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Assessment of neutralization of *Micrurus* venoms with a blend of anti-*Micrurus tener* and anti-ScNtx antibodies

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ABSTRACT

Background: *Micrurus* venoms contain two main groups of toxic protein components: short-chain α-neurotoxins (SNtx) and phospholipases type A_2 (PLA_2). In North America, generally, the *Micrurus* venoms have low abundance of SNtx compared to that of PLA_2s; however, both are highly toxic to mammals, and consequently both can play a major role in the envenomation processes. Concerning the commercial horse-derived antivenoms against *Micrurus* from the North America region, they contain a relatively large amount of antibodies against PLA_2s, and a low content of antibodies against short chain α-neurotoxins. This is mainly due to the lower relative abundance of SNtxs, and also to its poor immunogenicity due to their size and nature. Hence, *Micrurus* antivenoms made in North America usually present low neutralizing capacity towards *Micrurus* venoms whose lethality depend largely on short chain α-neurotoxins, such as South American *Micrurus* species.
Methods: Horses were hyperimmunized with either the venom of *M. tener* (PLA_2-predominant) or a recombinant short-chain consensus α-neurotoxin (ScNtx). Then, the combination of the two monospecific horse antibodies (anti-*M. tener* and anti-ScNtx) was used to test their efficacy against eleven *Micrurus* venoms.
Results: The blend of anti-*M. tener* and anti-ScNtx antibodies had a better capacity to neutralize the lethality of diverse species from North, Central and South American *Micrurus* venoms. The antibodies combination neutralized both the ScNtx and ten out of eleven *Micrurus* venom tested, and particularly, it neutralized the venoms of *M. distans* and *M. laticollaris* that were neither neutralized by monospecific anti-*M. tener* nor anti-ScNtx.
Conclusions: These results provide a proof-of-principle for using recombinant immunogens to enrich poor or even non-neutralizing antisera against elapid venoms containing short chain α-neurotoxins to develop antivenoms with higher effectiveness and broader neutralizing capacity.

1. Introduction

The World Health Organization (WHO) recognizes Snakebite as a Neglected Tropical Disease [1]. Millions of people are bitten every year and hundreds of thousands die or suffer permanent physical or psychological disabilities due to the deleterious effect of snake venoms [2,3]. Some reports have sketched out the importance of the synergy among components within spiders, scorpion and snake venoms, namely toxins [4–6]. Concerning human elapid envenomation, it has been documented the synergy among elapid venom components, mostly toxic peptides and proteins, such as α-neurotoxins (Type I and Type II neurotoxins) and cardiotoxins, which belong to a classification of the three-finger toxins (3Ftxs), and phospholipases A_2 [7,8]. The resulting envenomation can cause acute neurotoxicity ending in death by respiratory arrest: PLA_2 abrogates the neuromuscular communication by damaging the nerve terminals [9]; 3Ftxs, particularly α-neurotoxins, antagonize the nicotinic acetyl choline receptors at postsynaptic level and block the excitatory effect of acetyl choline [10].

To counter envenomation caused by elapid venoms, there are antivenoms constituted by antibodies obtained from hyperimmunized animals with whole venoms. To develop these

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polyspecific antivenoms, laboratories use either mixtures of coral venoms to immunize horses, or mix the monospecific horse-derived hyper-immune plasma or the monospecific antibodies [11]. Currently, antivenoms for *Micrurus* are manufactured in Mexico, Costa Rica, Colombia, Brazil and Argentina using the venom of regional species as immunogens. Together, these antivenoms are expected to have a coverage of ~80 species of coral snakes distributed from North to South America [11,12]. Still, a major flaw of antivenoms is their low efficacy and limited coverage. The problem lies in the low amount and low immunogenicity of some protein venom components, particularly 3Ftxs, generating an insufficient quantity of neutralizing antibodies [13]. Moreover, the low efficacy and narrow cross-reactivity of some antivenoms could also be related to the high inter-species and intra-species variability of the venom composition [14]. Hence, coral snake antivenoms efficacy is bounded to the venom(s) used for animal immunization, and the immune-dominance and abundance of key toxins within the venom [15]. To illustrate, if someone is accidentally bitten by a *Micrurus* species, which has significant variation in its venom composition (i.e. higher concentration of α-neurotoxins versus phospholipases A_2) respect to the species used for immunization, it could happen that the antivenom could become irrelevant, or have low efficacy to halt the envenomation symptoms. This is one of the reasons for regionalizing *Micrurus* antivenoms and focusing their effectiveness on a limited number of species and buffering the venom-variation effect [14].

Although some strategies have been described to improve coral snake antivenoms such as genetic immunization with synthetic multiepitope genes followed by booster doses with recombinant protein [16], or combined immunization protocols, using priming doses of *Micrurus* venom and booster doses of synthetic B-cell epitopes derived from *Micrurus* toxins [17], such strategies have not been probed at large animal scale. Therefore, to expand the coverage of *Micrurus* antivenoms, here we propose a strategy that includes a blending process of two monospecific polyclonal antibodies: one against a pure recombinant ScNtx and another against a predominant-PLA_2 venom (*M. tener*). In the present work, we systematically characterized the *in vitro* recognition, and the *in vivo* neutralization of the polyclonal monospecific antibodies, and the blend thereof -here called Ab-blend- against eleven *Micrurus* venoms. The resulting Ab-blend shows a broader species coverage and improvement in the neutralization efficacy when compared to monospecific antibodies.

2. Material and methods

2.1. Venoms and toxins

Venoms used in this study were from different sources. *Micrurus tener* (02.16.09) and *M. fulvius* (08.31.10) venoms were from National Natural Toxins Research Center (Kingsville, TX, US); *M. laticollaris, M. browni, M. diastema, M. distans* venoms were obtained manually by venom extraction of adult snakes at the "Instituto de Biotecnología" (UNAM, Mexico, permit SGPA/DGVS/01953/13). Briefly, venoms were recovered using 20 mM ammonium acetate buffer, pH 4.7, centrifuged at 17,600 g to remove cellular debris, lyophilized and stored at 4 °C until use.

M. nigrocinctus, M. surinamensis, M. mosquitensis, M. mipartitus and *M. dumerilii* venoms were kindly donated by Mr. Neils Santos from Guatemala, Prof. Adolfo de Roodt (Universidad de Buenos Aires, Argentina), Prof. Bruno Lomonte (Instituto Clodomiro Picado, UCR, San José, Costa Rica), Dra. Ligia-Luz Corrales (Universidad de Antioquia, Medellín, Colombia) and Prof. Francisco Ruiz and Dra. Ariadna Rodríguez (Universidad Nacional de Colombia, Bogota, Colombia), respectively. The recombinant short-chain neurotoxin (ScNtx) was expressed and purified following the protocol published by de la Rosa. et al. (2019).

The freeze-dried venoms were dissolved in PBS and were centrifuged at 17,600 g for 10 min and the supernatant was collected. The protein concentration was determined by Bradford [18]. For venoms and ScNtx, bovine serum albumin, and for antivenoms, horse IgG were used to construct the standard curves.

2.2. Animals

Mice of CD-1 strain (18–20 g) were from the animal house facilities at the Instituto de Biotecnología, UNAM, México. Adult castrated male horses (400–500 kg) were maintained at Ranch "Ojo de Agua", Puebla, México, (Certified by SAGARPA, No. B00.02.01.02.01.3598/2018). All animals received regular veterinary supervision and were maintained under good conditions and controlled environments. They received water and food *ad libitum*. Proper animal handling, in order to minimize distress and discomfort, was always conducted towards maximizing the animal welfare during experimentation according to Mexican legislation for the use of laboratory animals (Norma Oficial Mexicana, 1999, NOM-062-ZOO-1999). Additionally, animal handling was according to the Animal Care guidelines from the Bioethics Committee at the Instituto de Biotecnología, which supervised and approved the animal work (Ethical approval CB/IBt/Project # 385).

2.3. Immunization and hyperimmune serum production

The production of the horse-derived monospecific anti-ScNtx immunoglobulins is described in de la Rosa *et al.* (2019). To obtain equine hyperimmune plasma against Texas coral snake, three horses were immunized with *Micrurus tener* venom (02.16.09). Horses were subcutaneously immunized at the neck using a low dose, low volume, multi-site immunization protocol [19,20], starting with 3.7 μg/horse and ending with 3 mg/horse. The first six immunizations were separated by seven days then fortnightly until day 196 (see supplemental material, Table S1). As adjuvants, incomplete Freund's adjuvant (IFA; Sigma) alternated with aluminum hydroxide and magnesium hydroxide (Imject®Alum; Thermo Fisher) were used until the 14^{th} immunization (day 140). No adjuvants were used in the last four immunizations corresponding to days 154, 168,182 and 196 (see supplemental material, Table S1). Finally, horses were bled (100 mL), and sera corresponding to days 168, 182 and 196 were pooled for neutralization assays. This pooled horse anti-*M. tener* serum was fractionated using caprylic acid, following the protocol recommended in the WHO guidelines for the production, control and regulation of antivenoms [21,22]. Then, the horse immunoglobulins anti-*M. tener* were prepared to a final 50 mg/mL solution in PBS. The protein content of the anti-ScNtx (also previously fractionated using caprylic acid) was 50 mg/mL. Finally, the Ab-blend was obtained by mixing both monospecific immunoglobulin solutions anti-*M. tener* and anti-ScNtx in a 1:1 v/v ratio. Then, the Ab-blend was dialyzed against 0.5X PBS and lyophilized in aliquots of one mL and kept refrigerated till they were used for neutralization assays. For neutralization assays, the aliquots were dissolved in 0.5 mL of distilled H_2O to render a solution containing 50 mg/mL each of the monospecific immunoglobulins in 1X PBS.

2.4. Electrophoretic analysis of Micrurus venoms

Qualitative analysis of *Micrurus* venoms was done by reducing SDS-PAGE analysis with 15% acrylamide gels according to the method proposed by Laemmli [23]. The protein staining was done with Coomasie Brilliant Blue R-250.

2.5. Median lethal (LD_{50}) and effective doses (ED_{50})

Briefly, groups of three mice were injected by intravenous route (IV) and the LD_{50} was obtained from the plot analysis of mice mortality (at 48 h after injection) versus venom dose used. The LD_{50} was expressed in μg/mouse. For neutralization experiments, 3 × LD_{50} of whole *Micrurus* venom or ScNtx were pre-incubated 30 min at 37 °C with varying volumes of either anti-*M. tener* or anti-ScNtx and then injected by IV route. After 24 h, median effective dose (ED_{50}) was calculated from the plot of survival percent (at 48 h) versus antiserum dose. ED_{50} is defined as the volume of antiserum able to protect 50% of the mice challenged [24]. Negative control groups tested with 400 μL (50 mg/mL) of purified horse pre-immune immunoglobulins caused no mortality. The ED_{50} was expressed in μg of purified antibodies/mouse. The software Prism 4 v. 4.0 (GraphPad, Inc., San Diego, CA) was used to calculate the data by non-linear regression. Experiments were carried out following the guidelines published by WHO involved in the production, control and regulation of venoms and antivenoms [25].

2.6. ELISA assays

Horse immunoglobulins were tested by enzyme-linked immunosorbent assay (ELISA) for the presence of specific antibodies against either ScNtx or *Micrurus* venoms as previously described [26]. Flat bottom 96 MicroWell™ polystyrene microtiter plates (Maxisorp Nunc) were coated with 2 μg/mL of either ScNtx or individual *Micrurus* venoms. Anti-ScNtx, anti-*M. tener* or Ab-blend (50 mg/mL) were diluted 1:30 in the first column and 1:3 serial dilutions were made in subsequent columns using vehicle buffer (50 mM Tris-HCl, 0.5 M NaCl, 1 mg/mL gelatin, 0.05% Tween 20, pH 8). Finally, plates were incubated with peroxidase-conjugated rabbit anti-horse immunoglobulins ($5x10^{-4}$ μg/mL, Rockland) and developed using peroxidase chromogenic substrate (soluble BM Blue POD substrate, Roche). The colorimetric response was measured at 450 nm, and the results were plotted using GraphPad Prism v.4.0 with non-linear regression analysis [19].

2.7. Statistical analysis

The least significant difference method was used to determine whether statistically significant differences occurred among the mean values obtained using the software package Prism 4 (GraphPad Prism, v. 4.0, California, USA).

3. Results and discussion

3.1. Protein composition of eleven Micrurus venoms

Coral snakes present a large distribution in The Americas, from Southern United States to Argentina [27]. Despite the low morbidity, their bites are considered hazardous and life threatening [11]. Therefore, it is a priority that every country has effective antivenoms. Thus, medically important toxins, like α-neurotoxins and neurotoxic PLA_2s, could underpin the development of a better targeted antidote with wider species efficacy. Neurotoxins in *Micrurus* venoms are low molecular weight proteins (6–15 kDa). These correspond to two major protein super-families: Phospholipases A_2 (PLA_2) and three-finger toxins (3Ftx). Fig. 1 shows the overall protein content by SDS-PAGE of the eleven *Micrurus* venoms studied here. Besides the protein composition already reported by proteomic and/or transcriptomic assays [28–34]; it could be observed that the venoms from *M. distans*, *M. fulvius, M. laticollaris* and *M. dumerilii* have relatively larger content of PLA_2s (**red box in** Fig. 1) than 3Ftxs in the other venoms. This observation agrees with that reported by Lomonte *et al.* [14], who classified them as PLA_2–predominant venoms. Also, it can be observed that the venoms from *M. tener*, *M. diastema, M. browni, M. nigrocintus* and *M. mosquitensis* contain relatively slightly higher amounts of PLA_2s than 3Ftxs. Finally, the venom from *M. surinamensis* contains significantly more three finger toxins than PLA_2s. According to Lomonte *et al.* [14], *M. mipartitus* and *M. surinamensis* are 3Ftx–predominant venoms. It is relevant to note that this classification does not consider which components are truly toxic to mammals.

3.2. Neutralization of Micrurus venoms by using anti-ScNtx, anti-M. Tener and Ab-blend

One recombinant type I α-neurotoxin and eleven *Micrurus* venoms from different origins were challenged against horse antibodies. Horse-derived immunoglobulins raised against *Micrurus tener* (PLA_2–predominant) do not neutralize the lethality of ScNtx, *M. distans, M. laticollaris, M. mipartitus* or *M. surinamensis*. On the other hand, they do neutralize seven of the tested venoms: *M. tener, M. diastema, M. fulvius, M. nigrocinctus, M. browni, M. mosquitensis,* and *M. dumerilii*. It is important to point out that the amount of anti-*M. tener* antibodies necessary to neutralize *M. tener* (ED_{50} = 184.7 μL/mouse or 9.2 mg/mouse, equivalent to 243 μg of venom neutralized by 1 mL) and *M. diastema* (ED_{50} = 159.1 μL/mouse or 7.9 mg/mouse equivalent to 113 μg of venom/mL) was higher than that to neutralize the other five venoms. Conversely, the horse-derived anti-ScNtx only neutralizes ScNtx and the venom of *M. surinamensis.* Neither of the antibodies from anti-ScNtx nor anti-*M. tener* could neutralize the venom of *M. mipartitus.* Interestingly, the Ab-blend neutralizes both the short-chain α-neurotoxins and ten out of eleven *Micrurus* venoms, as shown in table 1**.** It is crucial to notice that the Ab-blend neutralizes the venoms of *M. distans* and *M. laticollaris* that were not neutralized by monospecific anti-*M. tener* nor anti-ScNtx.

The improvement of neutralization potency of the Ab-blend (compared to the monospecific Abs) is closely related to venom composition, specifically, to the relative abundance of toxic SNtx or PLA_2s. The venom of *M. fulvius*, which has been shown to lack lethal SNtxs [34] is neutralized by the anti-*M. tener* and the combination of antibodies causes a decrease in neutralization potency to approximately half (Table 1; Ratio = 0.4). This is a consequence of the dilution of anti-*M. tener* neutralizing antibodies with those from anti-ScNtx, irrelevant for neutralization of this venom. The opposite occurs with the venom of *M. surinamensis*, whose lethality is driven only by 3Ftxs, mostly SNtxs [31] (Table 1; Ratio = 0.9].

Among the venoms hereby analyzed, those where the neutralization potency of Ab-blend represents an improvement over two fold (Table 1; Ratio > 2) are PLA_2-predominant. Nonetheless, they contain at least one SNtx that is relevant for overall lethality. This has been described in detail for *M. laticollaris* [29], *M. tener* [28] and *M. diastema* [35]. Conversely, venoms from *M. browni* [36], *M. nigrocinctus* [30] and *M. dumerilii* [32], which are also PLA_2-predominant, have no or very low amounts of lethal SNtxs, and therefore, the effect of antibody combination seems detrimental (Table 1; Ratio < 1). It is important to note that all the lethal SNtxs reported for the neutralized venoms share above 78% sequence identity with the consensus ScNtx (Fig. 2).

Given the fact that North and Central American coral snake anti-venoms like NACSAV (Wyeth, USA), Coralmyn® (Bioclon, Mexico) and *Micrurus* Polyspecific (ICP, Costa Rica) are based on PLA_2-predominant venoms, they could expand their effectiveness and species coverage with the inclusion of antibodies obtained from anti-ScNtx. For example, Castillo-Beltran *et al.* (2019) reported a Colombian polyspecific anticoral antivenom, which was developed using the blend of four monospecific antivenoms raised against the venoms from *M. dumerilii, M. mipartitus, M. isozonus* and *M. surina-*

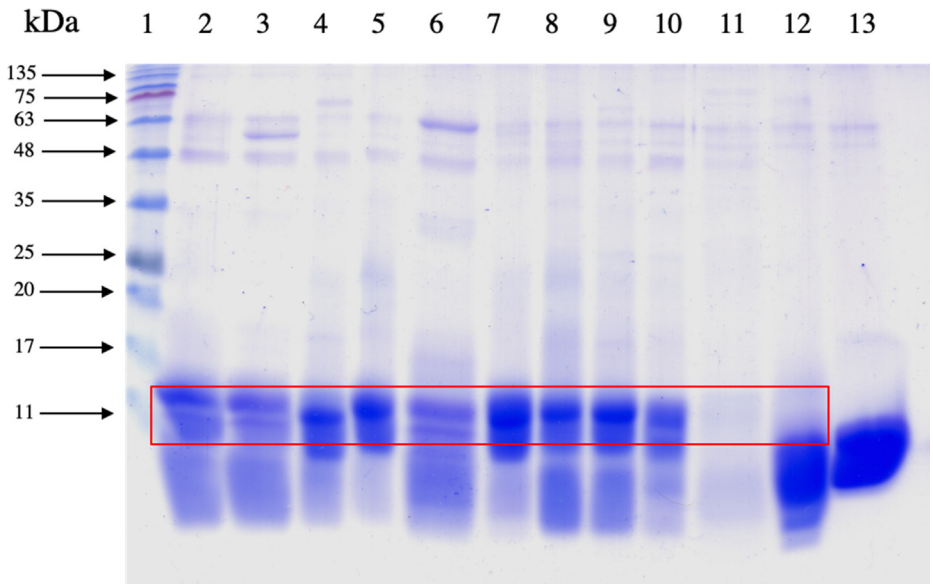

Fig. 1. 15% SDS-PAGE of *Micrurus* venoms under reducing conditions. 1. Molecular weight markers; 2. *M. tener* (10 μg); 3. *M. diastema* (10 μg); 4. *M. distans* (10 μg); 5. *M. fulvius* (10 μg); 6. *M. browni* (10 μg); 7. M. *laticollaris* (10 μg); 8. *M. nigrocintus* (10 μg); 9. *M. mosquitensis* (10 μg); 10. *M. dumerilii* (10 μg); 11. *M. mipartitus* (2 μg); 12. *M. surinamensis* (10 μg); 13. ScNtx (10 μg). The red rectangle shows the proteins of apparent molecular weight higher than 10 kDa, which most probably are PLA_2s. The proteins under 10 kDa are likely three finger toxins. The apparent molecular weight of ScNtx was taken as reference. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Median lethal (LD_{50}), effective dose (ED_{50}) and Ab-blend potency values of *Micrurus* venoms as well as median values of antivenom (EAv).

Venoms	LD_{50} μg/mouse (95% CI)	Anti-ScNtx ED_{50} in mg/mouse (95% CI)	Anti-*M. tener* ED_{50} in mg/mouse (95% CI)	Ab-blend ED_{50} in mg/mouse (95% CI)	Ratio	Ab-blend mg venom/mL AV (95% CI)
M. tener	15.0 (13.0–17.0)	NN[a]	9.2 (9.0–9.4)	2.6 (2.4–2.9)	**3.5**	1.7 (1.5–1.9)
M. diastema	6.0 (5.5–6.5)	NN[b]	7.9 (7.6–8.3)	2.6 (2.4–2.9)	**3.0**	0.7 (0.6–0.7)
M. distans	22.9 (19.0–27.6)	NN[a]	NN[a]	2.7 (2.6–2.8)	**> 3.7**	2.5 (2.4–2.6)
M. fulvius	4.0 (3.8–4.2)	NN[a]	0.7 (0.6–0.7)	1.4 (1.4–1.4)	0.4	0.9 (0.9–0.9)
M. browni	3.8 (3.5–4.1)	NN[a]	0.4 (0.4–0.4)	0.6 (0.4–1.0)	0.7	1.9 (1.1–2.8)
M. laticollaris	10 (9.1–11.4)	NN[b]	NN[b]	6.4 (6.2–6.7)	**> 3.1**	0.5 (0.4–0.5)
M. nigrocinctus	7.0 (5.0–9.0)	NN[b]	0.5 (0.4–0.6)	0.7 (0.4–1.2)	0.7	3 (1.7–5.2)
*M. mosquitensis**	< 20	NN[a]	1.8 (1.8–1.8)	0.8 (0.8–0.8)	**2.4**	2.5 (2.5–2.5)
*M. dumerilii**	< 20	NN[a]	0.7 (0.7–0.7)	1.0 (1.0–1.0)	0.7	2 (2–2)[c]
*M. mipartitus**	< 20	NN[a]	NN[a]	NN[a]	1	NN[a]
M. surinamensis	10 (9.5–10.5)	1.1 (1.0–1.2)	NN[a]	1.2 (1.0–1.6)	0.9	2.5 (1.9–3)
ScNtx	3.9 (3.8–3.9)	0.98 (0.95–1.0)	NN[b]	1.4 (1.4–1.5)	0.7	0.8 (0.8–0.8)

Anti-ScNtx, Anti-*M. tener* had a protein content of 50 mg/mL, and Ab-blend of 100 mg/mL.
*Because of the short supply of venoms, 20 μg of venom killed the mice (n = 3) in a period of 5 min, so the LD_{50} was set to < 20 μg/mouse; and mice were challenged with 20 μg of venom for calculating the ED_{50} determinations.
95% confidence intervals (CI) are shown in parentheses.
NN = no neutralization at a maximum of 10 (a) or 20 (b) mg of IgG (100% lethality).
3 × LD_{50} were used for ED_{50} determination, and for the mg venom/mL AV, except for *M. mosquitensis* and *M. dumerilii*, value used was 20 μg/mouse.
Ratio. ED_{50} of neutralizing monospecific (anti-ScNtx or anti-*M. tener*) serum over ED_{50} of Ab-blend. In the case of no-neutralizing sera, the maximum concentration tested was used to calculate ratio. Venoms with ratio > 1 are shown in bold.

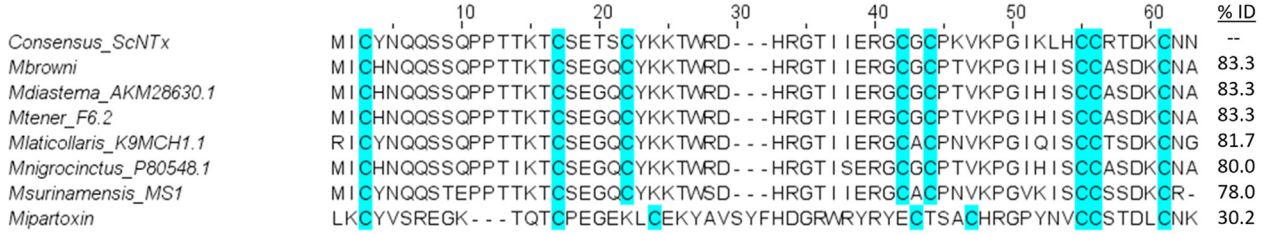

Fig. 2. Alignment of reported SNtxs from the *Micrurus* venoms analyzed in the present work. *% ID*. Percentage of sequence identity. Alignment performed in Jalview 2.10.4.

mensis. They showed that monospecific antisera, and the resulting polyspecific blend anti-serum, neutralized the species used as immunogens, and in a *para*-specific way the venoms of *M. medemi, M. lemniscatus* and *M. spixii* [37].

In this work, the venom of *M. mipartitus* was the only one not neutralized by any of the antibodies. This venom has been described to be 3Ftx-predominant [33]. The absence of neutralization may be explained by the low sequence identity of Mipartoxin-1 (30%), a 3Ftx which accounts for a lot of this venom's overall lethality [38], with ScNtx and with those type I α-neurotoxins present in the rest of the studied venoms (Fig. 2).

This result also provides a piece of evidence of the existence of at least two groups of SNtxs with different immunochemical properties in coral snake venoms: one compatible with the consensus ScNtx and another with Mipartoxin-1. The outcome is also in good agreement with Bolaños *et al.* (1978), who characterized a coral snake antivenom raised against *M. nigrocinctus* and *M. alleni*, and showed its inefficacy against *M. mipartitus* [39]. They then developed a monospecific *M. mipartitus* serum that cross-reacted against several venoms, suggesting that *M. mipartitus*, alongside *M. alleni* venom, is part of a unique serological group. This result gave way to a former Coral snake antivenom manufactured by The Instituto Clodomiro Picado [39]. So, here we suggest that *M. mipartitus* could be included as immunogen to improve the spectrum of efficacy of *Micrurus* antivenoms.

Regarding the ratio of the ED_{50} of neutralizing monospecific (anti-ScNtx or anti-*M. tener*) serum over ED_{50} of Ab-blend, an "antibody dilution" effect was observed for the venoms from *M. fulvius*, *M. browni*, *M. nigrocinctus*, *M. dumerilli*, *M. surinamensis* and the ScNtx. To avoid this, an alternative strategy could be the immunization of horses with mass concentration mixtures (1:1 or 1:2) of predominant-PLA_2 venoms (such as *M. tener* or other species) and three-finger toxins (such as ScNtx). However, this strategy does not warrant a high antibody response against α-neurotoxins, given their low immunogenicity. Furthermore, even with this effect the antivenom potency for the Ab-blend, measured as mg of venom neutralized per mL of antivenom, is better than some commercial antivenoms. For example, de Roodt et al. (2004) showed that 1 mL of an anti- antivenom (ANLIS, Argentina) could neutralize 0.05, 0.28 and 0.42 mg of *M. fulvius*, *M. nigrocinctus* and *M. surinamensis*, respectively. They also observed that Coralmyn® (Laboratorios Silanes, Mexico) could neutralize 0.49, 0.54 and 0.03 mg of the same venoms [24]. Additionally, Sanchez et al. (2008) reported that 1 mL of the North American Coral Snake Antivenom (NACSAV) (Wyeth, USA) could neutralize 0.24 and less than 0.08 mg of *M. fulvius* and *M. tener*, respectively, while the same volume of Coralmyn® neutralized 0.55 mg and 1.12 of the same venoms [40].

3.3. Recognition of Micrurus venoms and ScNtx by anti-ScNtx, anti-M. Tener and Ab-blend using ELISA

In order to support the previous observations, recognition of three-finger toxins and phospholipases A_2 within the venoms by anti-ScNtx, anti-*M. tener* and Ab-blend antibodies were evaluated (Fig. 3). The concept of IC_{50} is the concentration of an inhibitor where the response (or binding) is reduced by half. Here, the titers (IC_{50}) represent the serum concentration (μg/mL) required to obtain half of the colorimetric response. It is important to note that ELISA experiments allow the recognition of structural epitopes. In other words, the titers represent the needed concentration of antibodies (IC_{50} in μg/mL) to recognize structural similarities in short-chain α-neurotoxins related to ScNtx (for the anti-ScNtx), or structural similarities in 3Ftxs, PLA_2 or even other type of enzymes different to PLA_2 related to the venom components of *M. tener* (for the anti-*M. tener*). Table 2 summarizes the IC_{50} values.

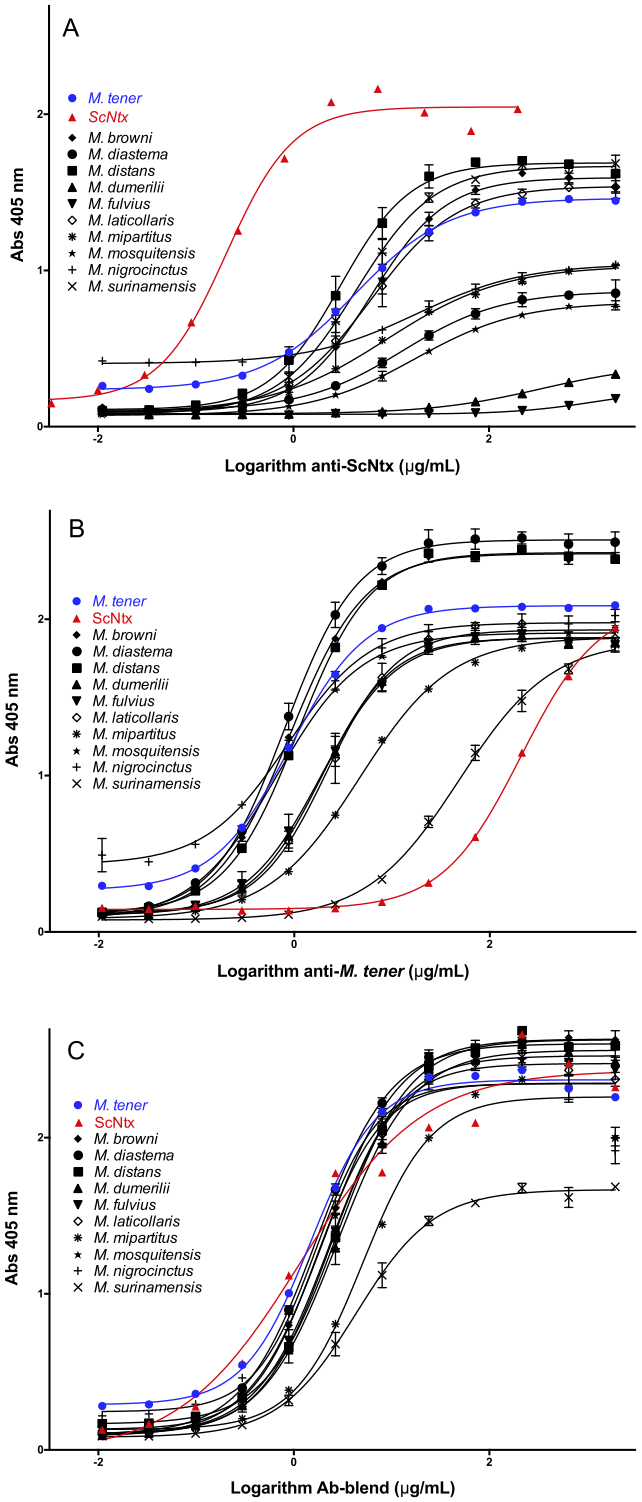

Fig. 3. Antibody titers against ScNtx and *Micrurus* venoms. A. Anti-ScNtx; B. Anti-*M. tener*; C. Ab-blend. Each point represents the average of two values.

Although ELISA assays are far from being used to predict antivenom neutralization, interesting data could be inferred from them. In our analysis, antibody recognition experiments strongly correlate with the neutralization assays. The venom of *M. fulvius*, whose lethality is driven by PLA_2s, is not recognized by the anti-ScNtx; on the other hand, it is recognized by anti-*M. tener* and Ab-blend. *M. surinamensis* represents the opposite case, being recognized by the anti-ScNtx and the Ab-blend; here, the use of Ab-blend repre-

Table 2
Summary of IC_{50} values for recognition of ScNtx and *Micrurus* venoms by antibodies from anti-ScNtx, anti-*M. tener* and Ab-blend.

	IC_{50} (μg/mL)		
Venom/Toxin	Anti-ScNtx	Anti-*M. tener*	Ab-blend
M. tener	4.2 (3.9–4.6)	0.9 (0.8–0.9)	1.4 (1.2–1.7)
M. diastema	13 (10.6–16.0)	0.8 (0.7–0.8)	1.7 (1.5–1.8)
M. distans	2.9 (2.5–3.5)	1.0 (0.9–1.1)	2.8 (2.6–3.0)
M. fulvius	1,233* (247–6,147)	1.8 (1.6–2.1)	2.3 (2.0–2.6)
M. browni	6.2 (5.1–7.6)	0.9 (0.8–0.9)	2.0 (1.9–2.1)
M. laticollaris	6.2 (5.7–6.8)	2.1 (1.8–2.5)	2.3 (2.0–2.5)
M. nigrocinctus	18.3 (14.5–23.0)	0.8 (0.7–1.0)	1.7 (1.2–2.3)
M. mosquitensis	17.5 (15.5–19.8)	0.6 (0.6–0.7)	1.7 (1.3–2.2)
M. dumerilii	341* (200–581)	1.9 (1.7–2.0)	2.7 (2.5–3.0)
M. mipartitus	9.0 (7.6–10.6)	4.6 (4.3–5.0)	5.0 (4.1–6.2)
M. surinamensis	4.3 (3.9–4.9)	48 (43–54)	4.3 (3.8–4.8)
ScNtx	0.2 (0.15–0.27)	208* (191–228)	1.1 (0.4–2.9)

95% confidence intervals (CI) are shown in parentheses. *Saturation was not reached in the dose–response curves (Fig. 3), so these values are only estimations of the IC_{50}s.

sents no neutralization improvement. The recognition of the venoms of *M. tener*, *M. distans* and *M. laticollaris* by the anti-ScNtx antibodies (Table 2) further confirms the presence of SNtxs with immunochemical similarities to the consensus ScNtx. This result supports our previous observation stating that, for these venoms, neutralization improvement upon antibody combination (Table 1) is a result of the better recognition and neutralization of lethal SNtxs and PLA2s. Conversely, the venoms of *M. nigrocinctus* and *M. dumerilli* are poorly recognized by the anti-ScNtx (Table 2), confirming the low abundance of such components, which accounts for the lack of neutralization improvement when using the Ab-blend (Table 1). For *M. browni, M. diastema* and *M. mosquitensis*, the relation between recognition and neutralization is less obvious, most likely due to the recognition of several venom components with little or no role in overall venom lethality.

Although the venom of *M. mipartitus* is recognized by antibodies from all sera, this could be due to the recognition of non-toxin 3Ftxs and PLA_2s, immunochemically similar to the ones present in *M. tener*. Another possibility is that antibodies are recognizing the most abundant components but have no effect on their neutralization.

4. Conclusion

In this work, we describe the efficacy of the combination of two types of horse-derived antibodies to improve the neutralization potency against *Micrurus* venoms. We have shown that the pool of antibodies that recognize SNtxs (anti-ScNtx) and antibodies that distinguish majorly PLA_2s (anti-*M. tener*) expand the neutralization towards *Micrurus sp*. To the best of our knowledge, it is unknown (not available in literature) if currently there are antivenom companies either public or private that perform a blend of different lots of antibodies, or if there is any regulation for performing this kind of lot mixtures. This report provides a proof-of-principle for taking advantage of recombinant immunogens to enrich poor or non-neutralizing antisera for developing commercial antivenoms with higher effectiveness and broader neutralizing capacity.

Author contributions

IA performed neutralization experiments and ELISAs. GR performed neutralization experiments and data analysis. FO performed the purification of IgGs from horse sera and executed the SDS-PAGE gel. AC performed the immunization of horses with *M. tener* venom. GR, AA and GC contributed to the study concept. GR, MBV and GC analyzed the data. AA and GC contributed to sample and funding acquisition. GR, MBV, AA and GC reviewed, edited and wrote the final manuscript. All authors discussed the results, contributed to critical revisions and approved the final manuscript.

Declaration of Competing Interest

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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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2020.12.052.

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