Toxicon 55 (2010) 307–319

Contents lists available at ScienceDirect

Toxicon

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

General biochemical and immunological characterization of the venom from the scorpion *Tityus trivittatus* of Argentina

Adolfo R. de Roodt [a,*], Fredy I.V. Coronas [b], Nestor Lago [c], María E. González [a], Rodrigo D. Laskowicz [a], Juan C. Beltramino [d], Silvina Saavedra [e], Raúl A. López [f], Gustavo J. Reati [g], Miriam G. Vucharchuk [h], Eduardo Bazán [i], Liliana Varni [j], Oscar D. Salomón [k], Lourival D. Possani [b]

[a] *Área Investigación y Desarrollo/Serpentario, I.N.P.B. – A.N.L.I.S. "Dr. Carlos G. Malbrán", Av. Velez Sarsfield 563, CP 1281, City of Buenos Aires, Argentina*
[b] *Instituto de Biotecnología, Universidad Nacional Autónoma de México, Avenida Universidad, 2001, Cuernavaca 62210, Mexico*
[c] *Patología Experimental, Departamento de Patología, Facultad de Medicina, Universidad de Buenos Aires, Buenos Aires, Argentina*
[d] *Hospital de Niños "Orlando Alassia", Ministerio de Salud de la Provincia de Santa Fe, Argentina*
[e] *Dirección de Zoonosis del Ministerio de Salud de la Provincia de Entre Ríos, Argentina*
[f] *Departamento de Zoonosis, Ministerio de Salud de la Provincia de Catamarca, Argentina*
[g] *Centro de Zoología Aplicada, F.C.E.F. y N., Universidad Nacional de Córdoba, Córdoba, Argentina*
[h] *Instituto de Animales Venenosos "Jorge W. Abalos", Ministerio de Salud de la Provincia de Santiago del Estero, Argentina*
[i] *Dirección de Epidemiología del Ministerio de Salud de la Provincia de La Rioja, Argentina*
[j] *Servicio de Sueros Terapéuticos, I.N.P.B. – A.N.L.I.S. "Dr. Carlos G. Malbrán", Buenos Aires, Argentina*
[k] *CENDIE – A.N.L.I.S. "Dr. Carlos G. Malbrán", Buenos Aires, Argentina*

ARTICLE INFO

Article history:
Received 19 February 2009
Received in revised form 11 August 2009
Accepted 11 August 2009
Available online 23 August 2009

Keywords:
Venom
Toxicity
Chromatographic separation
LD_{50}
Scorpion
Tityus trivittatus

ABSTRACT

Tityus trivittatus is the Argentinean scorpion reported to cause the majority of human fatalities in the country, however no systematic studies have been conducted with the venom of this species. This communication describes a general biochemical and immunological characterization of the venom obtained from *T. trivittatus* scorpions collected in the city of Buenos Aires and various provinces of Argentina: Catamarca, Cordoba, Entre Rios, La Rioja, Santa Fe and Santiago del Estero. These are places where human accidents were reported to occur due to this scorpion. For comparative purposes two types of samples were assayed: whole soluble venom obtained by electrical stimulation and supernatant from homogenized venomous glands. Two strains of mice (NIH and CF-1) were used for LD_{50} determinations by two distinct routes of administration (intravenously and intraperitoneally). Important variations were found that goes from 0.5 to 12 mg/kg mouse body weight. Samples of soluble venom were always more potent than Telson homogenates. More complex pattern was observed in homogenates compared to soluble venom, as expected. This was supported by gel electrophoretic analysis and high performance liquid chromatographic (HPLC) separations. Additionally, the HPLC profile was enriched in proteins resolved at similar elution times as other known toxins from scorpion venoms studied. Immune enzymatic assays were also conducted comparatively, using four different anti-venoms commercially available for treatment of scorpion stings (Argentinean antidote from INPB, two anti-venoms from Butantan Institute of Brazil and Alacramyn from the Mexican Bioclon Institute). Cross-reactivities were observed and are reported among the various venoms and anti-venoms used. Lung, heart, liver and pancreas pathological modifications were observed on tissues of intoxicated mice. It seems that

* Corresponding author. Tel.: +54 11 4301 2888; fax: +54 11 4302 2492.
E-mail address: aderoodt@uolsinectis.com.ar (A.R. de Roodt).

0041-0101/$ – see front matter
doi:10.1016/j.toxicon.2009.08.014

there are important variations on the venom compositions of the various samples studied and reported here, depending on the geographical area where the scorpions were captured. The results reported here are important for the clinical outcome of human accidents.

1. Introduction

Envenomation due to scorpion stings is a problem of growing medical importance in Argentina. In the period from 1993 to 1999 three deadly accidents in humans were registered (de Roodt et al., 2003), and in the period from 2000 to 2006, this number increased to twelve children (Saracco et al., 2006; de Roodt 2007).

The scorpion species responsible for the majority of these deadly accidents is *Tityus trivittatus*, here abbreviated *T. trivittatus*. Its geographical distribution goes from the North of the Patagonian region to the Northern provinces (Southern latitudes 40°–26°) (de Roodt et al., 2003). This scorpion belongs to the Buthidae family and is one of the six species of *Tityus* found in Argentina: *T. trivittatus*, *T. bahiensis*, *T. confluens*, *T. uruguayensis*, *T. paraguayensis and T. argentinus* (Ojanguren-Affilastro, 2005). *T. trivittatus* is a well adapted species to cohabit with humans dwellings, which increases the probability of accidents (Salomón and de Roodt, 2001; de Roodt et al., 2003; de Roodt 2007).

During the last years the Health Ministry of Argentina (Ministerio de Salud, 2004, 2005, 2008) reported a considerable increase on the total number of accidents and a fourth fold increment of deadly cases, due to stings by scorpions (de Roodt, 2007). Additionally, in recent years fatal events were registered in provinces where this scorpion, although known to occur, did not have record tracks of severe human envenomations. Examples of these geographical localizations are the provinces of Entre Rios, Santa Fe, Catamarca, La Rioja, San Juan and Tucumán. In these provinces, despite the potential risk associated with possible stings by *T. trivittatus* species, it was not considered a medical problem until recently, when deadly cases were registered to occur in children. Other provinces such as Buenos Aires, including the federal city of Buenos Aires, although having the presence of *T. trivittatus* species, have not reported severe human accidents, yet (de Roodt et al., 2003).

The treatment of scorpion envenomation consists in the application of the specific anti-venom, which is produced and freely distributed by the National Institute for Production of Biologicals of the Ministry of Health (INPB). Production of this anti-venom is difficult, since it depends on the capture of this species of scorpion for obtaining venom or venom glands for horse immunization procedures. This is a synanthropic species showing in some regions problems for correct taxonomic identification, apart from the rather low number of specimens that can be collected in the endemic areas. These facts not only limited the production of anti-venoms but hampered the conduction of systematic biochemical studies of its venom components, as well as the estimation of its toxicity and neutralization potency by the existent anti-venoms.

The toxicity symptoms caused by *T. trivittatus* venom vary according to distinct geographical regions and the clinical manifestations due to their stings seems to change abruptly in some provinces. It is thought that these changes might reflect changes in venom compositions, among other possible unknown factors. The human deaths in provinces where the stings were frequent but severe envenomation absent, strengths this observation.

In view of the above mentioned difficulties and due to the lack of information on the regional characteristics of this scorpion venom, the present communication reports toxicological and biochemical data from different samples of telson homogenates and/or soluble venom obtained by electrical stimulation of *T. trivittatus* scorpions from different provinces of Argentina. Among the data here considered are: the chromatographic and electrophoretic profiles, immunochemical reactivity and lethal potency of venom samples in mice. We observed very different lethal potencies and chromatographic profiles in the different samples even in those from the same province. The chromatographic pattern of the whole soluble venom showed clear differences regarding those observed from telson gland homogenates. The lethal potencies observed were in the range of potency described for Brazilian species of *Tityus*.

2. Material and methods

2.1. Venom

Venom was obtained from telson homogenates of scorpion of the provinces of Santiago del Estero (SdeE), Catamarca (Cat), La Rioja (LaR), Córdoba (Cba), Entre Ríos (ER), Santa Fe (SFe) and from the city of Buenos Aires (BsAs). Telsons were obtained from scorpions captured in different provinces (Cba, SdeE, SFe), dried *in situ*, and sent to the Institute in Buenos Aires for processing. In other cases (ER, Cat and LaR) the scorpions were obtained in the provinces but the telson extraction was done in our laboratory in Buenos Aires, using always similar methodologies. Telsons from BsAs were obtained in our laboratory from scorpions captured in the city. In all the cases the methodology to obtain the telsons was the same. Briefly, the scorpions were killed by freezing and immediately the telsons were cut and vacuum dried for two to six hours, depending on the number of telsons been processed. In our laboratory the telsons were dried again in the same way, prior making the homogenates. The venom from homogenates was obtained from the supernatant of centrifuged macerated telsons made by conventional methods (de Roodt et al., 2001; Ozkan et al., 2006). The pool of telsons used to prepare the homogenates from the different samples in all the cases ranged from 20 (minimum) to 160 telsons (maximum). Venom was diluted in NaCl 0.15 M,

aliquoted and stored at −20 °C until use. In addition, venoms of scorpions from SFe, ER, a pool of LR and Cat, pool of ER, SFe and Cba, and from BsAs were obtained by electrical stimulation of scorpions maintained in our laboratory. The number of animals used for these extractions was from 20 (minimum) to 220 (maximum) scorpions. Briefly, scorpions were sedated with CO_2 after which they were electrically stimulated in the articulation of the telsons with 12 V using a commercial battery. The venom collected in micropipettes was diluted in 0.15 M NaCl, centrifuged at 5000 × *g*, filtered through 0.22 μm (Minisart, Sartorius), aliquoted and stored at −20 °C until use. The protein concentration was determined by the method of Bradford (1976) using the Protein Assay Kit (BioRad).

2.2. Animals

Scorpions were maintained in plastic boxes with water *ad libitum* and were fed weekly with cockroaches, crickets or spiders.

The mice used to determine the lethal doses were 18–22 g body weight CF-1 or NIH strain. Animals were provided by the Animal Facilities of the National Institute for Production of Biologicals (INPB). The animals were kept under controlled environment and received food and filtered water *ad libitum*. The institutional ethical approval regarding the managing of animals was provided by the INPB, in agreement with the recommendations of the National Research Council (2002).

2.3. Lethal potency

Groups of five mice per dose level were inoculated by intravenous (i.v.) or intraperitoneal (i.p.) route with several dilutions of venom. After 48 h the number of animals surviving the protocol of inoculation were recorded and the median lethal dose (LD_{50}) was calculated, expressing either as the amount of venom that killed 50% of mice or the quantity of telsons used to prepare the homogenate that kills half of the mice under assay (de Roodt et al., 2001; Ozkan et al., 2006). Data were analyzed by the method of Spearman and Karber (World Health Organization, 1981).

The experiments were done with different samples of telson homogenates (SFe, ER, SdE, Cba, Cat, LR and BsAs) or milked venom of *T. trivittatus* from SFe, ER, Cba and pool of LR-Cat, SFe-ER-SdeE and ER-Cba-SFe. Data recorded were from experiments done in the Service of Therapeutic Sera or by the Research and Development Area/Serpentarium of the INPB.

2.4. Histopathological studies

The histopathological analysis was performed using animals (5–7 per geographical area) inoculated with 1.0 LD_{50} of telson homogenates from the following places: Cba, SdE, ER or SFe. For the two last areas similar experiments were also conducted by inoculation of 1.0 LD_{50} of whole soluble venom, extracted by electrical stimulation. A group of five mice was injected with 0.15 M NaCl and it was used as negative control. A general pathologic examination was conducted in all mice. Samples of lungs, heart, pancreas, liver and other selected organs were fixed in 10% formaldehyde in 0.15 M NaCl, 0.1 M phosphate buffer, pH 7.4. Paraffin-embedded tissues were sliced and stained with hematoxilin and eosin and PAS for light microscopic examination (Bancroft and Stevens, 1990). In order to make the morphometry of the images from the tissues, images were captured with a microscope Olympus CX31 with digital video camera Sony Hyper Had, using the program Image-Pro Plus for the measurement and position of reference previous calibration of the 25× objective as described by the manufacturer.

2.5. Electrophoretic analysis

Samples of soluble venom obtained by electrical stimulation (milked venom) and samples from the supernatant of telson homogenates of different regions were studied by SDS-PAGE as a routine control to determine gross similarities or differences in the samples obtained with both systems of venom extraction and specially the protein profile of the venom obtained by electrical stimulation. Briefly, 10 μg of proteins of telson homogenates (ER, Cba, SdeE, LR and SFe) or milked venom (SFe and ER) were applied to SDS-PAGE (12.5% polyacrylamide concentration in tris–glycine) analysis under non-reducing conditions as described by Laemmli (1970) using a Mini-Protean II electrophoresis system (BioRad). After running, the gels were fixed with methanol–acetic acid–formaldehyde solution for ten minutes, before staining with Coomassie Brilliant Blue R-250. Molecular mass markers were from Promega (Wide Range Molecular Mass).

It is known that gels with higher concentration of acrylamide (i,e. 15%) or gels prepared with a gradient of acrylamide concentration and/or using tricine (Schägger and von Jagow, 1987) are better for the separation of low molecular weight peptides. However, our experiments with 12.5% acrylamide clearly show the differences existing between components of the venom prepared from whole telson homogenates from those obtained by electrical stimulation. The samples from homogenates show more venom components of higher molecular weight forms, than that extracted by electrical stimulation.

2.6. Chromatographic profiles

Samples from telson homogenates of *T. trivittatus* of SFe, ER, Cba, SdE, LR and Cat and samples of milked venom from ER, SFe and BsAs were studied. Sample separation was performed by (HPLC), as earlier described by Coronas et al., (2003). Usually 2 mg of milked venom or telson homogenates were loaded onto the C18 reverse phase column of the HPLC system. The components were separated by a linear gradient of solution A (water in 0.12% trifluoroacetic acid, TFA) to solution B (acetonitrile in 0.10% TFA) for 60 min.

2.7. Immunochemical studies

2.7.1. Anti-venoms

The anti-venoms used were: 1) *Antiveneno escorpiónico* (henceforth A-*Tt*) of the INPB (this anti-venom is available in the format of 2 ml ampoules of $F(ab')_2$ fragments of equine immunoglobulin), batch 907 (Expiration date:

07-2003), prepared from serum of pre-immunized horses with telson homogenates from *T. trivittatus* 2) *Soro Anti-aracnídico* (henceforth AA) of the Instituto Butantan of Sao Paulo, Brazil (5 ml ampoules of $F(ab')_2$ fragments of equine immunoglobulins), produced with *T. serrulatus*, *Loxosceles gaucho* and *Phoneutria* sp milked venoms as immunogens, batch 950a d 921 (Expiration date 11-15-1998). 3) *Soro antiescorpión* (henceforth A-*Ts*) from the Instituto Butantan of Sao Paulo (5 ml ampoules of $F(ab')_2$ fragments of equine immunoglobulins), produced with milked venom of *T. serrulatus*, batch 0204040/E (Expiration date: 20-03-2005). 4) *Alacramyn NR*, an anti-venom against scorpions of the genus *Centruroides* (henceforth A-*Cn*), from Instituto Bioclón, México DF (liophilized presentation, to be reconstituted in a volume of 5 ml), produced in horses immunized with telson homogenates, batch [B-8 J-04] B-7E-06 (Expiration date: 06/2001)1. The protein content of the different anti-venoms was determined by the method of Bradford (1976) as described. It is worth mentioning that although the expiration dates of the various anti-venoms used in this study are not the same, they were always kept at low temperature (4 °C), which certainly prolongs their validity, especially for comparative biochemical characterization, not for human use. In this regard, recently the neutralizing capacity of anti-venoms stored for 15 years after the expiration data were reported (O'Leary et al., 2009), in agreement with previous literature showing that anti-venoms still maintain their neutralizing capacity for several years after the expiration date (World Health Organization, 1981).

2.7.2. Double immunoprecipitation

The immunoprecipitation analysis was performed using samples from milked venom or telson homogenates of *T. trivittatus* from Cba, ER, SFe and SdeE against different anti-venoms, using conventional methods (Margni, 1990). Briefly, 10 μl of each anti-venom was confronted against 10 μl of a telson homogenate or milked venom in a concentration of 0.8 mg/ml in 0.15 M NaCl. The gels were incubated for 48 h at room temperature, washed in 0.15 M NaCl for an additional 48 h, dried and stained with Amido Schwartz.

2.7.3. ELISA studies

ELISA tests were conducted with telson homogenates of scorpions from the provinces of LR, ER, SFe, a pool of telson homogenates from the different provinces and with milked venom from scorpions of ER, using standard procedures (Nishikawa et al., 1994). The anti-venoms used for developing the immuno-cross-reactivity are those mentioned above (see section Anti-venoms). Briefly, ELISA 96 wells plates (Falcon) were coated with 100 μl/well of 5 μg/ml of telson homogenates or whole soluble venom in 0.15 M HCO_3^- buffer pH 9.0 solution and blocked with 5% bovine serum albumin (BSA) in PBS pH 7.4. The plates were incubated with several dilutions of the different anti-venoms for 30 min at 37 °C, washed three times for 3 min each, and incubated with anti horse IgG labeled with peroxidase at a dilution of 1:5000. After washing, the plates were developed using *o*-phenylenediamine dihydrochloride (Sigma) plus H_2O_2 (Riedel–Muench) as substrate after which the reaction stopped and read at 490 nm and the effective concentration (EC_{50}) was determined. The EC_{50} is defined as the dilution in which the absorbance of each anti-venom reaches the 50% of the maximum absorbance. To determine the EC_{50}s of the different anti-venoms for the different samples, the dose–response curves were analyzed by non-linear regression (sigmoidal dose–response) using the software Prism3.0 (Graph Pad, Inc., CA).

2.8. Statistics

For the determination of the lethal potencies, the method of Spearman and Karber was used (WHO, 1979). The LD_{50}s and their 95% confidence intervals (into brackets) are indicated in Table 1. The ELISA curves were analyzed by non-linear regression using the software Prism3.0 (GraphPad Inc., CA). The EC_{50}s are indicated in Table 2 and their 95% confidence intervals are shown between brackets. When it was necessary the *t* Student test was used to compare results.

3. Results

3.1. Lethal potency

The lethal potencies of the venom from telson homogenates, expressed as $LD_{50,}$ ranged from 0.2 to 1.0 telson/mouse. For the whole soluble venom it varies from 10 μg to over 250 μg/20 g mouse weight, that is, 0.5 to over 12.5 mg/kg, showing that the milked venoms is more potent than the venom from telson homogenates ($p < 0.05$). These results were expected since the supernatant of whole venomous gland homogenates contains many irrelevant protein components that do not contribute to the lethality of the sample (see Discussion below). In this regard, samples from the same province showed difference potencies depending on the way the venom were obtained, either electrical stimulation or telson homogenates. Telson homogenates from Santa Fe showed an LD_{50} from 8 to 12 mg/kg, whereas the LD_{50} of the whole soluble venom was 1.75 mg/kg ($p < 0.05$). The same was observed with telson homogenates from Entre Rios, which was much higher (5.7 mg/kg) compared with milked venom (0.5–1.0 mg/kg; $p < 0.05$) from scorpions of the same place.

The LD_{50}s values obtained for animals injected intravenously were lower than those injected intraperitoneally in all the cases, meaning more lethal potency, although not in all the cases the differences were significant (see Table 1).

The differences found using two distinct strains of mice were not significant ($p < 0.05$). The samples of venom obtained from scorpions of the geographical area of Buenos Aires are the ones that show the lowest lethal potency. This would make sense with what is described in the introductory section, since until now no deadly cases of humans have been registered in this region.

3.2. Pathological studies

The histopathological lesions observed using venom from different geographical areas and from different types of venom sample preparations, are quite similar. Mice

Table 1
Mouse LD_{50} values of different *T. trivittatus* venoms from different regions of Argentina Lethal doses of different pools of *T. trivittatus* telson homogenates (Th) or whole soluble venom (WsV) obtained by electrical stimulation. The columns indicate the Geographical Area from where the venoms were obtained, the type of venom (Th or WsV), the route of administration (i.v. = intravenous, i.p. = intraperitoneal), the strain of mice used for the determination, the lethal potency expressed as LD_{50} in mg/kg (LD_{50} mg/kg) and as proportion of telson necessary to kill the half of the mice challenged (Telson/LD_{50}). The confidence intervals are expressed between brackets. When the dose of sample necessary to determine the LD_{50} was too high, the value is expressed as less than of the maximal value used to estimate the potency that did not kill the half of mice challenged. When the determinations were done in several samples from the same province, the range of values are expressed from the minimal ED_{50} to the maximal ED_{50} value and the number of samples studied are expressed into brackets as $n = X$. When it was not indicated, the values are from a single sample and the 95% confidence intervals are indicated into brackets. N.D. means not determined. When one sample was determined by different routes, it was indicated with an asterisk (*).

Geographical Area	Venom	Route	Strain of mice	DL_{50} (mg/kg)	Telson/DL_{50}
Buenos Aires	Th	i.p.	CF-1	> 11.85	From 0.95 to >1.00 (n = 2)
Buenos Aires	Th	i.p.	NIH	ND	>1.00
Córdoba	Th (*)	i.p.	NIH	From 4.00 to 10.0	From 0.25 to 0.38 (n = 2)
Córdoba	Th (*)	i.v.	NIH	3.45 (3.40–3.55)	0.33 (0.32–0.34)
Córdoba	Th (*)	i.v.	CF-1	2.60 (1.65–4.05)	From 0.25 to 0.67 (n = 3)
Córdoba	WsV	i.p.	CF-1	1.45 (1.15–1.80)	ND
Entre Ríos	Th	i.p.	NIH	5.70 (5.40–6.00)	0.26 (0.25–0.27)
Entre Ríos	WsV (*)	i.p.	NIH	1.03 (1.00–1.05)	ND
Entre Ríos	WsV (*)	i.v.	NIH	0.50 (0.45–0.55)	ND
La Rioja	Th	i.p.	CF-1	11.15 (6.95–17.8)	1.09 (0.68–1.75)
Santa Fe	Th	i.p.	NIH	11.45 (11.25–11.60)	0.63 (0.62–0.64)
Santa Fe	Th	i.v.	NIH	8.30 (1.15–59.0)	0.46 (0.10–11.30)
Santa Fe	WsV	i.v.	NIH	1.75 (0.75–1.20)	ND
Santiago del Estero	Th	i.v.	CF-1	5.40–11.80	From 0.16 to 0.35 (n = 11)
Santiago del Estero	Th	i.p	NIH	> 12.50 (n = 2)	ND
S. Fe–S. del Estero	WsV	i.v.	CF-1	1.55 (0.85–2.20)	ND
Catamarca–La Rioja	WsV	i.p.	CF-1	0.90 (0.60–1.15)	ND
E. Ríos–S. Fe	WsV	i.p.	CF-1	0.70 (0.50–1.00)	ND
E.Rios-S.Fe-Córdoba-La Rioja-Catamarca	WsV (*)	i.v	CF-1	0.76 (0.51–1.13)	ND
	WsV (*)	i.p	CF-1	0.99 (0.86–1.13)	ND

showed generalized congestion. Analysis of specific organs showed congested lungs in all cases studied and infiltration of mononuclear cells. Thickening of the alveolar septa and rupture of the alveolar structure and foci due to edema and hemorrhage were observed (See Fig. 1a.1 and its negative control Fig. 1b.1). Hearth tissue showed edema and focal fragmentation of myocardial fibers, some with cytoplasmic condensation and eosynophilia (Fig. 1a.2 and b.2). Pancreatic hemorrhage and signs of severe epithelial injury of acini were also observed in some samples of mice injected with material from the different regions (Fig. 1a.3 and b.3). In addition, the material from all treated mice showed liver esteatosis, sinusoidal congestion, vacuolization and hepatocyte necrosis (Fig. 1a.4 and b.4).

3.3. Electrophoretic profiles

Fig. 2 shows the results of electrophoretic separation of samples from various geographical areas and distinct sources of venom preparations. It is clear that usually the venom obtained by homogenization of entire telsons contains more protein of higher molecular weight forms, whereas the venom obtained by electrical stimulation contains mainly components of lower molecular weights. In the samples of whole soluble venom, in addition to the material around 8 kDa, there are other bands ranging from 10 do 35 KDa. They are present in all samples and possibly are non-toxic or have a lower toxicity (de Roodt et al., 2001). It is worth mentioning that most toxic peptides present in scorpion venoms are known to migrate faster than 10 kDa (Fig. 2).

3.4. Chromatographic profiles

Samples of venom from different sources were separated by HPLC as indicated in the section of Material and methods. Fig. 3 shows the various chromatogram profiles obtained. Although some similarities of retention times and relative concentration of certain components seems to occur, the overall profile is quite distinct. This is mainly seen when

Table 2
EC_{50} Reactivity by ELISA of the different anti-venom on the samples from different provinces. The EC_{50} (dilution in which is reached the half of the A_{490nm}) was estimated by non-linear regression. The confidence intervals are indicated in brackets. Note the highest reactivity of the A-*Tt* and AA regarding the other anti-venoms. TH: telson homogenate. Wsv: whole soluble venom. All the anti-venoms are the $F(ab')_2$ fragments of equine immunoglobulins. A-*Tt*: Suero "Antiescorpión" from the INPB (anti-*T. trivittatus*); A-*Ts*: Soro "Antiescorpiónico" from Butantan Institute; AA: Soro "Antiaracnidico" from Butantan Institute (antidote against scorpions and spiders); A-*Cn*: "Alacramyn" from Bioclon Institute of Mexico (against *Centruroides* scorpion venom); $F(a\acute{b})_2$: negative control, "Bivalent" anti-venom against the venom of snakes of the genus *Bothrops*.

Sample	A-*Tt*	AA	A-*Ts*	A-*Cn*	$F(a\acute{b})_2$
WsV from E. Rios	82.0 (47–144)	374 (302–463)	20 (12–33)	14 (9–22)	2×10^{-2} (2.3×10^{-5}–19.5)
Th from different provinces	872 (765–995)	1184 (905–1550)	122 (101–148)	106 (90–125)	1×10^{-2} (1.2×10^{-5}–8.7)
Th from Santa Fe	138 (101–187)	409 (325–514)	30 (25–36)	20 (13–32)	1.9×10^{-3} (1.7–2.1×10^{-3})
Th from E. Ríos	511 (371–705)	540 (363–802)	36 (31–42)	105 (85–131)	2×10^{-2} (2×10^{-7}–2564)
Th from La Rioja	414 (327–523)	330 (271–402)	64 (56–70)	97 (86–110)	7.3 (3.8–14.0)

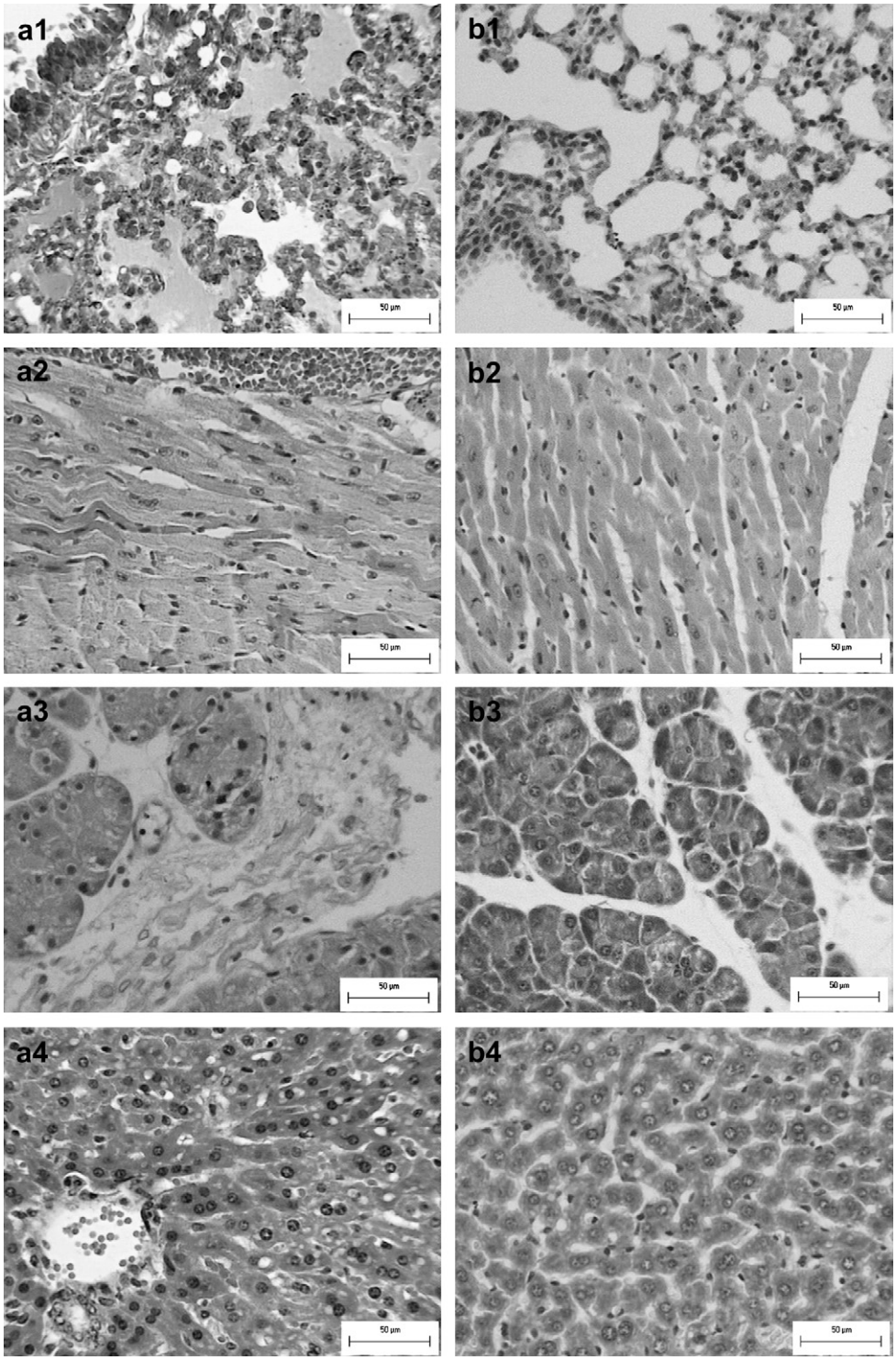

Fig. 1. Histopathological lessions of samples from mice inoculated *with T. trivittatus* venoms from different regions of Argentina. Fig. 1.a.1 Marked congestion and thickening of the septa, intra-alveolar edema and hemorrhage. Focal rupture of the alveolar structure in the lungs of mice injected with *T. Trivittatus*. (H&E, ×250). Control: Fig. 1.b.1. 1.a.2. Cardiac changes consisting in cardiac injury, represented by focal fragmentation of the myocardiac fibers and intrafibrillar edema (H&E, ×250). Control: Fig. 1.b.2. 1.a.3. Pancreatic hemorrhages and signs of severe epithelial injury of acini (H&E, ×250). Control: Fig. 1.b.3. 1.a.4. The liver showed esteatosis, sinusoidal congestion, vacuolization and hepatocyte necrosis (H&E, ×250). Control: Fig. 1.b.4.

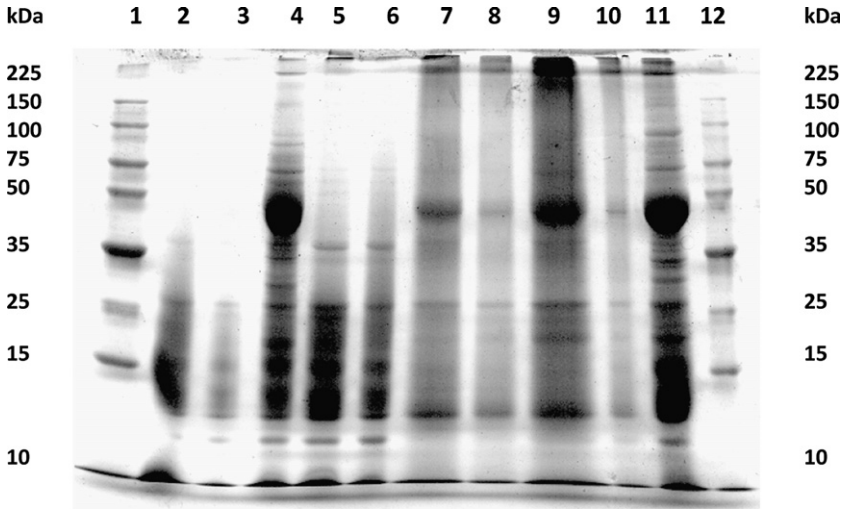

Fig. 2. Electropheretic separation of samples. A 12.5% acrylamde–bisacrylamide SDS-PAGE was run in Tris–Glycine buffer. Samples containing 10 μg of whole soluble venom or telson homogenates of *T. trivittatus* were run and stained with Coomasie Brilliant blue. The extreme right and left lanes (numbered 1 and 12) contain molecular weight markers (broad range, Promega), whose masses are indicated. Lane 2 contains soluble venom from Santa Fe; lane 3 soluble venom from Entre Ríos; lane 4 contains telson homogenates from Entre Ríos; lanes 5 and 6 correspond to soluble venom from Entre Ríos; lane 7 contains telson homogenate from Santa Fe; lane 8, telson homogenates from Santiago del Estero; lane 9, telson homogenates from Entre Ríos; lane 10 contains telson homogenates from Córdoba and lane 11 telson homogenates from La Rioja.

comparing the venom obtained by electrical stimulation with the samples obtained from gland homogenates, as expected. The supernatant from the centrifugation of telson extracts contains many components that elute at earlier retention times. On the first 20 min of HPLC separation there are many components that usually are not present when a less complex sample is applied, like that obtained by electrical stimulation. However it is worth mentioning that important components are separated around 30–40 min retention time. This is known to correspond mainly to peptides that were shown to be toxins that recognize Na^+-channels isolated from other species of *Tityus* scorpion venoms (Batista et al., 2007).

3.5. Immunochemical studies

Fig. 4 shows the results of double immunoprecipitation experiments done with selected samples of venom obtained by homogenization of telsons from scorpions collected in Cordoba, Entre Rios, Santa Fe and Santiago del Estero. The anti-venoms used were the anti-arachnidic from Butantan (AA), the specific anti *T. trivittatus* from Argentina (A-*Tt*) and the anti-*Centruroides* venom from Mexico (A-*Cn*).

Fig. 5 and Table 2 show the results of ELISA obtained by using the four anti-venoms described in the section of Material and methods and various combinations of venom samples from various places. It is worth mentioning that the protein content of the anti-venoms were: 45 ± 6 mg/ml for the specific A-*Tt*, 43 ± 3 mg/ml for the AA, 5.4 ± 0.1 mg/ml for the A-*Ts* and 4.5 ± 1.2 mg/ml for the A-*Cn*. The ELISAs studies showed an important reactivity of the three anti-*Tityus* anti-venoms, in which the reactivity using the heterologous A-*Cn* anti-venom is the lowest as observed in the immunoprecipitation studies (Fig. 5).

The reactivity of the anti-venoms is expressed as the EC_{50} in Table 2. As it can be seen in Table 2, the EC_{50} for the different anti-venoms in all the cases showed an important difference between the A-*Tt* and the AA regarding the A-*Ts* and A-*Cn* ($p < 0.05$), and in almost all the cases the reactivity between A-*Tt* and AA was similar ($p < 0.05$) (Fig. 5) and the confidence intervals overlap (Table 2). In all the cases the reactivity of the different anti-venoms was over the negative control ($p < 0.05$).

Although one of the anti-venoms used for immunochemical analysis contained a label showing that the product had an expiration data no longer valid for human use, it showed high immunochemical reactivity against the soluble venom and telson homogenates. In other words, the anti-venom was still good for the purposes here reported. The rationale applied in these cases is: if the anti-venom shows no precipitates or turbidity, if it was conserved in sterile conditions and more important yet maintained in refrigerated places, the neutralizing capacity is maintained. These observations are in agreement with data obtained with anti-venoms tested ten years after production, which still maintain their neutralizing potency around 80–90% regarding the original value (World Health Organisation, 1981). In addition, it was recently reported that anti-venoms are more robust than indicated on their label and maintain useful activity long past their nominal expiration dates, since it was found neutralizing capacity in samples inclusive after 15 years expiration (O'Leary et al., 2009).

4. Discussion

Although *T. trivittatus* is an Argentinean scorpion that has caused human fatalities in the country, little has been reported on its venom. For this reason, in the present communication a comparative analysis regarding venom composition of *T. trivittatus* collected in various

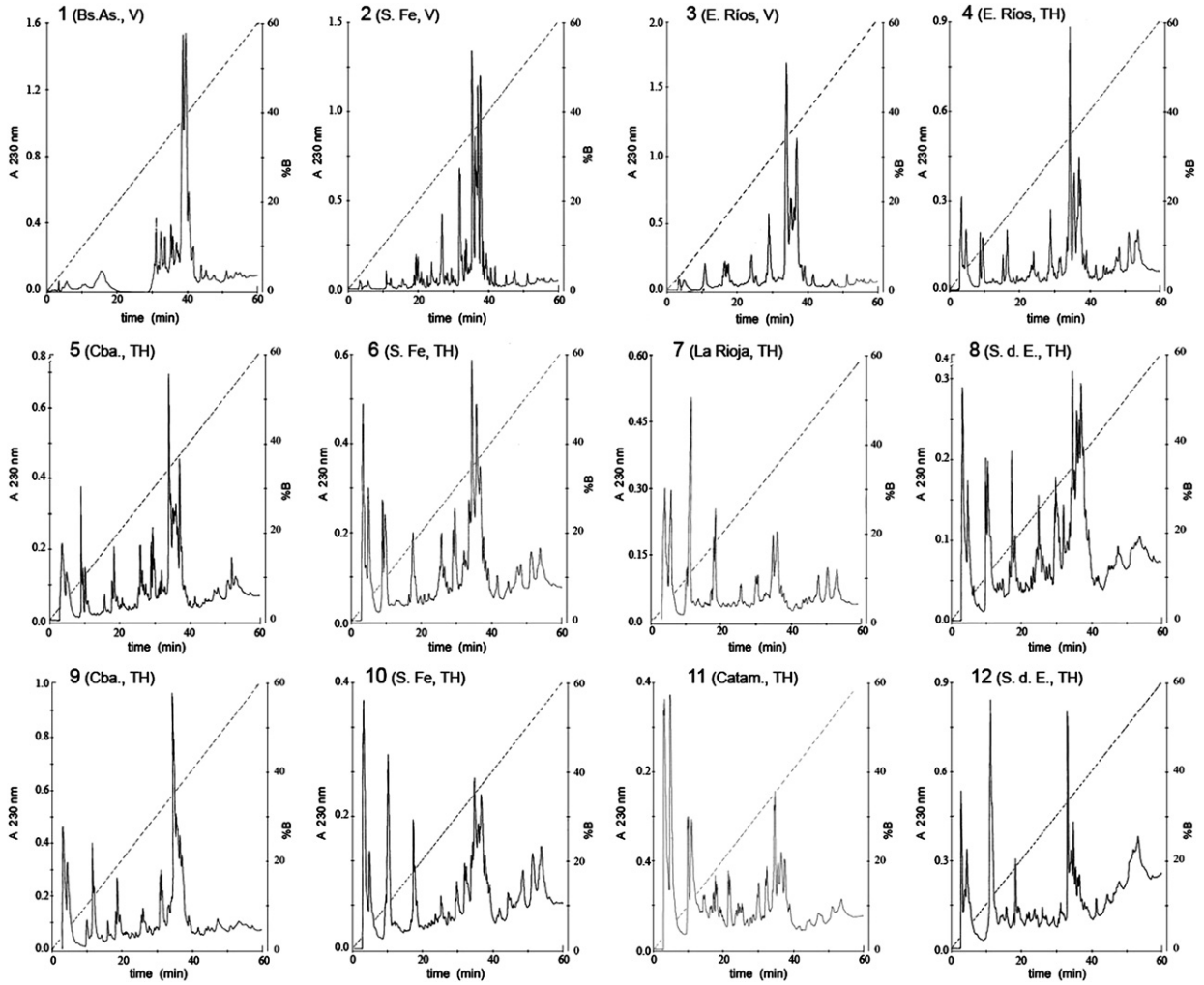

Fig. 3. HPLC separation of venom samples. Reverse phase HPLC separation of whole soluble venom and homogenates from telsons of *T. trivittatus* collected in the places indicated. The *Y* axes indicate the absorbance at 230 nm, the *X* axis the elution time of the fractions. The extreme right side indicates the gradient of acetonitrile used for separation. Samples 1, 2 and 3 are from whole soluble venom (V) of scorpions collected in Buenos Aires city (Bs.As.), Santa Fe (S. Fe) and Entre Rios (E. Ríos), respectively. Sample 4 to 12 are from telson homogenates (THs) of scorpions collected in Entre Ríos, Córdoba (Cba.), Santa Fe, La Rioja, Santiago del Estero (S. d. E.), Córdoba, Santa Fe and Catamarca (Catam.). Please note that are some repeated chromatograms from the same place, collected in different times.

geographical areas, together with a general biochemical and immunological characterization of the various samples of venom studied are reported. To the best of our knowledge, there is only a couple of reports dealing with the experimental toxicity of the telson homogenates (de Roodt et al., 2001; 2005), the characterization of peptides from this venom that affect K^+ channels (Coronas et al., 2003; Abdel-Mottaleb et al., 2006) and a congress communication describing the presence of a Na^+ channel specific peptide in the venom of *T. trivittatus* (Corornas et al., 2007), thought to be responsible for the toxicity symptoms observed in mammals (humans and mice). This is a peptide with high degree of similarity with toxin gamma isolated from the Brazilian dangerous species *T. serrulatus*, *T. bahiensis* and *T. stigmurus* (Becerril et al., 1996). Actually the LD_{50} reported here (around 0.3–12 mg/kg mouse body weight) for *T. trivittatus* is within the range of that found for the Brazilian species (Nishikawa et al., 1994; Kalapothakis and Chávez-Olórtegui, 1997). The venom of the Argentinean scorpion is less potent than the venoms of African and Asian species of scorpions, which have LD_{50}s in the range of 0.1–2.4 mg/Kg (de Roodt et al., 2006). The wider variation on the values of the LD_{50} reported here are certainly due to several factors. The most important one is the type of sample used for the estimation of the lethality test. Samples were obtained either by electrical stimulation of the scorpion (whole soluble venom) or by grinding the entire telsons (Bucherl, 1971; Sisson et al., 1990) and saving the supernatant of the homogenates after centrifugation. This is usually the way most manufacturers of anti-venoms do for immunization of horses for antidote production (Calderón-Aranda et al., 1996). However it is clear that the venom extracted by electrical impulses is more potent than the venom obtained from homogenates. Our data show that the lethal potency of telson homogenates from Santa Fe, ranged from 8 to 12 mg/kg, whereas the value of lethality of whole soluble

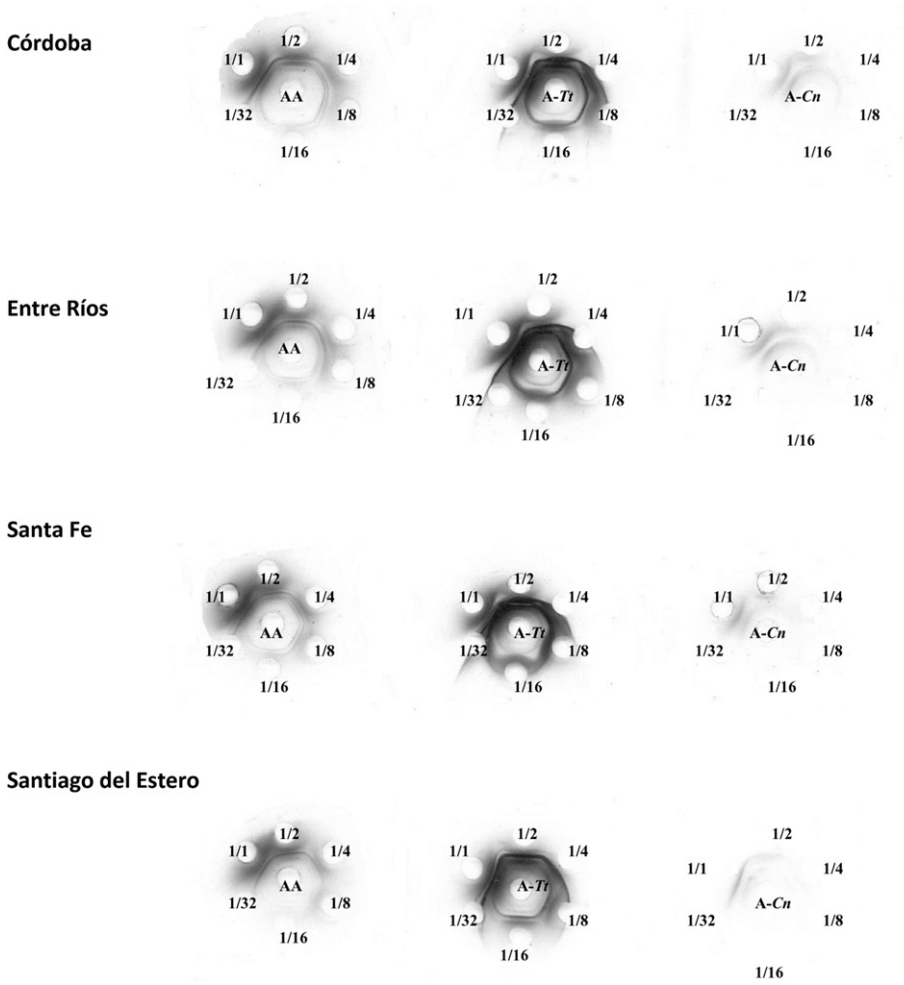

Fig. 4. Double immunodiffusion of venoms and anti-venoms. Samples containing telson homogenates from Cordoba, Entre Rios, Santa Fe and Santiago del Estero were tested against three anti-venoms: AA (left) is from the anti-arachnid of Butantan, A-*Tt* developed with *T. serrulatus* venom (center) is the specific anti *T. trivittatus* from the INPB and A-*Cn* is an anti-*Centruroides* anti-venom Alacramyn from Mexico.

venom showed a potency of 1.75 mg/kg ($p < 0.05$). The same can be observed with the telson homogenates from Entre Rios (5.7 mg/kg) when compared with the soluble venom from scorpions of this province (0.5–1.0 mg/kg, $p < 0.05$). In addition, if all the values are compared the p value between the toxicity of the whole venom and the telson homogenates is <0.0001 (see Table 1). This is expected since the homogenization of telsons can liberate in soluble format many proteins from the glandular cells that usually are not constituents of venom secretion extracted by electrical stimulation. This can certainly explain the higher molecular weight components found with the homogenate samples run in SDS-PAGE gels as shown in Fig. 2, when compared to the samples run with the whole soluble venom. Another factor found in this study is that there must have venom variations among the specimens collected in certain areas. For example the cities of Santa Fe and Paraná (Entre Rios), from where the majority of the scorpions from both provinces were obtained, are separated by the river Parana, but they are linked by a tunnel communicating both cities. The first human deadly case occurred first in Santa Fe, followed a few years late by a fatal accident in the other city (City of Parana). Although the population of scorpions seems to be very close, the potency of their venoms is slightly different (See Table 1). Children stung by scorpions on both places show symptoms of intoxication and need to be treated with anti-venom (Peirano et al., 2000; de Roodt 2007; Piola 2007). Another factor to be considered is that the venom of homogenized glands or whole soluble venom from scorpions of the provinces of the Center (Córdoba) and North West (La Rioja, Catamarca, S. del Estero) is in the range of those obtained from Santa Fe and Entre Rios. However, the samples of scorpions collected in Buenos Aires are less toxic ($t = 3.366$; $p < 0.0046$), in agreement with the historical data of scorpion sting in humans in this city (de Roodt et al., 2003).

It is interesting to observe that both strains of mice assayed for toxicity tests did not showed a great variability on the LD_{50}s registered (Table 1), contrary to what was reported for some strains of mice in Mexico (Alagón et al., 1988).

The route of administration of the venom seems to be relevant for the value of LD_{50} found. In same case, the

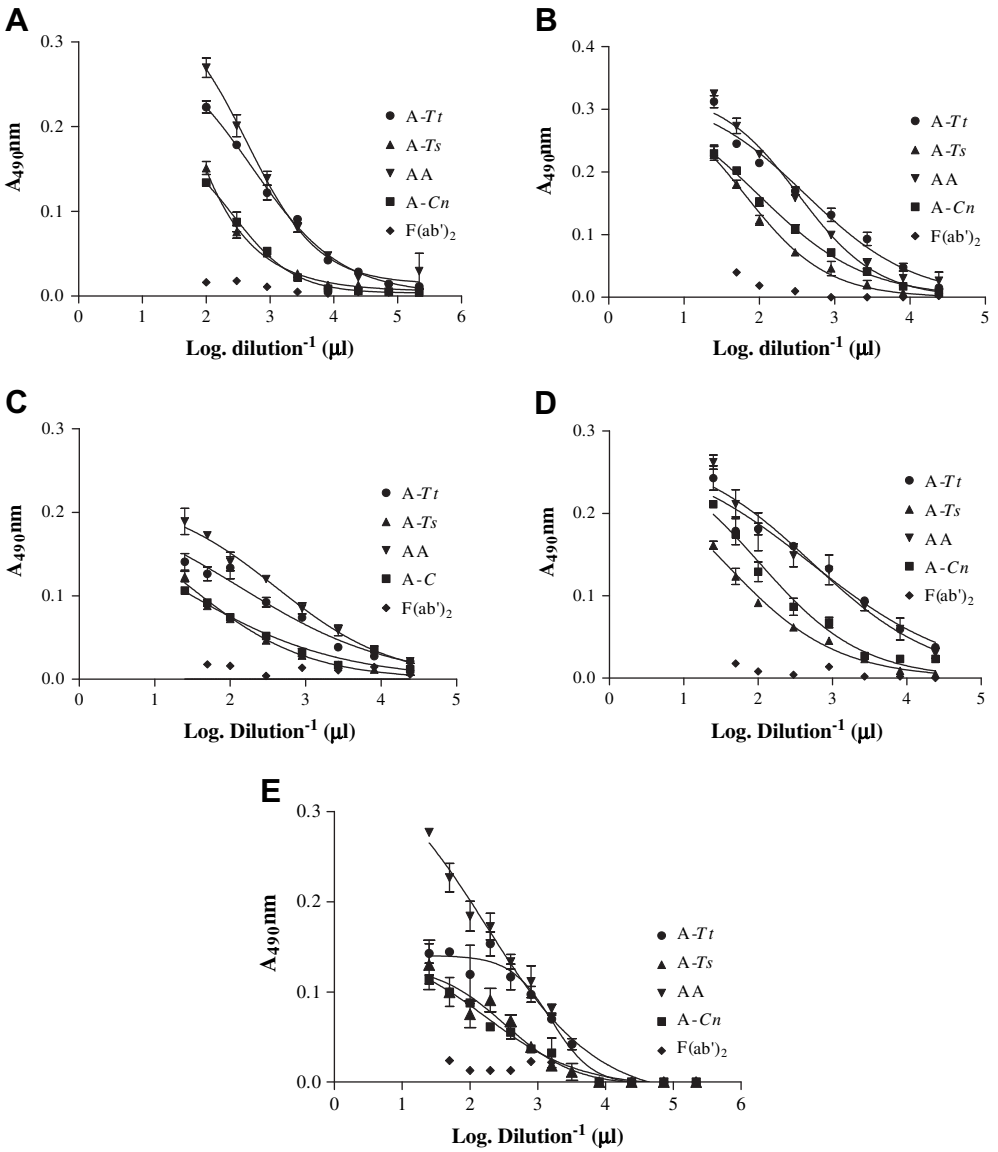

Fig. 5. ELISA titration of venoms from different places using anti-venoms. Samples of venom obtained by homogenates of telsons from *T. trivittatus* collected in various places were titrated using anti-venoms Antiescorpión from the INPB (●) against *T. trivittatus* (A-*Tt*); Antiescorpiónico from Butantan Institute (▲), antidote against *T. serrulatus* (A-*Ts*); Antiaracnídico from Butantan Institute (▼), antidote against scorpions and spiders (AA) and Alacramyn (■) from Bioclon Institute of Mexico, antidote against *Centruroides* scorpion venom (A-*Cn*). An irrelevant F(ab′)2 fragment , antidote against snake of the genus *Bothrops* was used as negative control (◆). In Fig. 5A the venom homogenate used was a mixture from different regions; Fig. 5B the sample is from homogenates coming from La Rioja; Fig. 5C is homogenates from Santa Fe; Fig. 5D is homogenates from Entre Rios and finally Fig. 5E is the only sample obtained by milking the scorpion (whole soluble venom) from Entre Rios.

intravenously injected venom seems to be more potent than the intraperitoneally injected. As it can be seen in Table 1, the telson homogenates from Cordoba and the whole soluble venom from Entre Rios show a high potency by i.v. route ($p < 0.05$ in both cases). These assays were done with venom from a single sample, using the two different routes. If all the values (i.p. versus i.v.), are considered the p value is <0.0001. However when considered separately the LD_{50}s of telson homogenates or the whole venom by these two different routes, the differences are not in all the cases of significance (the p value is >0.05) as shown in Table 1. In conclusion, the variations reported in the present study showed that the values of LD_{50} found for *T. trivittatus* are affected by the type of venom used, the place where the scorpions were collected and very possibly by the route of administration of the venom.

The chromatographic profiles of the various types of sample analyzed as shown in Fig. 3 also support some of the data found with the LD_{50}. There is a great variability on the profile of the HPLC separation of the samples obtained by homogenization. More components are visible, especially those that elute at earlier stage from the chromatogram. They

very likely correspond to more hydrophilic components, not necessarily toxic to mammals. Samples from electrical stimulated scorpions show to contain more peptides in the elution times around 30–40 min, where it is known that toxins that recognize Na^+-channels occur in other scorpion venoms (Batista et al., 2007). This is the elution time found for the gamma-like component of *T. trivittatus*, earlier reported by our group (Corornas et al., 2007).

The necropsy analysis of various tissues obtained from intoxicated mice, showed several histopathological lesions, but they all were very similar independently of the source of venom (Fig. 1). Among the most prominent observations were a generalized congestion and lesions in lungs, heart and liver damage with pancreatic lesions in some cases, similar to the results obtained previously when using a mixture of venoms from scorpions of different regions of the country (de Roodt et al., 2001) and with the lesions described for other *Tityus* venoms (Freire-Maia, 1990; D'Suze et al., 1999; 2004; Cupo et al., 2003; de Roodt et al., 2009). The pulmonary lesions observed in this study are consistent with those described in mice injected experimentally with venom of *Tityus confluens* and other *Tityus* (de Roodt et al., 2009) and with the venom of other Buthidae non American scorpions like *Buthus occitanus tunetanus*, *Androctonus australis Hector*, *Leiurus quinquestriatus haebraus*, *Parabuthus transvaalicus and Parabuthus granulatus* (de Roodt et al., 2006). In addition, the congestive pulmonary lesions are similar to those recently described in rats injected with venom of *Buthus occitanus tunetanus* (Ben Nasr et al., 2009).

Various samples of venom (either electrically obtained or from telson homogenates) were also used for immunochemical studies by means of two techniques: double immunodiffusion (Fig. 4) and enzymatic assays by ELISA (Fig. 5 and Table 2), in which either three or four anti-venoms mentioned in the section of Material and methods were tested: anti-scorpion of Argentina (A-*Tt*), anti-arachnid (AA) and anti-*Tityus serrulatus* (A-*Ts*) of Brazil and Alacramyn (A-*Cn*) against *Centruroides* of Mexico.

For the immunodiffusion only three anti-venoms were used and as it can be observed the strongest cross-reactivity was seen with the homologous anti-venom, the A-*Tt* from the Argentinean INPB, as expected since it is the homologous anti-serum (Fig. 4). But the extent of cross-reactivity with the anti-venom anti-arachnids of Brazil (AA) is quite comparable. This is not surprising since in previous work it was found that the AA anti-venom from Brazil was able to neutralize the venom of *T. trivittatus*. In Argentina the AA was successfully used to treat human stings by scorpions before the production of the specific anti-venom (de Roodt et al., 2001, 2003). It is also necessary to mention that both countries have scorpions that belong to the same genus and very similar components have been isolated from *T. trivittatus* of Argentina and *T. serrulatus* from Brazil (Coronas et al. 2003 and 2007). However the anti-venom from Mexico (A-*Cn*) showed less cross-reactivity, as also expected because the venom used to produce the antidote comes from a different genus of scorpion (*Centruroides*). The intensity of the bands observed is fainter, in part due to the fact that the concentration of protein from the anti-venom of Argentina (A-*Tt*) is 9 folds higher than the Alacramyn from Mexico. The cross-reactivity can also come from venom components other than toxins, such as hemocyanin or the enzyme hyaluronidase, which is ubiquitous in all these venoms. It would be interesting to conduct comparative neutralization assays using both anti-venoms (A-*Tt* and A-*Cn*).

Fig. 5 and Table 2 show the results of ELISA, using the four anti-venoms available and a variety of different combinations of sample venoms. Fig. 5A shows the results obtained using a mixture of homogenated venoms from the various regions studied. Fig. 5B was obtained with telson homogenates from La Rioja; Fig. 5C with telson homogenates from Santa Fe; Fig. 5D from telson homogenates of Entre Rios and Fig. 5E was obtained using whole soluble venom from scorpions of Entre Rios. A non relevant $F(ab')_2$ anti-venom was used as negative control. The most important observation from the 5 graphics showed that there is a significant cross-reactivity, especially between the various venoms used in the homologous anti-venom produced in Argentina. This again was somehow expected, but also a significant recognition was observed using the anti-arachnid antidote of the Butantan from Brazil. In this way, the EC_{50} values of the A-*Tt* and the AA did not show major differences confronted against the different samples being their curves and EC_{50}s very similar ($p < 0.05$) in almost all the cases. The Mexican anti-venom (A-*Cn*) and the specific A-*Ts* of Brazil had comparable results (see Fig. 5 and Table 2).

It is interesting to note that the specific anti-venom A-*Ts* of Butantan had lower reactivity than the AA anti-venom ($p < 0.05$). The latter is polyvalent anti-venom obtained by immunizing horses against electrical stimulated venom from *T. serrulatus*, and a mixture of milked venom from the spiders of the genera *Phoneutria* and *Loxosceles* (Ministerio de Saude, 1999). Certainly there is cross-reactivity of spider venom components with other components of *T. trivittatus* venom. However it is also necessary to clarify that the amount of protein content of the A-*Ts* anti-venom is about nine folds less concentrate than that of anti-arachnid AA.

This communication reports a general characterization of various types of venoms obtained from the scorpion of the species *T. trivittatus* collected in distinct regions of Argentina, showing some differences in venom composition and lethality, as well immunological characteristics using several anti-venoms commercially available and used by medical doctors. To the best of our knowledge, this is the first systematic record on the subject and it is relevant for the treatment of accidents by scorpions in Argentina. Medical doctors certainly will be benefit by reading this communication, because the results reported here can help taking decision on how to treat patients from the various geographical areas that are stung by *T. trivittatus*.

The toxicity of the venom from *T. trivittatus* in human envenomation seems to have grown in the last years in some regions of the country. In provinces in which the stings of these scorpions were cases without medical importance, abruptly became medical emergency. This happened in the provinces of Santa Fe, Catamarca, La Rioja, Entre Ríos and more recently in the province of San Juan. In these provinces more than six deaths related with this scorpion were recorded during the last five years (Piola 2007; de Roodt 2007). This means it is necessary to study

what is happening, both in terms of venom composition and in terms of human exposition to more often accidents.

The lack of information is one of the causes that make difficult to evaluate a possible change in scorpion venom toxicity. This type of information is relevant since the different studies of the venom showed differences among samples of venom collected in the same province and city. Only the record along the time of different data will help to detect modifications of the characteristics of the venoms.

The human deadly cases occurred due to scorpion stings very possibly can be attributed to a variation in the toxicity, but it is possible in addition, that a major frequency of contacts human-scorpion due to environmental changes, the growing or movement of scorpion populations or the colonization by humans in zones where this scorpion inhabits, could facilitate this contact. At present we do not have response for these questions.

Acknowledgements

This work was supported by the Ministry of Health of Argentina and by the collaboration of the Sanitary authorities from the provinces of Entre Ríos, La Rioja, Catamarca and Santiago del Estero. Authors are grateful with Dr. María del Carmen Diaz Ariza from the Faculty of Medicine of Barceló University, Dr. Miguel Angel Romero from the Hospital "Vera Barros", both from La Rioja and with M.Sc. Liliana Salas from the University of Catamarca by the provision of specimens of *T. trivittatus*. Authors acknowledged and are grateful to DVM Enrique Trabattoni from the Faculty of Veterinary Medicine of Esperanza, Santa Fe, for the facilitation to obtain milked venom from *T. trivittatus* specimens. The technical assistance of Mr. Victor M. Manzanelli and B.Sc. Laura Lanari (INPB), B.Sc. Diego Mendicino, and Dr. Roberto Tomassone (from Hospital "O. Alassia", Santa Fe) and to H.T. Claudio Mirabelli (Gema Biotech, Buenos Aires) by his technical assistance in the histopathologic studies, is deeply acknowledged. Authors are grateful with BSs. Sergio Rosset from the Faculty of Biological Sciences of the University of La Plata by his helpful advice in the revision of the statistics used in the work. This work was partially supported by grants from CONACyT, Mexican Government, (Fondo Salud number 014109) and DGAPA-UNAM (IN227507) to LDP.

Conflict of interest

None declared.

Appendix. Supplementary data

Supplementary data associated with this article can be found in online version at doi:10.1016/j.toxicon.2009.08.014.

References

Abdel-Mottaleb, Y., Coronas, F.V., de Roodt, A.R., Possani, l.D., Tytgat, J., 2006. A novel toxin from the venom of the scorpion *Tityus trivittatus*, is the first member of a new alpha-KTX subfamily. FEBS Lett. 580, 592–596.

Alagón, A.C., Guzmán, H.S., Martin, B.M., Ramírez, A.N., Carbone, E., Possani, L.D., 1988. Isolation and characterization of two toxins from the Mexican scorpion *Centruroides limpidus* limpidus Karsch. Comp. Biochem. Physiol. B. 1 (1), 53–161.

Bancroft, J.D., Stevens, A., 1990. In: Bancroft, J.D., Stevens, A. (Eds.), Theory and Practice of Hystological Techniques, third ed. Churchill Livingstone, Edinburgh.

Batista, C.V.F., Román-González, S., Salas-Castillo, S.P., Zamudio, F.Z., Gómez-Lagunas, F., Possani, L.D., 2007. Proteomic analysis of the venom from the scorpion *Tityus stigmurus*: biochemical and physiological comparison with other *Tityus* species. Comp. Biochem. Physiol. 146, 147–157.

Becerril, B., Corona, M., Coronas, F.I.V., Zamudio, F., Calderon-Aranda, E.S., Fletcher Jr., P.L., Martins, B.M., Possani, L.D., 1996. Toxic peptides and genes encoding toxin gamma of the Brazilian scorpions *Tityus bahiensis* and *Tityus stigmurus*. Biochem. J. 313, 753–760.

Ben Nasr, H., Serria H., Chaker S., Riadh B., Zouheir S., Kamel J., Tarek R., Khaled Z., 2009. Some biological effects of scorpion envenomation in late pregnant rats. Exp. Toxicol. Pathol., (Epub ahead of print).

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

Bucherl, W., 1971. Classification, biology and venom extraction of scorpions. In: Bucherl, W., Buckley, E. (Eds.), Venomous Animals and their Venoms, vol. III. Academic Press, New York, p. 341.

Calderón-Aranda, E.S., Dehesa-Dávila, M., Chávez-Haro, A., Possani, L.D., 1996. Scorpion stings and their treatment in Mexico. In: Bon, C., Goyffon, M. (Eds.), Envenomings and Their Treatments. Editions Fondation Marcel Mérieux, Lyon, France, pp. 311–320.

Coronas, F.V., de Roodt, A.R., Portugal, T.O., Zamudio, F.Z., Batista, C.V., Gómez-Lagunas, F., Possani, L.D., 2003. Disulfide bridges and blockage of Shaker B K(+)-channels by another butantoxin peptide purified from the Argentinean scorpion *Titiyus trivittatus*. Toxicon 41, 173–179.

Corornas, F.I., Diego-García, E., Zamudio, F., de Roodt, A.R., Restano-Cassulini, R., Possani, L.D., 2007. Cloning and Peptide Sequencing of a Gamma-like Toxin from the Argentinian Scorpion *Tityus trivittatus*. Pan American Congress of the International Society on Toxinology, Juriquilla, Queretaro, Mexico.

Cupo, P., Azevedo-Marquez, M., Hering, S.E., 2003. Escorpionismo. In: Costa Cardoso, J.L., Siqueira Franca, F.O., Fan Hui, Wen, SanfAna Málaque, C.M., Haddad Jr., V. (Eds.), Animais Peconhentos No Brasil. Biología, Clínica e Terapeutica Dos Acidentes. Sarvier–Fapesp, Sao Paulo, pp. 198–208.

de Roodt, A.R., 2007. Situación en los países. Argentina. In: Consulta Técnica Sobre Animales Venenosos en Latinoamérica. PAHO, Sao Paulo, Brazil, p. 22.

de Roodt, A.R., Beltramino, J.C., Reati, G.J., Amoroso, M., Vucharchuk, M., Tomassone, R., Manzanelli, V.M., Laskowicz, R.D., Mendicino, R., 2005. Toxicity of telsons of *Tityus trivittatus* from different regions of Argentina. Acta Toxicol Argent 13 (Suppl.), 117.

de Roodt, A.R., García, S.I., Salomón, O.D., Segre, L., Dolab, J.A., Funes, R.F., de Titto, E.H., 2003. Epidemiological and clinical aspects of scorpionism by *Tityus trivittatus* in Argentina. Toxicon 41, 971–977.

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. Journal of Natural Toxins 10, 99–109.

de Roodt, A.R., Lago N.R., Estevez J., Scarlatto E., Laskowicz R.D., Paniagua J.F., García H., Manzanelli V.M., Alagon A., 2006. Toxicity of five scorpion venoms from the old world, and its immunological reactivity with American scorpion antivenoms. In: XV World congress on animal, plant and microbial toxins. Glasgow, Scotland, Abstract book, pp. 340.

de Roodt, A.R., Lago, N.R., Salomón, O.D., Laskowicz, R.D., Neder de Román, L.E., López, R.A., Montero, T.E., del V, Vega V., 2009. A new venomous scorpion responsible for severe envenomation in Argentina: *Tityus confluens*. Toxicon 53, 1–8.

D'Suze, G., Comellas, A., Pesce, L., Sevcik, C., Sánchez-de León, R., 1999. *Tityus discrepans* venom produces a respiratory distress syndrome in rabbits trough and indirect mechanism. Toxicon 37, 173–180.

D'Suze, G., Salazar, V., Diaz, P., Sevcik, C., Azpurua, H., Bracho, N., 2004. Histopathological changes and inflammatory response induced by *Tityus discrepans* scorpion venom in rams. Toxicon 44, 851–860.

Freire-Maia, L., 1990. Pharmacology of *Tityus serrulatus* scorpion venom. Mem. Inst. Butantan 52, 51–57.

Kalapothakis, E., Chávez-Olórtegui, C., 1997. Venom variability among several *Tityus serrulatus* specimens. Toxicon 35, 1523–1529.

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

Margni, R.A., 1990. Inmunoquímica. Cap. 34, third ed. El Ateneo, Buenos Aires.

Ministerio de Salud y Ambiente de la Nación, 2004. Boletín Epidemiológico Nacional. Envenenamiento por Animales Ponzoñosos. Ch. 9. Ministerio de Salud y Ambiente de la Nación, Buenos Aires. pp. 67–75.

Ministerio de Salud, March 2005. Boletín Epidemiológico Periódico, vol. 19. Ministerio de Salud de la Nación, Buenos Aires, pp. 9, 14.

Ministerio de Salud, 2008. Boletín Epidemiológico Periódico, vol. 39. Ministerio de Salud de la Nación, Buenos Aires, September–October 2007 pp. 6–19.

Ministerio de Saúde, Fundacao Nacional de Saúde 1999. Manual de Diagnóstico e Tratamento de Acidentes por Animais Peconhentos. Brasilia.

National Research Council, 2002. In: Academia Nacional de Medicina (Ed.), Guía Para el Cuidado y Uso de Los Animales de Laboratorio. Institute of Laboratory Animal Resources, Commission of Life Sciences, México DF.

Nishikawa, A.K., Caricati, C.P., Lima, M.L.S.R., Dos Santos, M.C., Kipnis, T.L., Eickstedt, V.R.D., Knysak, I., Da Silva, M.H., Higashi, H.G., Dias Da Silva, W., 1994. Antigenic cross-reactivity among the venoms from several species of Brazilian scorpions. Toxicon 32, 989–998.

Ojanguren-Affilastro, A.A., 2005. Estudio monográfico de los escorpiones de la República Argentina. Revista Ibérica de Aracnología 11, 75–241.

O'Leary, M.A., Kornhauser, R.S., Hodgson, W.C., Isbister, G.K., 2009. An examination of the activity of expired and mistreated commercial Australian antivenoms. Trans. R. Soc. Trop. Med. Hyg. Epub ahead of print.

Ozkan, O., Adiguzel, S., Yakistiran, S., Filazi, A., 2006. Study of the relationship between *Androctonus crassicauda* (Olivier, 1807; Scorpiones, Buthidae) venom toxicity and telson size, weight and storing condition. J. Venom. Anim. Toxins Incl. Trop. Dis 12, 297–309.

Peirano, S., Vainstub, V., Tomassone, R., 2000. Envenenamiento Grave Por Escorpión en Pediatría. En Libro de Resúmenes del 32. Congreso Argentino de Pediatría, Salta, abstract book, pp. 399.

Piola, J.C., 2007. Cambios regionales en la Toxicología Clínica: 30 años. Curso Internacional de Emergencias Toxicológicas, Lima, Perú, 4–7 de julio, 2007. <http://www.cepis.ops-oms.org/bvstox/e/cursos/peru_julio07/dia06/07_Piola.pdf>.

Salomón, O.D., de Roodt, A.R., 2001. Scorpions: spontaneous report in 2 reference centers in the city of Buenos Aires, 1997–2000. Medicina (Buenos Aires) 61, 391–396.

Saracco, A.S., de Roodt, A.R., Cabrera, A.M., García, S.I., 2006. Escorpionismo. Boletín de la Asociación Toxicológica Argentina 20, 17–23.

Sisson, W.D., Polis, G.A., Watt, D.D., 1990. Venom collection and purification. Cap. 11. In: Polis Eds, A.G. (Ed.), The Biology of Scorpions, second ed. Stanford University Press, pp. 459–461, pp. 587.

Schägger, H., von Jagow, G., 1987. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal. Biochem. 166, 368–379.

World Health Organization, 1981. Progress in the Characterization of Venoms and Standarization of Anti-venoms. Offset Publication, WHO, Geneva.