

SHORT REPORT

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Antivenomics as a tool to improve the neutralizing capacity of the crotalic antivenom: a study with crotamine

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Abstract

Background: Snakebite treatment requires administration of an appropriate antivenom that should contain antibodies capable of neutralizing the venom. To achieve this goal, antivenom production must start from a suitable immunization protocol and proper venom mixtures. In Brazil, antivenom against South American rattlesnake (*Crotalus durissus terrificus*) bites is produced by public institutions based on the guidelines defined by the regulatory agency of the Brazilian Ministry of Health, ANVISA. However, each institution uses its own mixture of rattlesnake venom antigens. Previous works have shown that crotamine, a toxin found in *Crotalus durissus* venom, shows marked individual and populational variation. In addition, serum produced from crotamine-negative venoms fails to recognize this molecule.

Methods: In this work, we used an antivenomics approach to assess the cross-reactivity of crotalic antivenom manufactured by IVB towards crotamine-negative venom and a mixture of crotamine-negative/crotamine-positive venoms.

Results: We show that the venom mixture containing 20% crotamine and 57% crotoxin produced a strong immunogenic response in horses. Antivenom raised against this venom mixture reacted with most venom components including crotamine and crotoxin, in contrast to the antivenom raised against crotamine-negative venom.

Conclusions: These results indicate that venom databases and antivenomics analysis provide a useful approach for choosing the better venom mixture for antibody production and for the subsequent screening of antivenom cross-reactivity with relevant snake venom components.

Keywords: Antivenom production, Antivenomics, *Crotalus durissus*, Crotamine, Crotoxin, Geographic venom variation

Background

For over a century, antivenoms have remained the only effective treatment for snakebite. An important technical consideration in the production of antivenoms is to use suitable mixtures of venoms (as antigens) in order to produce neutralizing antibodies against the venom of the intended species. Although antivenom administration constitutes an effective therapy against envenomation, the occurrence of inter- and intraspecies venom variability has

led to the need for a more robust understanding of venom composition and antivenom efficacy.

The preparation of representative mixtures of venoms from snake species with a broad geographic distribution is not easy, particularly if there is no venom-based assessment to facilitate the selection of appropriate venom-producing specimens [1, 2]. In Brazil, six subspecies of *Crotalus durissus* are currently recognized (*C. d. dryinas*, *C. d. marajoensis*, *C. d. ruruima*, *C. d. terrificus*, *C. d. cascavella*, and *C. d. collilineatus*), with each inhabiting distinct ecosystems and displaying a wide geographical distribution [3–5]. All of these subspecies are capable of producing lethal envenomation in humans, since their venoms exhibit systemic neuro- and myotoxic activities.

Envenomation symptoms are often attributed to the presence of crotoxin and crotamine, although marked

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differences in the concentration of these toxins among venoms have been documented. For example, crotoxin, a neurotoxic phospholipase A₂ (PLA₂), is the main toxin of *C. durissus* venom and accounts for 70–90% of its venom proteome [6–10]. On the other hand, significant variation has been observed for crotoamine at both individual and population levels, since it accounts from 2 up to 22% of *C. durissus* proteome [9, 11–13]. There is also a positive correlation between the concentration of crotoamine present in venom and the level of crotoamine gene expression (ranging from 1 to 32 copies per haploid genome) [12].

The Vital Brazil Institute (IVB) is one of three Brazilian institutions that manufacture antivenoms, the others being the Butantan Institute and Ezequiel Dias Foundation (FUNED). Although the crotalic antivenom produced by the three institutions follows the guidelines defined by Brazilian National Health Surveillance Agency (ANVISA), each institution uses its own crotalic antigens. ANVISA has determined that immunization should use crotoamine-positive venom obtained from specimens that cover the geographical distribution of *C. durissus* [5]. However, determination of the LD₅₀ is the only quality control measure required for the venoms.

The use of antivenomics to evaluate antivenom efficacy was first described in an investigation of the immunoreactivity of the polyvalent antivenom produced by the Costa Rican Clodomiro Picado Institute (ICP) against *Bothriechis lateralis* and *Bothriechis schlegelii* venoms [14]. Subsequently, antivenomics has emerged as a logical extension of venom studies and has been applied to numerous medically relevant species [1, 15, 16]. In addition, antivenomics protocols have been extensively revised and improved, and used in pre-clinical studies to assess the efficacy of antivenoms and their potential clinical applicability across the geographical range of a species [1, 2, 17–20].

In a previous study, we applied a first generation antivenomics approach to examine the immunoreactivity of crotalic antivenom against subspecies of Brazilian rattlesnakes. The results indicated that the crotalic antivenom was devoid of antibodies capable of recognizing and binding to crotoamine [9]. This finding suggested either that the venom used in the production of the crotalic antivenom was devoid of crotoamine, or that the low molecular mass of crotoamine (4.8 kDa) meant that this cationic polypeptide could be a poor immunogen in horses. In order to explore further this question, the current study applied a second generation of antivenomics approach to examine the cross-reactivity of the crotalic antivenom produced at IVB using a pool of crotoamine-negative and crotoamine-positive venoms. Our results showed that using the proper immunogenic pool, all components from *Crotalus* venom can be recognized.

Material and methods

Venoms and antivenoms

The venoms of *C. d. terrificus* were obtained from captive specimens maintained at the Regional Ophiology Center of Porto Alegre (NOPA) and IVB. Crotoamine-positive venom (batch 2014CDU00301) was extracted from 26 adult specimens (10 males and 16 females) housed at NOPA. These snakes were collected primarily in Protásio Alves city, in the southern Brazilian state of Rio Grande do Sul. Crotoamine-negative venom (batch 2014CDU00201) was extracted from 44 adult specimens of both genders maintained by IVB. The latter snakes were originally collected near Juiz de Fora in the state of Minas Gerais. Following venom extraction, samples were centrifuged at 1000 g to remove cell debris, lyophilized and stored at –20 °C.

In accordance with the guidelines of the Brazilian Pharmacopeia [21], and before preparing the mixture of venoms for immunization, the median lethal doses (LD₅₀) for the crotoamine-positive (batch 2014CDU00301) and crotoamine-negative (batch 2014CDU00201) venoms were determined as a quality control. The data available from internal registers of IVB indicated an LD₅₀ of 153 µg/kg, accessed via intraperitoneal (i.p.) route, for the crotoamine-positive venom (batch 2014CDU00301) and an LD₅₀ of 73 µg/kg, i.p., for the crotoamine-negative venom (batch 2014CDU00201). The mixture of venoms for immunization was obtained by combining equal amounts of crotoamine-positive and negative venoms.

The crotalic antivenom used in this study was produced at IVB (batches SAC085204b and SAC155204F), based on the guidelines of Brazilian Pharmacopeia, and the instructions of ANVISA [5, 21]. This antivenom was of equine origin and consisted of purified F(ab')₂ fragments. Antivenom SAC085204b, which expired in 2011, was from the same batch used in our previous antivenomics study [9]. The expiry date of the antivenom batch SAC155204F is October, 2018.

RP-HPLC venom fractionation

Venom composition was assessed by reversed-phase high-performance liquid chromatography (RP-HPLC) using a Shimadzu Prominence HPLC system. Pooled crotoamine-positive (batch 2014CDU00301) and pooled crotoamine-negative (batch 2014CDU00201) venom samples were resuspended in 200 µL of 0.1% TFA and applied to a Teknokroma Europa C₁₈ column equilibrated with solvent A (0.1% trifluoroacetic acid – TFA). Bound proteins were eluted with discontinuous gradient of solvent B (0.1% TFA in 100% of acetonitrile) at a flow rate of 1 mL/min. For RP-HPLC, we used the same gradient conditions applied in the previous proteomic characterization of *C. d. terrificus* [9]. The elution conditions were: isocratic at 5% B for 10 min, followed by a

gradient of 5–15% B for 20 min, 15–45% B for 120 min and 45–70% B for 20 min, with a final isocratic step of 70% B for 5 min. The elution profile was monitored at 214 nm in all experiments. Specific toxin families were identified by comparison of the chromatographic profile of each fraction with the RP-HPLC results from previous venom analyses of *C. d. terrificus* [8, 9].

Antivenomics

A second-generation antivenomics method was used, as previously described by Pla et al. [17]. Briefly, 1 mL of NHS-activated Sepharose 4 Fast Flow resin (GE Healthcare) was washed with 10–15 mL of 1 mM HCl and then packed into a column. The column was equilibrated with 2 mL of coupling buffer (0.2 M NaHCO₃, 0.5 M NaCl, pH 9.3) at pH 7–8. Fifty milligrams of F(ab')₂ fragments purified from crotalic antivenom was then loaded onto the column and incubated for 4 h at room temperature, according to the manufacturer's instructions. Unbound F(ab')₂ was washed from the column with equilibration buffer, collected and analyzed by SDS-PAGE. The amount of bound F(ab')₂ was determined by quantifying the unbound antibody densitometrically after SDS-PAGE, using a standard curve obtained by loading known amounts of F(ab')₂ molecules (1–5 µg) from the original antivenom. The efficiency of coupling (based on densitometric analysis) was >90% for both antivenoms.

After the removal of unbound F(ab')₂, unreacted groups of the resin were blocked by incubation with 1 mL of 0.1 M Tris-HCl, pH 8.0 on an orbital shaker, overnight at 22–25 °C. The columns were subsequently washed alternately with three volumes of 0.1 M acetate buffer/0.5 M NaCl, pH 4–5, and 0.1 M Tris-HCl, pH 8.5. This treatment was repeated six times.

Before incubation with the venoms, the columns were equilibrated with five volumes of phosphate-buffered saline (PBS). For the immunoassay, 300 µg of *C. d. terrificus* venom, representing a venom:antivenom ratio of 1:150, was dissolved in 1 mL of PBS and applied to the column followed by incubation for 4 h at 25 °C on an orbital shaker. After the incubation, the columns were washed five times with PBS and the unbound material was collected. Immunobound proteins were eluted with 5 mL of buffer (0.1 M glycine, pH 2.0), and neutralized with neutralization buffer (1 M Tris-HCl, pH 9.0). Venom proteins from the immunoaffinity column prepared with antivenom SAC085204b were fractionated by RP-HPLC using a Teknokroma Europa C₁₈ column on a Shimadzu Prominence HPLC system, whereas venom proteins from the immunoaffinity column prepared with antivenom SAC155204F were fractionated by RP-HPLC using a Shimadzu (10Avp) HPLC system. Proteins were eluted by washing the columns isocratically with 5% B

for 5 min, followed by a gradient of 5–25% B for 5 min, 25–45% B for 60 min and 45–70% for 10 min, with a final isocratic step of 70% B for 5 min at a flow rate of 1 mL/min. Protein detection was performed at 214 nm.

Results

In a previous study, we reported that the antivenom raised against crotoamine-negative *C. d. terrificus* venom failed to recognize crotoamine in crotoamine-positive venom [9]. To investigate the reason for this lack of immunoreactivity, the present study used a second-generation antivenomics protocol to assess the cross-reactivity of a new batch of antivenom (SAC155204F) raised against a mixture of crotoamine-positive and crotoamine-negative venoms.

Initially, we antivenomics to confirm our previous finding regarding the lack of cross-reactivity between SAC085204b and crotoamine. Figure 1 confirms the absence of immunoreactivity. To explore further the lack of immunoreactivity, we immunized horses with a mixture containing equal amounts of crotoamine-positive and crotoamine-negative venoms. Figure 2 shows the profiles of each venom type and the mixture of both. Based on comparison of the elution time of each fraction from RP-HPLC (Fig. 2) with the RP-HPLC results obtained during previous *C. d. terrificus* venom characterization [8, 9], we identified the HPLC peaks as: 1 – crotoamine, 2 – disintegrin, 3 – crotoxin acid chain, 4 to 7 – crotoxin basic chain, and 8 – low expressed toxins including D49-PLA₂, serine protease (gyroxin), C-type lectin (convulxin) and PIII-metalloproteases.

Each batch of venom was obtained from specimens collected in the same geographical region as the populations of rattlesnakes were previously characterized by our venomics protocols [9]. Crotoamine-positive venom (batch 2014CDU00301, LD₅₀ = 153 µg/kg) was provided by NOPA and collected from 26 specimens of *C. d. terrificus* captured mainly in the state of Rio Grande do Sul (Fig. 2a). Crotoamine-negative venom (batch 2014CDU00201, LD₅₀ = 73 µg/kg) was provided by IVB and collected from 44 specimens found in the state of Minas Gerais (Fig. 2b).

The pooled venoms used in horse immunization were designed to provide a strong immune response against both crotoamine and crotoxin (Fig. 3), and contained approximately 20% and 57% of these toxins, respectively (Fig. 2c). The data of antivenomics analysis clearly demonstrated immunoreactivity towards crotoamine, as well as crotoxin, from both venom types (Fig. 3b and e). Immunoreactivity was also observed against toxins that were expressed at lower concentrations and accounted for approximately 6% of the overall protein content of the immunizing pool (Fig. 3c and f, fraction 8). This group of toxins, which includes D49-PLA₂, serine

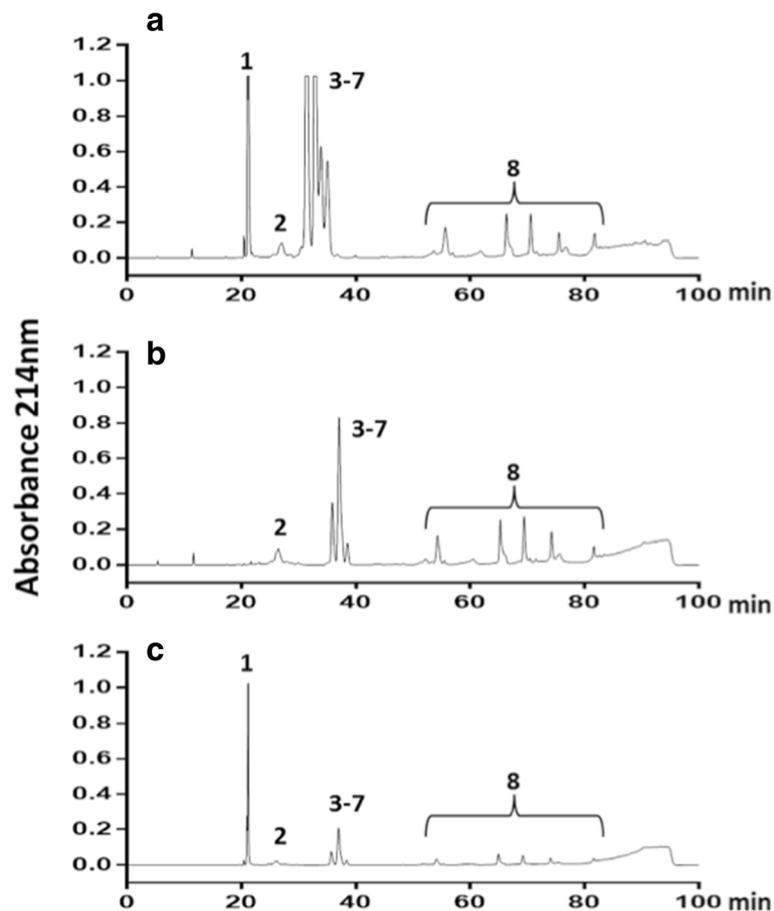


Fig. 1 Antivenomics analyses of crocotalic antivenom manufactured by IVB. **a** About 300 μg of crocotalic venom separated by RP-HPLC using second-generation antivenomics protocols [17]. The elution conditions were: isocratic gradient with 5% of 0.1% TFA in 100% of acetonitrile (solvent B) for 5 min followed by a 5–25% B for 5 min, 25–45% B for 60 min and 45–70% B for 10 min, with a final isocratic step of 70% B for 5 min at a flow rate of 1 mL/min. **b** and **c** the RP-HPLC profiles of retained and non-retained venom toxins on anticrocotalic (batch SAC085204b) affinity column, respectively. Protein families associated with HPLC peaks: 1 –crotamine, 2 – disintegrin, 3–7 – crotoxin, and 8 – fractions of low expression toxins including D49-PLA₂, serine protease (gyroxin), C-type lectin (convulxin) and PIII-metalloproteases

proteases (gyroxin) and P-III metalloproteases, is conserved in others subspecies of *C. durissus* [8, 9].

Discussion

Despite significant intraspecific venom variability, the beta-neurotoxin crotoxin is the main lethal component in *C. durissus* venom. Its LD₅₀ values calculated in mice are between 60 and 180 $\mu\text{g}/\text{kg}$, depending on the route of administration (intravenous, subcutaneous etc.). Following crocotalic envenomation, crotoxin is responsible for the neurotoxicity and local and systemic myotoxicity that leads to acute nephrotoxicity and renal failure [22–26]. On the other hand, the main effect of crotamine is to induce skeletal muscle spasms via interaction with Na⁺ channels [27, 28]. In animal models, crotamine also induces strong paralysis of the hind limbs [29].

Crotamine has a significantly lower toxicity (LD₅₀ 6.8 mg/kg, i.e., two orders of magnitude higher than

crotoxin) suggesting that it contributes more to prey immobilization than to lethality. However, crotamine has also been shown to contribute to myotoxic, cytotoxic and hemolytic activities that could potentially contribute to the nephrotoxicity often observed after envenomation by *C. durissus* [22, 26, 28, 30, 31]. Crocotalic antivenom would be expected to neutralize crotamine and its activities. A major limitation of antivenom therapy after snakebite is that antivenoms with low neutralizing capacity must be administered at higher doses, with severe cases of envenomation requiring high amounts of antivenom [26]. Regarding the production and quality control of antivenoms, each batch must contain similar amount of IgG-derived molecules capable of neutralizing all of the toxic compounds in the venom of a certain specific species.

The venom LD₅₀ values reported herein agree with the crotoxin/crotamine concentration for each type of

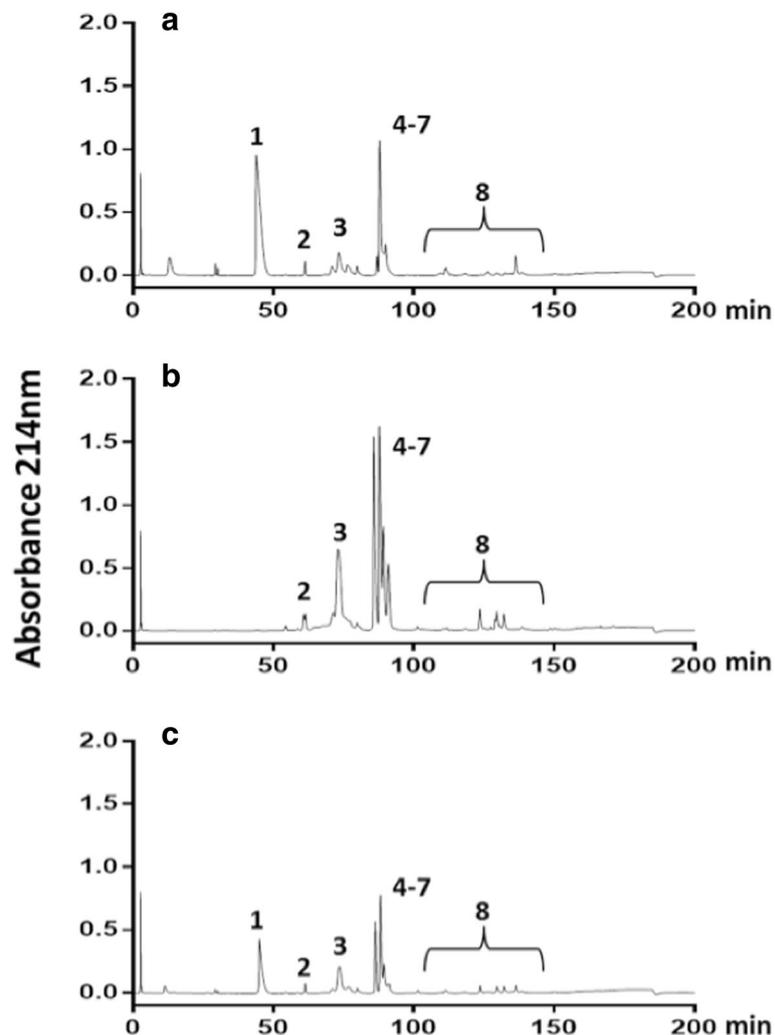


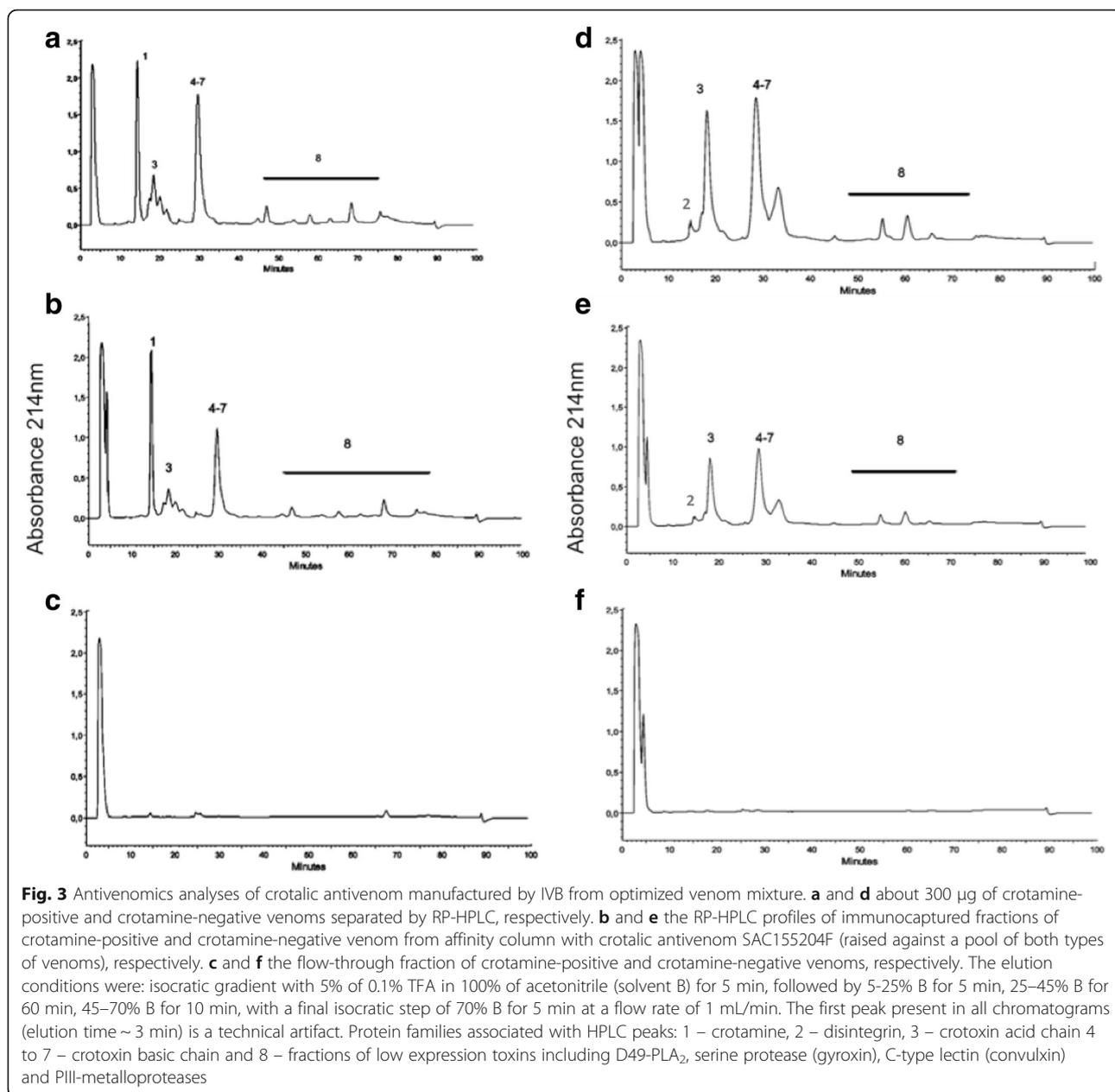
Fig. 2 Analyses of *Crotalus durissus terrificus* venoms by RP-HPLC. The venoms of *C. d. terrificus* (batches 2014CDU00301 and 2014CDU00201) were pooled from (a) crotammine-positive specimens collected in southern and (b) crotammine-negative specimens collected in southeastern Brazil, respectively. The pooled venom for immunization was obtained by combining (c) the same ratio of crotammine-positive and crotammine-negative venoms. The elution conditions were: isocratic gradient with 5% of 0.1% TFA in 100% of acetonitrile (solvent B for 10 min followed by 5–15% B for 20 min, 15–45% B for 120 min and 45–70% B for 20 min, with a final isocratic step of 70% B for 5 min at a flow rate of 1 mL/min. Protein families associated with HPLC peaks were: 1 – crotammine, 2 – disintegrin, 3 – crotoxin acid chain, 4 to 7 – crotoxin basic chain, and 8 – fractions of low expression toxins including D49-PLA₂, serine protease (gyroxin), C-type lectin (convulxin) and PIII-metalloproteases

venom. The high content of crotoxin present in crotammine-negative venom likely explains the low LD₅₀ value observed for this venom when compared to the LD₅₀ of crotammine-positive venom. Although venom LD₅₀ values may be similar, they provide little information about the overall venom composition since venoms often vary in their content of crotammine and other venom compounds (e.g., serine proteases, D49-PLA_{2s}, and metalloproteinases).

Although methods such as HPLC, SDS-PAGE and ELISA have been proposed to identify and quantify crotammine [32, 33], the ANVISA guidelines neither require standardization of the crotammine concentration in

venom pools, nor do they propose any analytical method for the identification and quantification of this toxin. The current ANVISA regulation recommending the use of crotammine-positive venoms for immunization, without knowledge of the actual concentration of this compound in the venom, is problematic and can lead to fluctuations in antibody concentrations among batches.

The results presented in the current study show that the use of pools of venom with low crotammine content may stimulate a weak immune response for this molecule. In addition, crotammine-negative venoms may contain a greater amount of isoforms of the acidic chain of crotoxin, as well as other low expression toxins (Fig. 2b,



fractions 8), including D49-PLA₂, a myotoxin that accounts for 18% of the venom proteome of the northeastern Brazilian rattlesnake *C. d. cascavella* [9]. Herein, we propose that the standardization of a minimal concentration of toxic compounds, especially crotoamine and crotoxin in the *C. d. terrificus* venom pools used for immunization, is a valuable and necessary procedure to ensure quality and reproducibility among batches of crotoalic antivenom from each manufacturing institution.

Conclusions

The results of the present study demonstrate the usefulness of antivenomics analyses for choosing the

appropriate *C. d. terrificus* venoms for antivenom production. Our findings also show that by using a mixture containing equal amounts of crotoamine-positive and crotoamine-negative *C. d. terrificus* venoms, we were able to produce an equine antivenom that successfully recognized crotoamine in crotoamine-positive venom.

Abbreviations

ANVISA: National Health Surveillance Agency; FUNED: Ezequiel Dias Foundation; i.p.: Intraperitoneal; ICP: Clodomiro Picado Institute; IVB: Vital Brazil Institute; LD₅₀: Median lethal doses; NOPA: Regional Ophiology Center of Porto Alegre; PBS: Phosphate-buffered saline; PLA₂: Phospholipase A₂; RP-HPLC: Reversed-phase high-performance liquid chromatography; TFA: Trifluoroacetic acid

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Authors' contributions

RTA participated in data collection and experimental design. PC, LBM and FP participated in data collection. MLA and MLMA supervised the collection of *Crotalus* venom. RBZ controlled the experiments, interpreted results and reviewed the article. CCN performed data collection, experimental design, interpreted results, wrote the article draft, reviewed and edited the article. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

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