactans* and *L. hesperus* from North America (Daly et al., 2001) and that of anti-*L. tredecimguttatus* from Europe and Asia on venom of *L. mactans* from North America (Keegan, 1955). However, at least in this case, the use of the specific therapeutic antivenoms seems to be the best choice due to the neutralizing potency showed and the health status of the surviving animals.

This is the first comparative study on the variation of the toxicity of *Latrodectus* venoms in South America and on the immunochemical reactivity and neutralizing capacity of American anti-*Latrodectus* antivenoms on several regional samples of black widows spider venoms.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.toxicon.2017.02.029.

References

Ábalos, J.W., 1980. Las arañas del género Latrodectus en la Argentina. Obras Centen. del Mus. La Plata 6, 29–51.

Bittner, M.A., 2000. Alpha-latrotoxin and its receptors CIRL (latrophilin) and neurexin 1 alpha mediate effects on secretion through multiple mechanisms. Biochimie 82 (5), 447–452.

Bradford, M.M., 1976. A rapid sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dyebinding. Anal. Biochem. 72, 248–257.

Casas, N., de Roodt, A., García, E., Fandiño, E., 2013. Situación Epidemiológica de envenenamiento poranimales venenosos-ponzoñosos. Rev. Argent. Zoonosis Enfermedades Infecc. Emergentes 12, 22.

Casasola, A., Ramos-Cerrillo, B., de Roodt, A.R., Carbajal, A., Alagón, A., Stock, R.P., 2009. Paraspecific neutralization of the venom of African species of cobra by an equine antiserum against *Naja melanoleuca*: a comparative study. Toxicon 53, 602–608.

Conover, W.J., 1999. Practical Nonparametric Statistics, third ed., vol. 1999. John Wiley & Sons, New. 592 pp.

Daly, F.F., Hill, R.E., Bogdan, G.M., Dart, R.C., 2001. Neutralization of *Latrodectus mactans* and *L. hesperus* venom by red back spider *L. hasseltii* antivenom. J. Clin. Toxicol. 39 (2), 119–123.

de Roodt, A., Clement, H., Dolab, J., Litwin, S., Hajos, S., Boyer, L., Alagón, A., 2014. Protein content of antivenoms and relationship with their immunochemical reactivity and neutralization assays. Clin. Toxicol. 52, 594–603.

de Roodt, A.R., Estévez Ramírez, J., Litwin, S., Magaña, P., Olvera, A., Alagón, A., 2007. Toxicity of two North American Loxosceles (brown recluse spiders) venoms and their neutralization by antivenoms. Clin. Toxicol. 45, 678–687.

de Roodt, A.R., Gimeno, E., Portiansky, E., Varni, L., Dolab, J.A., Segre, L., Litwin, S., Vidal, J.C., 2001. A study on the experimental envenomation in mice with the venom of *Tityus trivitattus* Kraepelin 1898 (Scorpiones, Buthidae) captured in Argentina. J. Nat. Toxins 10 (2), 99–109.

de Roodt, A.R., Lanari, L.C., García, S.I., Costa de Oliveira, V., Casas, N., de Titto, E.H., 2013. Accidentes y óbitos por envenenamiento por animales venenosos-ponzoñosos en Argentina en el período 2000-2011. Acta Toxicológica Argent. 21 (Suppl. l.), 100.

de Roodt, A.R., Laskowicz, R., López, R., Lanari, L., Giambelucca, L., Costa de Oliveira, V., Vurcharchuc, M., Regner, P., Rocco, D., González, A., 2012. Potencia letal del veneno de diferentes especies de *Latrodectus* de diferentes regiones de Argentina. Acta Toxicológica Argent. 20 (Suppl. l.), 69–70.

de Roodt, A.R., Laskowicz, R.D., Manzanelli, V.M., Estevez, J., 2006. A Study on "Black Widow" Spiders from Argentina. XV World Congress on Animal, Plant and Microbial Toxins. Abstract Book, Glasgow, Scotland, p. 368.

de Roodt, A.R., Litwin, S., Dokmetjian, J.C., Vidal, J.C., 2002a. A reduced immunization scheme to obtain an experimental anti – *Loxosceles laeta* ("Violinist spider") venom. J. Nat. Toxins 11 (3), 193–203.

de Roodt, A.R., Salomón, D.O., Lloveras, S.C., Orduna, T.A., 2002b. Envenenamiento por arañas del genero *Loxosceles*. Med. (Buenos Aires) 62, 83–94.

Dias da Silva, W., Tambourgi, D.V., 2011. Comment on "Preclinical assessment of the neutralizing capacity of antivenoms produced in six Latin American countries against medically-relevant *Bothrops* snake venoms". Toxicon 57, 1109–1110.

Garb, J.E., González, A., Gillespie, R.G., 2004. The Black Widow spider genus *Latrodectus* (Araneae:Theridiidae): phylogeny, biogeography, and invasion history. Mol. Phylogenetics Evol. 31, 1127–1142.

González, A., 1985. Arañas ponzoñosas de Argentina. Géneros Latrodectus, Loxosceles Phoneutria. Bol. Acad. Nac. Med. 5–19.

Grishin, E.V., 1998. Black widow spider toxins: the present and the future. Toxicon 36 (11), 1693–1701.

Isbister, G.K., Fan, H.W., 2011. Spider bite. Lancet 378, 2039–2047.

Isbister, G.K., White, J., 2004. Clinical consequences of spider bites: recent advances in our understanding. Toxicon 43, 477–492.

Keegan, H.L., 1955. Effectiveness of *Latrodectus tredecimguttatus* antivenin in protecting laboratory mice against effects of intraperitoneal injections of *Latrodectus mactans* venom. Am. J. Trop. Med. Hyg. 4 (4), 756–764.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head bacteriophage T4. Nature 227, 680–685.

Maretic, Z., 1971. Latrodectism in mediterranean countries. Including south Russia, Israel and north Africa. In: Bucherl, W., Buckley, E. (Eds.), Venomous Animals and Their Venoms, vol. III. Academic Press, New York, pp. 299–309. Vol. 2, Chapter 53.

Maretic, Z., Stanic, M., 1954. The health problem of arachnidism. Bull. World Health Org. 11, 1007–1022.

Margni, R.A., 1990. Inmunoquímica, third. ed. El Ateneo, Buenos Aires.

Martino, O., Mathet, H., Masini, R.D., Ibarra Grasso, A., Thompson, R., Gondell, C., Bosch, J., 1979. Emponzoñamiento humano provocado por venenos de origen animal. Secretaría de Salud de la República Argentina, Buenos Aires.

Ministerio de Salud, 2012. Guía de prevención, diagnóstico, tratamiento y vigilancia epidemiológica del envenenamiento por arañas. Ministerio de Salud, Buenos Aires, Argentina. ISBN 978-950-38-0124-6. 110 pp.

National Research Council, 2002. Guía para el cuidado y uso de los animales de laboratorio. In: Commission of Life Sciences. Academia Nacional de Medicina,. Institute of Laboratory Animal Resources, México DF.

Ozkan, O., Adiguzel, S., Yakistiran, S., Filazi, A., 2006. Study of the relationship between *Androctonus crassicauda* (Oliver 1897, Scorpiones, Buthidae) venom toxicity and telson size, weight and storing condition. J. Venom. Anim. Toxins Incl. Trop. Dis. 12 (2), 297–309.

Ozkan, O., Kar, S., Güven, E., Ergun, G., 2007. Comparisson of proteins, lethality and immunogenic compounds of Androctonus crassicauda (Olivier 1807) (Scorpiones:Buthidae) venom obtained by different methods. J. Venom. Anim. Toxins Incl. Trop. Dis. 13 (4), 844–856, 2007.

Theakston, R.D.G., Reid, H.A., 1983. Development of simple standard assay procedures for the characterization of snake venoms. Bull. World Health Organ. 61, 949–956.

Towbin, H., Stahelein, T., Gordon, J., 1979. Electrophoretic transfer of protein from polyacrilamide gels to nitrocelulose sheets: procedure and some applications. Proc. Natn. Acad. Sci. U.S.A. 76, 4350–4354.

Ushkaryov, Y.A., Rohou, A., Sugita, S., 2008. α-Latrotoxins and its receptors. Handb. Exp. Pharmacol. 184, 171–206.

Van Renterghem, C., Iborra, C., Martin-Moutot, N., Leianova, V., Ushkaryov, Y., Seagar, M., 2000. Alpha-latrotoxin forms calcium-permeable membrane pores via interactions with latrophilin or neurexin. Eur. J. Neurosci. 12 (11), 3953–3962.

Volynski, K.E., Meunier, F.A., Lelianova, V.G., Dudina, E.E., Volkova, T.M., Rahman, M.A., Manser, C., Grishin, E.V., Dolly, J.O., Ashley, R.H., Ushkaryov, Y.A., 2000. Latrophilin, neurexin, and their signaling-deficient mutants facilitate alfpha-latrotoxin insertion into membranes but are not involved in pore formation. J. Biol. Chem. 275 (52), 41175–41183.

World Health Organization, 1981. Progress in the Characterization of Venoms and Standardization of Antivenoms. Offset Publication, WHO, Geneva.

World Health Organization, 2010. Guidelines for the Production, Control and Regulation of Snake Antivenom Immunoglobulins. WHO, Geneva, 134 pp.

World Spider Catalog, 2017. World Spider Catalog. Natural History Museum Bern online at. http://wsc.nmbe.ch. version 18.0, accessed on 2017.