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BASIC RESEARCH

Cross neutralization of coral snake venoms by commercial Australian snake antivenoms

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ABSTRACT

Context: Although rare, coral snake envenomation is a serious health threat in Brazil, because of the highly neurotoxic venom and the scarcely available antivenom. The major bottleneck for antivenom production is the low availability of venom. Furthermore, the available serum is not effective against all coral snake species found in Brazil. An alternative to circumvent the lack of venom for serum production and the restricted protection of the actually available antivenom would be of great value. We compared the Brazilian coral snake and mono and polyvalent Australian antivenoms in terms of reactivity and protection.

Methods: The immunoreactivity of venoms from 9 coral snakes species were assayed by ELISA and western blot using the Brazilian *Micrurus* and the Australian pentavalent as well as monovalent anti-*Notechis*, *Oxyuranus* and *Pseudechis* antivenoms. Neutralization assays were performed in mice, using 3 LD_{50} of the venoms, incubated for 30 minutes with 100 µL of antivenom/animal.

Discussion: All the venoms reacted against the autologous and heterologous antivenoms. Nevertheless, the neutralization assays showed that the coral snake antivenom was only effective against *M. corallinus*, *M. frontalis*, *M. fulvius*, *M. nigrocinctus* and *M. pyrrhocryptus* venoms. On the other hand, the Australian pentavalent antivenom neutralized all venoms except the one from *M. spixii*. A combination of anti-*Oxyuranus* and *Pseudechis* monovalent sera, extended the protection to *M. altirostris* and, partially, to *M. ibiboboca*. By adding *Notechis* antivenom to this mixture, we obtained full protection against *M. ibiboboca* and partial neutralization against *M. lemniscatus* venoms.

Conclusions: Our findings confirm the limited effectiveness of the Brazilian coral snake antivenom and indicate that antivenoms made from Australian snakes venoms are an effective alternative for coral snake bites in South America and also in the United States were coral snake antivenom production has been discontinued.

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Introduction

Coral snakes are the main representatives of the elapid family in the Americas.[1] Despite the fact that bites are rare, accidents caused by these snakes are considered dangerous, due to the high toxicity of the venom that contains a lethal combination of pre- and post-synaptic neurotoxins. The major symptoms of envenomation by coral snakes are caused by progressive neuromuscular blockade which can lead to respiratory paralysis and death.[2]

As with other snake accidents, antivenom is the most specific treatment for coral snake envenomation. In Brazil, most of the accidents are caused by *Micrurus corallinus* and *M. frontalis,* which inhabit highly populated regions of the country.[3,4] The only commercial elapidic antivenom available is the one produced by Instituto Butantan or Fundação Ezequiel Dias using a mixture of equal amounts of venoms from the aforementioned species. There is evidence, however, that this antivenom is not efficient against other Brazilian *Micrurus* spp. venoms.[5] Although the inclusion of venoms from other species would be an alternative, due to characteristics such as fossorial habit and ophiophagous diet, it is very challenging to obtain and keep these snakes in captivity, with survival rate rarely exceeding one year.[6] Likewise, *Micrurus* coral snakes have an average dry venom yield of 14 mg/extraction,[7] which results in the need of snake collections comprised of numerous specimens in order to obtain sufficient amounts of venom for horse immunization. These limitations in maintenance, the small size of

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venom glands and the low venom yield, have been the major factors jeopardizing the production of the Brazilian elapidic antivenom. In fact, the quantity of venom available for antivenom generation is not enough to supply the national demands.

Moreover, the situation in the United States is far more critical. Since 2003, ANTIVENIN®, the only antivenom licensed in the U.S. for local coral snake bites is no longer manufactured by Wyeth pharmaceuticals, leading to the situation where patients are being intubated and ventilated while the toxin wears off, increasing the morbidity and mortality.[8–11] Although FDA has approved to continue using the expired antivenom, at some point, it will no longer be available. The development of an efficient and broad spectrum coral snake antivenom would, therefore, be a solution for this neglected health threat on both sides of the equator.

Several studies have shown that elapids from other parts of the world share related venom components with coral snakes.[12–15] As a result, it sounds reasonable to assume that an antivenom raised against another elapid venom might present some cross-reactivity with coral snake venom and, eventually, protect against its lethality. Indeed, Wisniewski et al. [16] showed that the Australian commercial tiger snake (*Notechis scutatus*) antivenom prevented death from North American *Micrurus fulvius fulvius* venom in a murine model. If this cross-neutralization extends to other coral snake venoms, the limitations in antivenom production would be eliminated, as Australian elapids are easy to keep in captivity and yield far more venom. Since the Australia/New Guinea polyvalent snake antivenom (produced by CSL Limited) contains antibodies not only against the venom of *Notechis scutatus*, but also to other four Australian elapids (*Pseudechis australis*, *Pseudonaja textilis*, *Acanthophis antarcticus* and *Oxyuranus scutelatus*), it is conceivable that this commercial antivenom would present some cross reactivity against American (North and South) elapidae snakes, comprised by *Micrurus* spp. It would be an enormous accomplishment if this hypothesis was confirmed, culminating in the development of an alternative and efficient elapidic antivenom for the treatment of coral snake envenomation from both sides of the hemisphere.

In the present work, we describe that a commercial elapidic antivenom or a formulation composed at least by a mixture of antivenoms against the venoms of the Australian elapids *Oxyuranus scutellatus*, *Pseudechis australis* and *Notechis scutatus* efficiently neutralizes most of the American coral snake venoms.

Material and methods

Venoms

Nine different *Micrurus* venoms were used for this work. Venoms from *M. frontalis*, *M. corallinus*, *M. lemniscatus*, *M. altirostris*, *M. spixii*, *M. ibiboboca* were provided by Instituto Butantan. Venoms from *M. fulvius*, *M. pyrrhrocryptus* and *M. nigrocinctus* were from the bank of venoms of the Laboratory of Toxinopathology (Labtoxpat) of the Faculty of Medicine – University of Buenos Aires.

Antivenoms

Five different equine $F(ab')_2$ antivenoms were used for this work: the Brazilian coral snake antivenom, obtained from Butantan Institute, SP, Brazil; the Australian polyvalent antielapidic antivenom (CSL's ANG Polyvalent Snake Antivenom, manufactured by CSL Limited, Australia); individual antielapidic sera against the venoms of *Oxyuranus scutellatus*, *Pseudechis australis* and *Notechis scutatus,* manufactured by CSL Limited, Australia.

Electrophoresis and western-blot

Samples of 20 μg of *Micrurus* venoms were solubilized in sample buffer and run in four different 15% SDS-PAGE gels and were transferred to nitrocellulose membranes (GE Healthcare, Piscataway, NJ) using a semidry transfer system and a current of 0.85 mA/cm^2. Following transfer, the membranes were stained with Ponceau red and photographed (see Supplementary Material). After an overnight blocking at 4 °C with 10% (v/v) nonfat dry milk diluted in PBS-Tween (0.5% Tween 20 in Phosphate Buffered Saline, PBS-T), each of the membranes were incubated for 90 min, at constant agitation and at room temperature, with a 1:3000 dilution of one of the antisera. Free, nonbound primary antibodies were removed with three times wash for 30 min in PBS-T. Goat anti-horse IgG-HRP antibodies (Sigma Aldrich) were used as secondary antibody at a dilution of 1:5000. Membranes were incubated with ECL Prime® detection reagent (GE Healthcare) according to manufacturer's instruction.

Enzyme linked immunosorbent assay (ELISA)

After an overnight incubation at 4 °C with 100 μL of *Micrurus* venoms diluted in Carbonate-Bicarbonate Buffer, pH 9.6, for a final concentration of 10 μg.ml^{-1}, 96-well microtiter plates were blocked with 5% Bovine Serum Albumin (BSA), diluted in PBS-T Buffer. Increased dilutions of each antisera, starting at 1:2000 and using a twofold dilution factor, were added to the wells, followed by 1 h incubation at 37 °C. Bound antibodies were detected by a 1:5000 dilution of commercial peroxidase-conjugated anti-horse IgG (Sigma Aldrich). Detection was performed with 8 mg o-phenylelediamine (OPD) diluted in 20 mL of 0·2 M citrate-phosphate buffer, pH 5·0, in the presence of 10 μL of 30% H_2O_2. Reaction was stopped by adding 50 μL of 4M H_2SO_4 to each well. The absorbance was measured at 492 nm.

Animals and lethal dose challenge assays

Groups of five female Balb/c mice, weighting around 20 g (18–22g) were used for lethal dose challenge assays, in accordance with Butantan's animal handling ethical committee. Neutralizing capacity of antisera against lethal doses of *Micrurus* venoms was assessed by challenging each animal with three LD_{50} of each venom previously incubated for 30 min at 37 °C with either antivenom or physiological saline alone., at a 1:1 (V/V) ratio. Each animal was injected with

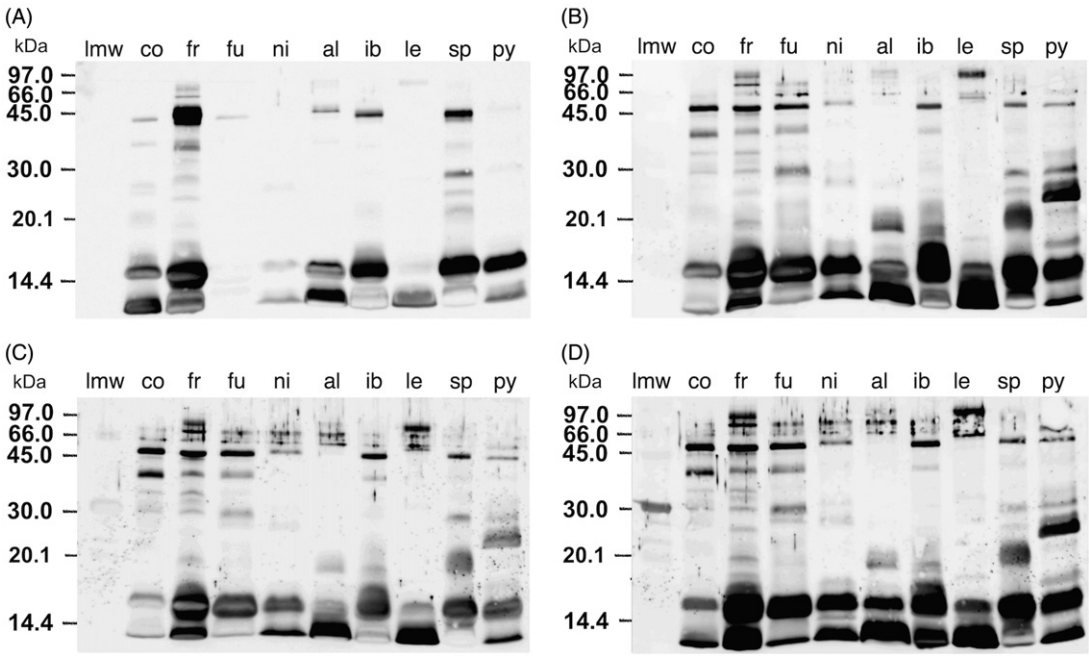

Figure 1. Western blot of the 9 venoms against (A) Butantan Institute antivenom; (B) CSL pentavalent antivenom; (C) *Oxyuranus scutellatus* + *Pseudechis australis* CSL antivenoms; (D) *Oxyuranus scutellatus* + *Pseudechis australis* + *Notechis scutatus* CSL antivenoms. co: *Micrurus corallinus*; fr: *Micrurus frontalis*; fu: *Micrurus fulvius*; ni: *Micrurus nigrocinctus*; al: *Micrurus altirostris*; ib: *Micrurus ibiboboca*; le: *Micrurus lemniscatus*; sp: *Micrurus spixii*; py: *Micrurus pyrrhochrytus*.

200 µL of a solution consisting of three LD_{50} dissolved in 100 µL saline, incubated with 100 µL antivenom or saline. The venom/antivenom mixture, for each group of five animals, was prepared as a single 1400 µL solution containing 21 LD_{50} dissolved in 700 µL physiological saline and 700 µL antivenom. Each animal was injected with 200 µL of this solution. The number and time of deaths was recorded until 48 h after the challenge. Lethal doses for each *Micrurus* venom were as follows: *M. altirostris*: 9 µg;[5] *M. corallinus*: 7 µg;[5] *M. frontalis*: 22 µg;[5] *M. fulvius*: 10 µg;[12] *M. ibiboboca*: 76 µg;[5] *M. lemniscatus*: 13 µg;[5] *M. nigrocinctus*: 17µg;[12] *M. pyrrhocryptus*: 26 µg [12] and *M. spixii*: 6 µg.[17] All values are expressed as LD_{50} per 20 g mouse.

Results

In order to investigate the potential of Australian antivenoms as a substitute of the Brazilian coral snake antivenom, the cross-reactivity of these antivenoms was assayed using nine different *Micrurus* spp. venoms as antigens (Supplemental Figure). The results obtained with this very first experiment were quite impressive since every single *Micrurus* venom tested were detected by antibodies present on the Australian antivenom (Figure 1(A)).

On the other hand, as expected, the reactivity obtained when testing the Brazilian coral snake antivenom was far more subtle (Figure 1(B)). This same immunochemical cross-reactivity could be observed in nonreducing and nondenaturing conditions, as venoms and sera were also tested in an ELISA assay (Figure 2) suggesting that the Australian polyvalent antielapidic sera could probably be able to neutralize lethal doses of all venoms we investigated.

Indeed, when these nine *Micrurus* venoms were submitted to an *in vivo* antivenom neutralization assay (Table 1), the results we obtained were evident showing the potential use of this commercial antivenom to treat *Micrurus* spp envenomation. Surprisingly, only one venom (*M. spixii*) was not neutralized. The lack of correlation between immunoreactivity and neutralization of this venom might be ascribed to qualitative and/or quantitative differences in venom composition.

Next, since the Australian polyvalent snake antivenom (CSL Limited) has antibodies to five different venoms and since individual antivenoms against two of these snakes (*O. scutelatus*, *P. australis*) were available, the cross-reactivity and neutralization using a mixture of these two antivenoms, which was named *OsPa* was investigated. The results obtained were also remarkable, with a cross-reactivity almost similar to the one observed previously with the polyvalent antivenom (Figure 1(C)) combined with an almost identical neutralization capability. The *OsPa* mixture did not neutralize lethal doses of *M. lemniscatus* venom and showed a partial neutralization of *M. ibiboboca* venom (Table 1).

Finally, as a *Notechis scutatus* antivenom was already described as presenting neutralizing effects against *M. fulvius* venom,[16] this antivenom was added to the *OsPa* formulation in order to investigate if this addition would be sufficient to restore the neutralization capability observed with the commercial polyvalent one. This mixture, which was named *OsPaNs*, displayed a cross-reactivity that clearly resembles the one observed when using the CSL pentavalent antivenom (Figure 1(D)). However, this formulation was still not sufficient to duplicate those first neutralizing effects, providing only partial protection against *M. lemniscatus* (Table 1).

Discussion

Distributed throughout the tropical and subtropical regions around the world, the Elapidae family consists of 325

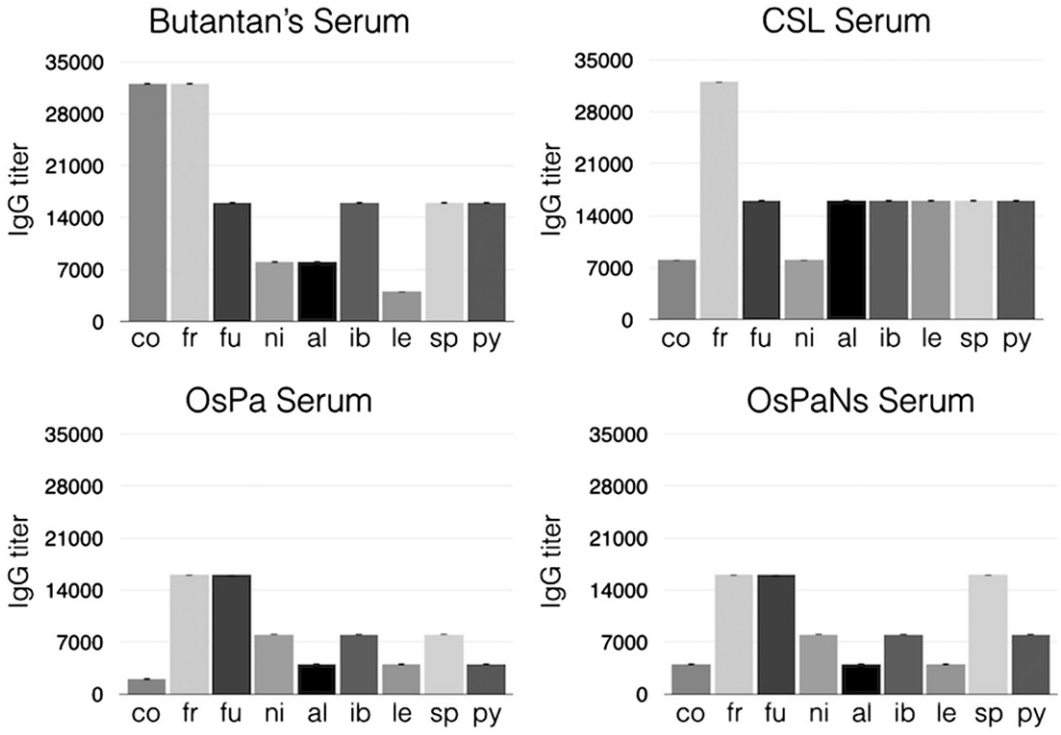

Figure 2. Titration of the antivenoms by ELISA. The titer was established as the highest antivenom dilution, in which OD 492 nm value were >0.1. Assays were carried out in triplicate. co: *Micrurus corallinus*; fr: *Micrurus frontalis*; fu: *Micrurus fulvius*; ni: *Micrurus nigrocinctus*; al: *Micrurus altirostris*; ib: *Micrurus ibiboboca*; le: *Micrurus lemniscatus*; sp: *Micrurus spixii*; py: *Micrurus pyrrhochrytus*.

Table 1. Neutralization assay against different *micrurus* spp. snake venoms.

	Serum used for venom neutralization				
Snake venom	Serum from naïve mice	*Butantan antielapidic* serum	CSL antielapidic serum	OsPa serum	OsPaNs serum
M. corallinus	5/5	0/5	0/5	0/5	0/5
M. frontalis	5/5	0/5	0/5	0/5	0/5
M. fulvius	5/5	0/5	0/5	0/5	0/5
M. nigrocinctus	5/5	0/5	0/5	0/5	0/5
M. pyrrhocryptus	5/5	0/5	0/5	0/5	0/5
M. altirostris	5/5	5/5	0/5	0/5	0/5
M. ibiboboca	5/5	5/5	0/5	3/5	0/5
M. lemniscatus	5/5	5/5	0/5	5/5	2/5
M. spixii	5/5	5/5	4/5	4/5	[a]

OsPa: *Oxyuranus scutellatus* + *Pseudechis australis antivenom*; Ns: *Notechis scutatus*.
[a]Neutralization of OsPaNs antivenom was not performed against *M. spixii* venom, as CSL serum itself did not provide protection.
Results are shown as total deaths/number of animals per group. For this assay, before intraperitoneal injection, 21 LD_{50} of venom diluted in 700 µL saline were incubated, for 30 min, with the same volume of serum. Each animal was injected with 200 µL of this mixture. Animals were monitored every 6 h for a total of 48 h.

potentially lethal neurotoxic proteroglyphous snakes that exhibit a wide range of sizes. In the Americas, the elapids are represented by different species of coral snakes (genera *Micrurus, Leptomicrurus* and *Micruroides*), which are not aggressive and, for most species, brightly colored with red, yellow or white, and black rings. Despite the low incidence of accidents involving these snakes, envenomation with members of this family do occur and can be severe or even lethal due to the high neurotoxicity of their venom, which induces peripheral nervous depression in ways similar to curare poisoning, with muscle paralysis and vasomotor instability.[2]

As with any snakebite envenoming accident, the treatment for coral snake bites involves the administration of antivenom immunoglobulins generated by horse immunization with snake venoms.[18,19] However, due to limitations in captivity maintenance [6] and low venom yield,[7] the production of the Brazilian elapidic antivenom is jeopardized and its availability is not enough to supply the national needs. Furthermore, the situation in the United States is far more critical: due to its very limited commercial value; since 2003, the production of the only FDA licensed antivenom (ANTIVENIN®, Wyeth Pharmaceuticals) was interrupted, leading to registered cases of patients needing to be intubated and ventilated as circulating toxins wear off.[9,11] This antivenom shortage is not restricted to the American continent. In a recent statement, Doctors Without Borders (MSF) said stocks of Fav-Afrique, the antivenom used to treat bites from 10 different types of snake across Sub-Saharan Africa, will expire by June 2016 and there are no effective replacements. The drug's manufacturers, Sanofi Pasteur, stopped producing Fav-Afrique in 2014, saying competitors had priced them out of the market.[20]

Figure 3. Geographical distribution of the coral snake species which venoms were investigated in this study.

Antivenoms cross reactivity has been explored by several authors. Sherman and Minton [21] demonstrated the general principle of antivenom paraspecificity and even nowadays, Australian sea snake antivenom is used for most sea snake bites around the world, despite only two venoms being used for its manufacture. Kornhauser et al. [22] demonstrated in a series of *in vitro* experiments that the Australian tiger snake antivenom was able to neutralize the inhibition of twitches of the chick biventer cervicis nerve-muscle preparation caused by *Naja haje* (Egyptian cobra) venom. Referring specifically to coral snake venoms, Wisniewski et al. [16] demonstrated that an antivenom produced by horse immunization with the venom from *Notechis scutatus* (a.k.a. Australian Tiger Snake) was capable of neutralizing the lethality of *M. fulvius* coral snake venom in mice.

These results show, for the first time, that the Australian polyvalent snake antivenom, as produced by CSL Limited, Australia can neutralize lethal doses of venoms from *M. corallinus*, *M. frontalis*, *M. fulvius*, *M. nigrocinctus*, *M. pyrrhocryptus*, *M. altirostris*, *M. ibiboboca* and *M. lemniscatus* in the mouse model used. Most of these venoms, as described elsewhere,[5] could not even be neutralized by the Brazilian coral snake antivenom. According to these data, a novel antigen formulation, using Australian snake venoms could be used to produce antivenoms for the treatment of coral snake bites, circumventing the chronical shortage of antivenom in Brazil and other countries. However, the value of these neutralization assays must be viewed with caution since they were performed using a mouse model as described. Although neutralization of other toxic activities of elapid venoms could

be determined such as myotoxicity or hemolysis,[23–26] these activities were only experimentally observed in some species of *Micrurus* [26] and were not clinically observed in envenomation caused by Brazilian coral snakes.[27,28] The neutralizing effects observed in this animal model need to be tested in human clinical trials for safety and efficacy for coral snake envenomation as standard procedure for any new need to be tested in human clinical trials for safety and efficacy for coral snake envenomation, a standard procedure for any new.[29] Indeed, despite the usefulness of the neutralization assays for the determination of preclinical utility of the antivenoms,[23] only well designed clinical assays can give information enough on their clinical effectiveness.[30]

Conclusions

In the present work, we show that Australian antivenoms, besides their use for the treatment of Australian snake envenomations and their effectivity against *Naja haje venom*,[22] could become an effective alternative for the treatment of Pan-American coral snake bites (Figure 3), a milestone toward a universal antielapidic antivenom. However, safety, clinical and efficacy studies have to be performed prior to its use for coral snake envenomations.

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Disclosure statement

The authors report no declarations of interest.

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