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Detection and quantification of a β-neurotoxin (crotoxin homologs) in the venom of the rattlesnakes *Crotalus simus, C. culminatus* and *C. tzabcan* from Mexico

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ABSTRACT

Snake venom may vary in composition and toxicity across the geographic distribution of a species. In the case of the three species of the Neotropical rattlesnakes *Crotalus simus, C. culminatus* and *C. tzabcan* recent research has revealed that their venoms can contain a neurotoxic component (crotoxin homologs), but is not always the case. In the present work, we detected and quantified crotoxin homologs in venom samples from three species distributed across Mexico, to describe variation at the individual and subspecific levels, using slot blot and ELISA immunoassays. We found that all *C. simus* individuals analyzed had substantial percentages of crotoxin homologs in their venoms (7.6–44.3%). In contrast, *C. culminatus* lacked them completely and six of ten individuals of the species *C. tzabcan* had low percentages (3.0–7.7%). We also found a direct relationship between the lethality of a venom and the percentage of crotoxin homologs it contained, indicating that the quantity of this component influences venom lethality in the rattlesnake *C. simus*.

1. Introduction

Viperid venoms are composed of a large number of inorganic and organic molecules, the latter include proteins, some of which are responsible for generating physiopathology when envenomation occurs (Gutiérrez, 2002; Gutiérrez et al., 2009). Sixty-three protein families have been reported in viperid venoms (Tasoulis and Isbister, 2017). However, it is known that the most important families are snake venom metalloproteases (SVMPs), snake venom serine proteases (SVSPs) and phospholipases A_2 (PLA_2) (Calvete, 2017; Durban et al., 2017; Lomonte et al., 2012; Tasoulis and Isbister, 2017). Together, these families usually represent more than 70% of the protein composition. Viperid venoms can be generally classified into type I (low lethal potency, high enzymatic activity) and type II (high lethal activity, low enzymatic activity) (Mackessy, 2008) and there are venoms that behave as intermediate, like some populations of *C. s. scutulatus* and *C. simus* (high lethality and high enzymatic activity) (Borja et al., 2018; Castro et al., 2013).

One of the families of proteins with greatest diversity of toxic activities is PLA_2s, where two groups have been described based on the presence or absence of enzymatic activity (Gutiérrez et al., 2008; Gutiérrez and Lomonte, 2013; Kini, 2003, 2005; Kini and Evans, 1987, 1989; Lomonte et al., 2003; Lomonte and Rangel, 2012). Among those with catalytic activity is a group of neurotoxins that have been called crotoxin homologs because they are similar to crotoxin, a PLA_2 that was first purified and crystallized from the venom of the South American rattlesnake *C. durissus terrificus* (Slotta and Fraenkel-Conrat, 1938). Crotoxin is a β-neurotoxin that inhibits the release of the neurotransmitter acetylcholine at the neuromuscular junction, producing possibly lethal, neurotoxic effects (Faure et al., 1991, 1993; 1994; Rangel-Santos et al., 2004; Slotta and Fraenkel-Conrat, 1938). The protein is a heterodimeric complex united by non-covalent bonds, consisting of a basic PLA_2 (CB) (M.W. 14,350 Da, pI 8.2) with neurotoxic and enzymatic activity and a non-toxic acidic protein called crotapotin (CA), which consists of three disulfide-linked polypeptide chains (M.W. 9490 Da, pI 3.4), whose function is to direct CB to its

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target site (Faure et al., 1993, 1994; 2011; Gutiérrez, 2002). CA increases the lethal potential of CB and each complex may include 4 isoforms, whose identities directly influence the toxicity of the venom (Canziani et al., 1983; Faure et al., 1991, 1993).

The venoms of *C. d. durissus, C. d. terrificus* and *C. d. ruruima,* for example, have been reported to have more than 50% of crotoxin (Calvete et al., 2010). More recently, crotoxin homologs have been reported in the venoms of other species of the genus *Crotalus*, including some populations of *C. scutulatus scutulatus* (Mojave toxin) named as "type A"; and the population lacking of this toxin is named "type B" (Borja et al., 2014, 2018; Cate and Bieber, 1978; Dobson et al., 2018; Massey et al., 2012; Strickland et al., 2018). Other examples are *C. viridis concolor* (concolor toxin) (Mackessy et al., 2003), *C. tigris* (Mojave-like toxin) (Minton and Weinstein, 1984; Weinstein and Smith, 1990), *C. vegrandis* (vegrandis toxin) (Chen et al., 2004) and *C. horridus* (canebrake toxin) (Glenn et al., 1994). Similar components have been described in the venoms of a few non-*Crotalus* viperids, like *Bothriechis nigroviridis* (nigroviriditoxin) (Lomonte et al., 2015) and *Ophryacus sphenophrys* (sphenotoxin) (Neri-Castro et al., 2019). Neurotoxic venoms tend to have median lethal doses (LD_{50}) from 10 to 100 times lower when compared to venoms without neurotoxic components (Borja et al., 2018; Castro et al., 2013; Glenn et al., 1982; Mackessy, 2008, 2010a, 2010b; Rivas et al., 2017; Strickland et al., 2018). Populations with presence and absence of crotoxin homologs have been described in venoms of *C. s. scutulatus* and *C. lepidus* and research indicates that this can also be the case for *C. tzabcan* (Borja et al., 2014, 2018; Castro et al., 2013; Durban et al., 2017; Rivas et al., 2017; Saviola et al., 2017).

The rattlesnake *Crotalus simus* is distributed from Mexico to Costa Rica and typically inhabits semiarid regions, including tropical dry forest, chaparral, tropical deciduous forest and pastures. Previously, *C. simus* was classified as part of the *C. durissus* group, which includes snakes from North, Central, and South America. Campbell and Lamar (2004) then separated the *C. durissus* complex into three species: *C. totonacus*, *C. durissus* and *C. simus*. They further divided *C. simus* into three subspecies: *C. s. simus*, *C. s. culminatus* and *C. s. tzabcan* (Campbell and Lamar, 2004). Finally, in 2005, Wüster (Wüster et al., 2005) and collaborators proposed to elevate the three subspecies described by Campbell to species level: a) *C. simus*, which is distributed along the Atlantic slope, from central Veracruz in Mexico to western Honduras, and along the Pacific coast from the Isthmus of Tehuantepec to central Costa Rica; b) *C. culminatus*, which is found from southern Michoacan to the Isthmus of Tehuantepec and c) *C. tzabcan*, which consists of populations from the Yucatan Peninsula and northern Belize and Guatemala.

The venoms of these three species of the *C. simus* complex were recently characterized by Castro et al. (2013) who reported intraspecific variation in their biological and biochemical activities. They observed that there were marked differences in venom lethality among them three species: *C. simus* venoms showed the lowest median lethal doses (LD_{50} 0.18–0.65 μg/g), and *C. culminatus* the highest (LD_{50} 3.42–15.9 μg/g), whereas *C. tzabcan* showed intermediate lethality (LD_{50} 0.47–8.21 μg/g). Also, proteomic analysis showed that crotoxin homologs made up 14.3% of the total *C. simus* venom, while *C. culminatus* venom lacked them completely (Castro et al., 2013) and *C. tzabcan* contained 3% (Durban et al., 2017).

Variation in venom composition over a species' geographic distribution is an integral part of intraspecific variation. Clinically, these geographical differences can have a great impact, because envenomations could present different symptomatology depending on the region (Mackessy, 2008). It is also important to know whether there is a geographic variation in venoms used as immunogens, because this might affect whether antivenom quality remains the same for different antivenom batches (Gutiérrez et al., 2017). The wide distributional range of the rattlesnake *C. simus* makes this species a good model for venom variation studies. This research would also increase our knowledge regarding the species' biology and provide information to improve the selection of venoms used for immunization of animals during antivenom production (Calvete, 2017; Chaves et al., 1992; Gutiérrez et al., 2010b, 2009; Lomonte et al., 2008).

The aim of the present work was to detect and quantify crotoxin homologs in the venom of different organisms of the three species of the *C. simus* complex: *C. simus*, *C. culminatus* and *C. tzabcan*.

2. Materials and methods

2.1. Venoms

Crotalus simus venom samples used were collected under permit from the Secretaría de Medio Ambiene y Recursos Naturales (SEMARNAT). Other venom samples came from the collection UMA TSAAB KAN (identification number UMA-IN-0183-YUC-10). *C. scutulatus scutulatus* pools of type A venom, used as a positive control for the presence of crotoxin homologs and type B venom, used as a negative control, were from the National Natural Toxins Research Center (NNTRC, Kingsville, Texas, USA) and include material from two or more individuals. *C. durissus terrificus* venom, used to purify the sub B of crotoxin and as a positive control, was from captive snakes kept at the Instituto Carlos Malbrán, Buenos Aires, Argentina. We assigned identification numbers to all venom samples. All the individual venoms from *C. simus*, *C. culminatus* and *C. tzabcan* are part of the IBt-UNAM venom bank, (for more details see Castro et al., 2013).

2.2. Protein quantification

Venoms were quantified using the bicinchoninic acid method (BCA). We followed the methods described in the manual of the commercial Pierce™ BCA Protein Assay kit.

2.3. Electrophoresis

Samples of 15 μg of each venom were analyzed using SDS-PAGE 15% under denaturing and reducing (2-mercaptoethanol) conditions. Gels were dyed using Coomasie 0.2% R-250. We included molecular weight markers (Biolabs) in each gel (Laemmli, 1970).

2.4. Crotoxin purification

Crotoxin was purified from *C. durissus terrificus* venom by size-exclusion chromatography (Aird et al., 1990). We used a glass column 196 cm in length and 0.9 cm in diameter, packed with Sephadex G-75 (Sigma). The buffer used was 20 mM ammonium acetate with 6 M urea, pH 4.7, with a flow of 16 mL/h. Fraction 3 was lyophilized and then passed through an ion-exchange chromatographic column (Mono Q 5/5 μm) on an FPLC system (AccesoLab) as previously described by Rangel-Santos et al. (2004), to yield highly purified crotoxin B subunit (CB).

2.5. Production and purification of polyclonal antibodies (pAbs)

Three rabbits from the New Zealand strain were immunized over three months in intervals of fifteen days, with increasing doses of CB (10–300 μg/rabbit) alternating with incomplete Freund's adjuvant and alum (aluminum hydroxide and magnesium hydroxide, Thermo Fisher). The serum of the three rabbits was mixed and the antibodies were purified by affinity chromatography in a Sepharose 4B resin activated with cyanogen bromide (Sigma) and coupled to CB (5 mg).

2.6. Production of monoclonal antibodies (mAb)

Two groups of five mice from the Balb/C strain were immunized via intraperitoneal injection with CB. We started with 1 μg of toxin with

incomplete Freund's adjuvant, and immunized once a week, intercalating with alum, until we reached 5 μg of toxin over a 2-months period. Spleen lymphocytes from the immunized mice were fused with murine myeloma cells from the cell line SP2/0 Ag14 ATCC. Antibodies from an established hybridoma were purified using affinity chromatography in a Sepharose column coupled to protein A (Invitrogen™) (Valdés et al., 2001).

2.7. Detection of crotoxin homologs by slot blot

The slot blot analysis was performed with 50 μg of venom in PBS (native conditions) on a polyvinylidene fluoride membrane (Merck Millipore), using a Hoefer chamber (Amersham Pharmacia Biotech). We used the monoclonal antibody for detection antibody (1 μg/mL), and goat anti-mouse (diluted 1:4000) coupled to alkaline phosphatase (Millipore) as the secondary antibody. Finally, we used BCIP/NBT buffer (Invitrogen) to obtain a colorimetric reaction.

2.8. Detection of crotoxin homologs by western blot

We used a Western blot to analyze 2 μg of venom under reducing conditions (2-mercaptoethanol) on a nitrocellulose membrane (Merck Millipore). For the recognition antibody, we used our polyclonal rabbit anti-crotoxin antibody, using a concentration of 5 μg/mL. As a secondary antibody, we used goat anti-rabbit IGg (diluted 1:4000) coupled to alkaline phosphatase (Millipore). Again, we obtained a colorimetric reaction using BCIP/NBT Invitrogen reagents, according to the manufacturer's protocols.

2.9. Enzyme-linked immunosorbent assay (ELISA)

We measured the antibody titres generated in mice or rabbit using indirect ELISA and sandwich-type ELISA to quantify the percentage of crotoxin homologs in *C. simus* venoms, as explained below:

Indirect ELISA: 96-well plates (Nunc MaxiSorp®) were sensitized with 100 μL of 5 μg/mL CB in 0.05 M carbonate-bicarbonate buffer (pH 9.5), incubating for 2 h at 37 °C. Then, the wells were washed three times with TBST pH 8 (50 mM Tris/HCl, 150 mM NaCl, 0.5% Tween 20). Next, the wells were blocked with gelatin (50 mM Tris/HCl pH8, 0.5% gelatin, 0.2% Tween 20) at 37 °C for 2 h. We repeated the washing and then incubated for 1 h at 37 °C with the serum sample of interest in 1:3 consecutive dilutions. The wells were washed again and then incubated for 1 h at 37 °C with the secondary antibody diluted 1:4000 (anti-mouse or anti-rabbit, depending on the analysis), coupled with horseradish peroxidase (HRP) (Merck Millipore). Finally, the plate was incubated for 10 min with ABTS (2,2′-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt-colorimetric method) for detection.

Sandwich-type ELISA: we used the same buffers as in the indirect ELISA and all incubations were done at 37 °C. 96-well plates (Nunc MaxiSorp®) were sensitized with 100 μL of 1 μg/mL 4F6 monoclonal antibody for 1 h. After washing, the wells were blocked for 2 h with gelatin (200 μL of 0.5% gelatin in 50 mM Tris, 0.2% Tween 20, pH 8.0). We then generated a standard curve with CB at a starting concentration of 2 μg/mL and 1:3 consecutive dilutions. Sample venoms were placed in known concentrations diluted in the same way and incubated for 1 h. Next, we added 100 μL of 1 μg/mL polyclonal rabbit anti-crototoxin antibody to each well, incubated for 1 h, washed and incubated with goat anti-rabbit antibody coupled to HRP (diluted 1:4000) (Millipore) for another hour. Finally, the colorimetric reaction was obtained using ABTS, as before. Absorbances were quantified in an ELISA reader (Magellan R) at 405 nm. To determine the concentration of crotoxin homologs in sample venoms, the values were interpolated from the CB standard curve, fitted to a sigmoidal dose-response (variable slope) nonlinear regression, using the software GraphPad Prism v. 6.0 (GraphPad Software).

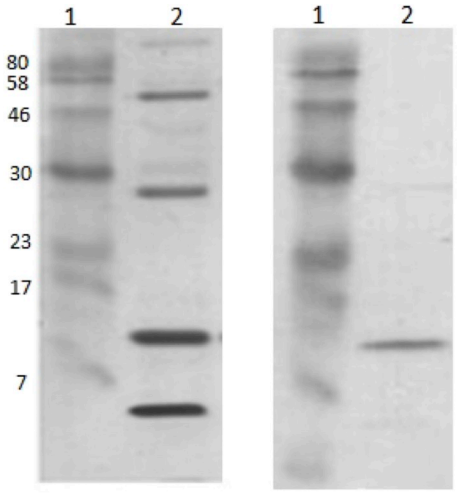

Fig. 1. SDS-PAGE 15% (left) and Western blot (right) of *C. durissus terrificus* venom, incubation with 5 μg/mL antibodies obtained from the immunopurification of polyclonal rabbit serum. 1. Molecular weight marker; 2. *C. durissus terrificus* venom 20 μg (SDS-PAGE) and 2 μg (Western blot).

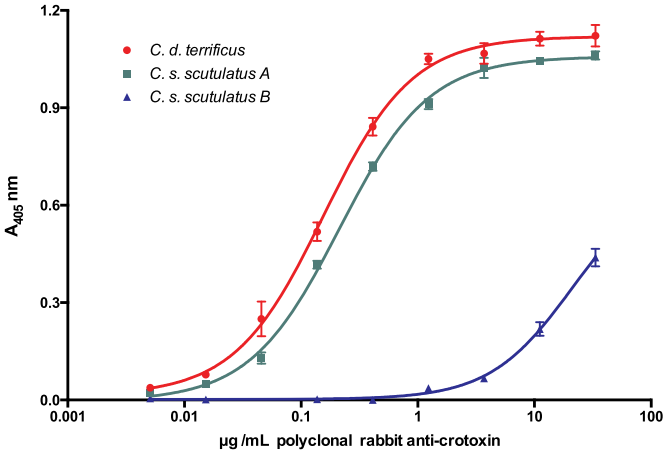

Fig. 2. Recognition curves of polyclonal rabbit anti-crotoxin with (red circle) *C. d. terrificus* venom (C+), (green square) *C. s. scutulatus* type A (C+), and (blue triangle) *C. s. scutulatus* type B (C-). The titers are 0.15 ± 0.006 μg/mL for *C. d. terrificus*, 0.21 ± 0.01 μg/mL for *C. s. scutulatus* type A and 20.1 ± 7.8 01 μg/mL for *C. s. scutulatus* type B. Bars represent mean ± SD of triplicates.

3. Results

3.1. Detection of crotoxin by polyclonal antibodies from rabbit

As expected, the obtained polyclonal antibodies recognized CB (14 kDa) from *C. durissus terrificus* venom (Fig. 1). Also, Fig. 2 shows an indirect ELISA, demonstrating recognition of *C. d. terrificus* and *C. s scutulatus* type A venoms, used as positive controls (C+), but not of type B venom of *C. s. scutulatus*, used as a negative control (C-). Therefore, polyclonal antibody recognizes 134 times more *C. d. terrificus* and 96 times more *C. s. scutulatus* type A compared to the venom of *C. s. scutulatus* type B.

3.2. Detection of crotoxin by monoclonal antibody 4F6

Regarding the production of monoclonal antibodies (mAb), we found one with a concentration of 240 μg/mL in ascites fluid, which we labelled 4F6. This antibody recognized the two positive controls (*C. d. terrificus* and *C. s. scutulatus* type A), while presenting no cross-reactivity with the PLA_2s from *C. s. scutulatus* type B venom (Fig. 3).

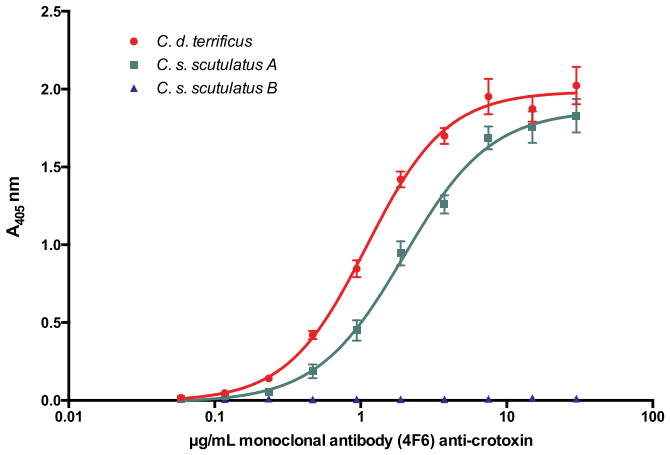

Fig. 3. Recognition curve of monoclonal antibody 4F6 with venom from (red circle) *C. d. terrificus* (C+), (green square) *C. s. scutulatus* type A (C+), and (blue triangle) for *C. s. scutulatus* type B (C). The titers are 1.09 ± 0.01 µg/mL for *C. d. terrificus* and 1.97 ± 0.23 µg/mL for *C. s. scutulatus* type A. Bars represent mean ± SD of triplicates.

Detection of crotoxin homologs was carried out using the slot blot technique under native venom conditions (non-reducing). The mAb 4F6 recognized these components in the venom of the 11 *C. simus* samples analyzed and showed no cross-reactivity with the negative control, nor with any protein from *C. culminatus* venoms. Furthermore, the mAb recognized these neurotoxins in 6 samples from *C. tzabcan* (samples: IBt 212, IBt 218, IBt 217, IBt 211, IBt 216, and IBt 207), but not in 4 samples from the same species (samples: IBt 255, IBt 254, IBt 214, and IBt 253) (Fig. 4).

3.3. Quantitative analysis of crotoxin homologs

In accordance with the observations made using the slot blot technique, no crotoxin homologs were found using ELISA in any of the venoms from the species *C. culminatus* (Table 1).

The limit of quantification (LoQ) of our ELISA was 3.5 ng/mL. With this technique, we determined that the venom of the positive control (*C. d. terrificus*) contained 44.8%, type A venom from *C. s. scutulatus* contained an average of 23.8%, and we did not detect these components at all in type B venom from that same species. Table 1 shows the mean percentage of crotoxin homologs in venoms from the three species of *C. simus*, according to their geographic distributions. Venom from individuals of *C. simus* collected in the state of Veracruz had the highest mean percentage of these neurotoxins (32.8%), followed by the single sample from Oaxaca (14.5%), whereas venoms from individuals collected in Chiapas and Quintana Roo had the lowest average percentage of crotoxin homologs (9%). Individual IBt 225 stood out as having the venom with the highest concentration of these proteins (44.3%) (Table 1). We did not find them at all in the venoms of four *C. tzabcan* individuals (IBt 255, IBt 254, IBt 214, and IBt 253), one of which was collected in Quintana Roo and the rest in Yucatán (Table 1).

4. Discussion

4.1. Polyclonal and monoclonal antibodies

The recognition of *C. d. terrificus* venom by previously purified polyclonal antibodies (under reducing conditions) was evaluated by western blot. We only observed recognition of the band corresponding to the basic subunit of crotoxin. Additionally, we carried out an ELISA to evaluate our antibodies' ability to recognize native proteins from *C. d. terrificus* venom and from *C. s. scutulatus* "A" venom as positive controls. We also tested type B venom from *C. s. scutulatus* as a negative control. We observed that the antibodies recognized PLA_2s from both positive controls. Yet, the antibodies recognized non-neurotoxic PLA_2s from *C. s. scutulatus* population B with low intensity, 134 times lower than *C. d. terrificus* venom and 96 times less than *C. s. scutulatus* type A. The basic subunit of crotoxin has approximately 50% sequence identity with non-neurotoxic PLA_2s (Aird et al., 1986). However, this identity refers to the linear sequence so the probability of cross-recognition is likely lower given that many epitopes are a result of three-dimensional structure (Sela et al., 1967). Additionally, although a great variety of antigenic sites are presented to the animal immune system during immunization, there are particular epitopes antigens that may substantially stimulate the production of antibodies, some of which may be associated with the active site of a toxin (Oshima-Franco et al., 1999). A great variety of cross recognition levels have been found with antibodies to heterologous and homologous PLA_2s, including lack of cross-reactivity among certain species within the same family. These differences reflect variation in the antigenic sites of enzymes belonging to the same family (Nair et al., 1980). For example, polyclonal antibodies obtained against non-neurotoxic PLA_2s did not recognize the basic subunit of Mojave toxin, showing that, despite high sequence identity, the two classes of PLA_2s are antigenically different (Rael et al., 1986).

We evaluated the recognition pattern of the obtained mAb 4F6 using indirect ELISA. It showed cross-reactivity with Mojave toxin, but it did not recognize non-neurotoxic PLA_2s from type B *C. s. scutulatus* venom. Rael and collaborators observed the same phenomenon in 1986, when they obtained a mAb that recognized the basic subunit of crotoxin and showed no cross-reactivity with non-neurotoxic PLA_2s from the same venom (Rael et al., 1986). They therefore concluded that, despite high sequence identity between neurotoxic and non-neurotoxic PLA_2s, it is probable that the three-dimensional configuration of CB is different from that of other PLA_2s. It is also worth mentioning that the mAb from Rael et al. recognized several isoforms of crotoxin. Their work did not evaluate whether the antibody showed recognition of the different isoforms that may exist in the venoms of *C. d. terrificus* and *C. simus*. However, it is very possible that such recognition occurs, since the sequence identity between the crotoxin isoforms of *C. d. terrificus*, *C.*

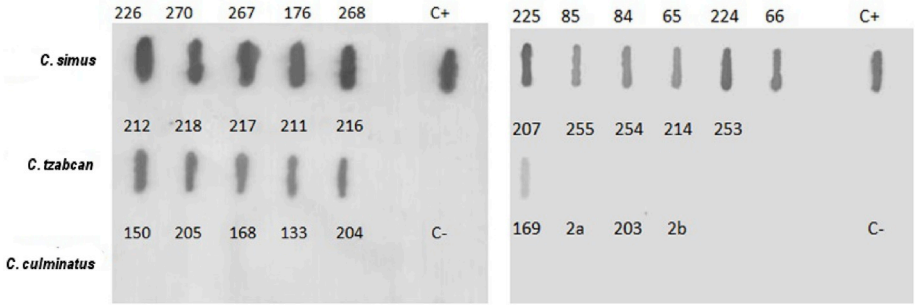

Fig. 4. Slot blots of native venom (50 µg) of different individuals from the species *C. simus*, *C. tzabcan*, and *C. culminatus*; *C. d. terrificus* (C+); and *C. s. scutulatus* type B (C-). Incubation with mAb 4F6 anti-crotoxin (2 µg/mL).

Table 1
Median lethal dose (μg/g mouse) and percentage of crotoxin homologs found in venoms of *C. simus, C. culminatus* and *C. tzabcan*.

Species	Sample ID	Locality	LD_{50}	Crotoxin homologs	
			(μg/g mouse)	Detection[a]	% ± SD
C. simus	IBt 226	La Tinaja, Veracruz	0.18 (0.15–0.21)	+	38.5 ± 0.7
	IBt270	Actopan, Veracruz	0.20 (0.18–0.21)	+	21.6 ± 0.73
	IBt267	Puente Nacional, Veracruz	0.21 (0.20–0.21)	+	26.3 ± 0.88
	IBt 176	Santo Domingo, Oaxaca	0.20 (0.20–0.21)	+	14.5 ± 0.4
	IBt 268	Actopan, Veracruz	0.26 (0.15–0.27)	+	26.2 ± 0.4
	IBt 225	Tinajas, Veracruz	0.26 (0.26–0.26)	+	44.3 ± 0.24
	IBt 085	Chiapa de Corzo, Chiapas	0.26 (0.21–0.30)	+	7.6 ± 0.5
	IBt 084	Chiapa de Corzo, Chiapas	0.30 (0.26–0.32)	+	6.3 ± 0.42
	IBt 065	Copainalá, Chiapas	0.31 (0.30–0.31)	+	8.4 ± 0.8
	IBt 224	Playas del Conchal, Veracruz	0.32 (0.31–0.32)	+	39.7 ± 0.8
	IBt 066	Chiapa de Corzo, Chiapas	0.65 (0.61–0.67)	+	13.7 ± 0.4
C. tzabcan	IBt 212	Solidaridad, Quintana Roo	0.80 (0.71–0.91)	+	7.7 ± 0.45
	IBt 218	Chetumal, Quintana Roo	0.91 (0.89–0.93)	+	4.4 ± 0.34
	IBt 217	Chetumal, Quintana Roo	1.08 (0.96–1.20)	+	3.0 ± 0.16
	IBt 211	Solidaridad, Quintana Roo	1.19 (1.08–1.31)	+	4.6 ± 0.74
	IBt 216	Chetumal, Quintana Roo	1.79 (1.71–1.87)	+	5.0 ± 0.43
	IBt 207	Solidaridad, Quintana Roo	1.99 (1.84–2.16)	+	7.3 ± 0.45
	IBt 255	Mérida, Yucatán	4.68 (4.42–5.00)	–	0
	IBt 254	Mérida, Yucatán	7.21 (5.29–8.79)	–	0
	IBt 214	Solidaridad, Quintana Roo	7.89 (7.47–8.31)	–	0
	IBt 253	Mérida, Yucatán	8.21 (8.00–8.42)	–	0
C. culminatus	IBt 150	Puente de Ixtla, Morelos	3.42 (3.37–3.47)	–	0
	IBt 205	Chilpancingo, Guerrero	3.47 (3.37–3.63)	–	0
	IBt 168	Tepalcingo, Morelos	7.95 (7.79–8.10)	–	0
	IBt 133	Alpuyeca, Morelos	8.53 (8.42–8.68)	–	0
	IBt 204	Tlaltizapán, Morelos	9.31 (9.10–9.58)	–	0
	IBt 169	Tepalcingo, Morelos	9.84 (9.86–10.0)	–	0
	Csim2A	Villa de Ayala, Morelos	15.0 (14.3–15.8)	–	0
	IBt 203	Tlaltizapán, Morelos	15.5 (15.0–16.0)	–	0
	Csim2B	Villa de Ayala, Morelos	15.9 (14.7–17.3)	–	0

[a] Detection by slot blot, venoms with presence for crotoxin homologs (+), venoms with absence of crotoxin homologs (−).

simus (Costa Rican and Mexico populations), and Mojave neurotoxin is between 98% and 100% (Calvete et al., 2012; Durban et al., 2017; Faure et al., 1991, 1994; Massey et al., 2012), suggesting that the structural epitopes of all three toxins are similar.

4.2. Qualitative analysis by slot blot

Due to the wide geographic distribution and high levels of genetic divergence among species of the *C. simus* complex, these species represents an interesting model to study variation in venom composition (Castro et al., 2013). We did not observe cross-reactivity with non-neurotoxic PLA_2s from type B *C. s. scutulatus* venom, nor was there reactivity with venom proteins of *C. culminatus*. These results are consistent with the proteomic results of Castro et al. (2013). Additionally, we determined that all *C. simus* individuals studied and six of the ten *C. tzabcan* individuals studied had crotoxin homologs in their venom. These experiments were not performed with polyclonal antibodies because they showed ELISA recognition (low) to the venom of *C. s. scutulatus* type B whose venom lacks crotoxin. In slot blot experiments the results are qualitative (presence or absence) so antibodies that have cross reactivity to non-neurotoxic PLA2s, even if it is low, could give unreliable results, while monoclonal antibodies are specific for crotoxin homologs.

4.3. Quantitative analysis of crotoxin homologs

The purpose of quantifying crotoxin homologs in venoms from three species was to relate these quantities to the median lethal dose of each venom, as proposed by Calvete et al. (2012), who report that the species with the highest percentage of crotoxin have low LD_{50}s. We thus worked with the same adult individuals from which Castro et al. (2013) had already obtained intravenous LD_{50} values (Table 1), as well as biological and biochemical activities and proteomes of pools from *C. simus* and *C. culminatus* venoms. As a result of our quantification, we observed that venom from *C. simus* individuals had the highest average concentration of crotoxin homologs (7.6%–44.3%), followed by 6 individuals of *C. tzabcan* (3.0%–7.7%). However, 4 *C. tzabcan* individuals and all of the 9 individuals of *C. culminatus* we evaluated lacked the neurotoxin, which is consistent with the slot blot results.

In 2010, Calvete and collaborators performed a proteomic and biological analysis of the venom of the species *C. simus* and the *C. durissus* complex, which are distributed in Central and South America, respectively. They proposed that the concentration of crotoxin in venom is directly related with its lethal activity (Calvete et al., 2010). In general, the venoms that have crotoxin homologs have higher lethal potency than those that lack them. However, there are cases where the percentage of these components do not appear to be the only factor increasing lethality, for example, the venoms of individuals IBt 225 and IBt 085 both had LD_{50} values of 5 μg/g mouse, but they had very different percentages of crotoxin homologs: 44.3% and 7.6%, respectively. There may be several reasons for this discrepancy, one of which has to do with the ratio of CB and CA in each venom, given that the relative levels of CA may increase the lethality (Canziani et al., 1983). Another potential explanation may involve the isoforms that each venom contains; these differ slightly in their molecular structure, as well as in their chromatographic properties and electrophoretic mobility. The isoforms of crotoxin homologs CB from different species (i.e. *C. s. scutulatus*, *C. d. terrificus*, *C. oreganus concolor* and *C. tzabcan*) often differ in a small number of amino acid residues (typically 1 to 5) and, in some cases, these differences modify the enzymatic and pharmacological properties of the toxin. The LD_{50} of different isoforms ranges from 0.07 to 0.45 μg/g (Faure and Bon, 1988; Faure et al., 1993). Finally, variation in non-neurotoxic components of the venoms, like SVMP, can also influence venom lethality.

In our study, we also found that the lethality (LD_{50}) of snake venoms not containing crotoxin homologs (those of *C. culminatus* and four

individuals of *C. tzabcan*) is 26–100 times lower when compared to the lethality of individuals that have this protein. Previously, the same relationship was reported in populations of the snake *C. s. scutulatus*, where type B venom (which does not contain crotoxin) has 20 times lower lethality in comparison with type A venom (Glenn et al., 1982). The causes for this difference in the presence of neurotoxins have been poorly addressed and some authors attribute the variation in venom composition to local adaptations, which confer advantages such as specialization on different prey species. However, recent research has described that variation between neurotoxic and haemorrhagic specimens within the same species is due to very marked differences in haplotypes of PLA_2 and SVMPs complex genes (Dowell et al., 2018).

4.4. Intraspecific variation

The variation in venom toxicity we observed also exhibited a geographic pattern, as has been described in several snake species (Borja et al., 2013, 2018; Glenn et al., 1982, 1994,; Gutiérrez et al., 2010a; Salazar et al., 2007; Strickland et al., 2018; Sunagar et al., 2014). We found that *Crotalus simus* from eastern Mexico, in the state of Veracruz had the highest average concentration of crotoxin homologs (14.5%–44.3%), while those from Chiapas presented lower proportions (6.3%–13.7%). Taxonomic studies suggest that the populations of the state of Veracruz is a different species from those of South (Chiapas) and Central America (Wüster et al., 2005), however, this has not yet been clarified due to the low number of samples analyzed in the aforementioned study. Six individuals of *C. tzabcan* from the state of Quintana Roo had low neurotoxin concentrations (3%–7.7%), in contrast, three individuals from the state of Yucatán and one from Quintana Roo did not present crotoxin homologs (Fig. 5 and Table 2). This is interesting because we now know that there are positive and negative individuals for crotoxin homologs. However, with the few samples we have we cannot delimit populations or correlate with external factors, so a larger number of samples should be studied along this species distribution.

In the states of Morelos and Guerrero, we did not find neurotoxin in any of the samples belonging to *C. culminatus* but this species also includes populations in the state of Oaxaca, where it overlaps with the distribution of *C. simus*. It is thus important to increase our sampling to determine whether there are populations of *C. simus* without crotoxin homologs, or individuals of *C. culminatus* with neurotoxic venoms. This

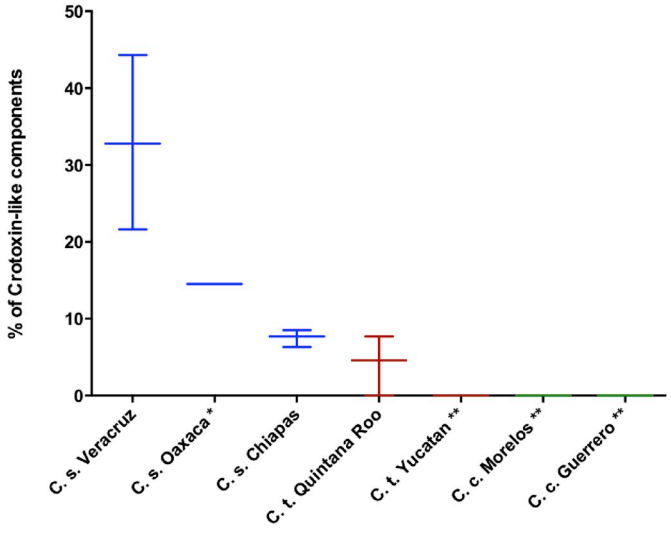

Fig. 5. Intervals of crotoxin homologs percentage for different individuals per state of origin. Horizontal lines represent mean of all data and error bars represent minimum and maximum percentages. *Data of a single individual. **crotoxin quantifications were zero.

Table 2
Percentage of crotoxin homologs (mean and range) in venoms from individuals of the three species of *Crotalus simus*, according to their geographic distributions.

Species	Locality	n	% Crotoxin (Mean)	% Crotoxin (Range)
C. simus	Veracruz	6	32.8	21.6–44.3
	Oaxaca	1	14.5	14.5
	Chiapas	4	7.7	6.3–8.5
Total		11	22.0	6.3–44.3
C. tzabcan	Quintana Roo	7	4.6	0–7.7
	Yucatán	3	0	0
Total		10	3.2	0–7.7
C. culminatus	Morelos	8	0	0
	Guerrero	1	0	0
Total		9	0	0

phenomenon, as previously mentioned, has been reported in the subspecies *C. s. scutulatus*, where there is a marked geographic variation in venom composition (Glenn et al., 1982).

There are two possible reasons why venom may vary across a species' geographic distribution. The first occurs in nearby or sympatric populations, as in the case of *C. scutulatus*, where there are two divergent populations without significant morphological differences, but which differ by the presence or absence of Mojave toxin. There is evidence that populations with type A and type B venoms were historically isolated, however, there is currently no physical barrier between these populations, and, in fact, intermediate populations have been described (Borja et al., 2018; Glenn et al., 1982; Massey et al., 2012; Schield et al., 2018; Strickland et al., 2018; Wilkinson et al., 2018; Zancolli et al., 2018). The second type of venom variation has been seen in isolated populations. Small, isolated populations tend to have homogeneous venoms. In contrast, large populations tend to conserve many venom components when isolated but also show variation in the total spectrum of venom, related to the time since isolation (Chippaux et al., 1991). The particular case of the *C. simus* complex has not been previously studied. Nevertheless, previous researchers have proposed that Mexico is the center of diversification for rattlesnakes, and that their diversity, also reflected in the composition and toxicity of their venoms, is a consequence of physiographic and climatic fluctuations over the past 50 million years (Flores-Villela, 1993; Wüster et al., 2005). The species of the genus *Crotalus* have been proposed to, originally, possess the genes for subunits A and B of crotoxin homologs (because the ancestor that arrived through the north of America had them) and then some were lost over the course of time (Calvete et al., 2010; Calvete, 2017; Dowell et al., 2016). On the other hand, the intraspecific variation in the case of *C. simus* of Costa Rica and Mexico is ontogenetic, where the concentrations of crotoxin are greater in neonates than in adults (Calvete et al., 2010; Castro et al., 2013; Durban et al., 2017, 2013; Lomonte et al., 1983), Still, it is necessary to carry out studies with a greater number of samples of both juvenile and adult specimens of various species, to further analyze the regulation mechanisms that have generated the described variation.

5. Conclusions

In the present study, we obtained important information regarding the presence/absence of crotoxin homologs across the geographic distribution of the *C. simus*, *C. culminatus* and *C. tzabcan,* describing a significant interspecific as well as intraspecific variation in venom composition. This type of analysis may be used to identify and quantify crotoxin homologs in venoms of other species of snakes, which may help to predict their neurotoxic properties and improve hospital treatment of patients bitten by snakes. On the other hand, the identification of species or populations of viperids with neurotoxins will help to evaluate antivenoms and eventually improve them. The ELISA technique used here, has important advantages such as the analysis of

multiple samples at the same time, and being relatively cheap and fast.

Ethical statement

All animal work was performed according to the guidelines approved by the bioethics committee of the Instituto de Biotecnología, UNAM.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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