Effectiveness of Two Common Antivenoms for North, Central, and South American *Micrurus* Envenomations

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ABSTRACT

*Micrurus* snakes (coral snakes) may produce severe envenomation that can lead to death by peripheral respiratory paralysis. Only few laboratories produce specific antivenoms, and despite the cross-reactivity found in some *Micrurus* species venoms, the treatment is not always effective. To test two therapeutic antivenoms against the venom of four species of *Micrurus* from Southern America, North of South America, Central America, and North America, the determination of the lethal potency of the venoms, the study of some biochemical and immunochemical characteristics, and the determination of the neutralizing activity of both antivenoms were studied. North American and South American antivenoms neutralized well venoms from *Micrurus* species of the corresponding hemisphere but displayed lower effectiveness against venoms of species from different hemispheres. It was concluded that the neutralization
INTRODUCTION

Accidents by venomous snakes represent a health problem worldwide that varies in severity in different regions, ranging from a sanitary problem of scarce significance in the North of Europe to a very important sanitary problem in the whole of Africa (1).

In America the snakes responsible for the highest number of envenomations in humans belong to the Viperidae Family (pit vipers, lance-headed vipers). Information from Argentina, Brazil, Colombia, Costa Rica, and Mexico indicates that these snakes are responsible for over 95% of the accidents (2–8).

The remaining small percentage of accidents by venomous snakes is due to the American Elapids, represented by the Genus Micrurus, Micruroides, and Leptomicrurus. Most of the accidents are due to Micrurus species, owing mostly to their distribution range (9), from Patagonia (M. pyrrhocryptus) to the United States (M. fulvius). The American Elapids are named coral snakes because of their bright red color that contrasts with the black, white, or yellow bands along their body.

Coral snake venoms are extremely toxic and the bite of Micrurus constitutes a medical emergency because of high risk of death due to their high neurotoxicity. These venoms produce loss of muscle strength and, in general, death by respiratory paralysis of peripheral origin in animals and humans. The neurotoxicity of these venoms can be produced by a postsynaptic action (alpha neurotoxins, e.g., M. frontalis), block of the end-plate receptors (alpha neurotoxins), and inhibition of evoked acetylcholine release by the motor nerve endings (presynaptic-like action e.g., M. coralinus), or, venoms that block endplate receptors (alpha neurotoxins) and depolarize the muscle fiber membrane (cardiotoxins or myotoxic phospholipases A2, i.e., M. nigrocinetus and M. fulvius (10). However, envenomation by these snakes is not frequent, and the accidents are uncommon for several reasons, among which we can summarize the following: Elapid venomous apparatus (proteroglyphous) is not as efficient as the viper’s (solenoglyphous) in venom delivery; the small size of the snake’s mouth and the inability to maintain a large aperture of the jaws hinders utility to bite a human; there is a need for some time to inject a high amount of venom by their unsealed venomous conducts; coral snakes are not aggressive, in fact they are rather shy snakes; and they live mostly underground. For these reasons, accidents by Micrurus are most common in snake handlers in serpentariums or in children in the forest, who take the snakes to play because of their nonaggressive character and very attractive colors. In the North of Argentina it is not unusual for children from the communities of the forest to play with coral snakes.

However, even if not frequent, accidents by Micrurus do happen and do constitute a medical emergency. As has been mentioned, venoms from Micrurus spp. are rich in α-neurotoxins that bind to alpha subunits of acetylcholine receptors at the myoneural plate, leading to muscular and respiratory paralysis and death (5,6,10,11). Although experimentally it has been documented that some Micrurus venoms may produce myotoxicity and local lesions (12–15), the paramount feature of the coral snake toxicity is the neurotoxicity, and all the cases are considered serious because of the high risk of death.

The only specific treatment for Micrurus envenomation is the application of the specific antivenom. Although Micrurus antivenoms are produced by several laboratories like Wyeth (United States), Bioclon (México), Clodomiro Picado Institute (Costa Rica), Butantan Institute (Brazil), and the Instituto Nacional de Producción de Biológicos (Argentina) (3,5,6,16–18), they are not always available for treatment, even for use in the same country, and sometimes its production is discontinued. This lack of regular availability is due to various technical causes, among which is the difficulty in the obtention of coral snakes for venom extraction (in some regions of South America it is necessary to hunt the snakes in the jungle); the demands of maintaining Micrurus snakes in captivity in good state of health and feeding (19); and the small
amount of venom that can be extracted per snake (20). These facts make the production of Micrurus antivenom more difficult than the production of viper antivenom.

If α-neurotoxins are responsible for the toxicity of these venoms and all coral snakes have this type of toxins (11) and considering that cross-reactivity among several venoms of Micrurus has been reported (21–24), it may be hypothesized that the use of a given Micrurus antivenom could be used to treat envenomation caused by other species of Micrurus. However such statement seems not to be entirely true, since lack of neutralization of venoms from Micrurus by antivenom produced with venom from snakes of the same country has been also reported (25).

In spite of the above, and as a first step to the obtention of an effective polyvalent anti-Micrurus antivenom to be used in several regions of America, we studied the neutralizing capacity of two antivenoms of therapeutic use against the venoms of Micrurus snakes of different regions.

One of the antivenoms was from North America and another from South America. Both antivenoms were tested against venoms from Micrurus species from North America (M. fulvius), Central America (M. nigrocinctus), and South America (M. surinamensis and M. pyrrhocryptus) in order to evaluate their neutralizing capacity.

MATERIALS AND METHODS

Venoms

Venoms from healthy specimens of M. fulvius (La Florida), M. nigrocinctus (Costa Rica), and M. surinamensis (Leticia, Colombia) were provided from the Bank of Venoms of Biocloín Institute, México DF. Venom from M. pyrrhocryptus (Argentina) was provided from the Centro Zootoxicológico de Misiones, Oberá, Misiones, Argentina. All the venoms were obtained by manual extraction, and immediately frozen at −20°C and lyophilized. The venoms were aliquoted and stored at −20°C until use.

Determination of Lethal Potency

Mice (CF-1 strain, 18–22 g, 5 to 8 animals per dose level) were injected by i.p. route with different amounts of venom in 0.15 M NaCl. From the number of surviving animals 48 h after the injection, the LD50 was calculated by non-linear regression using the combined Prism and Stat-Mate softwares (GraphPad, Inc., San Diego, CA). It was defined as the amount of venom that produce the death of 50% of the challenged mice (26,27).

Electrophoretic Study

Samples prepared under non-reducing conditions were separated on a vertical slab of 12.5% acrylamide gel using the discontinuous buffer system described by Laemmli (28). For molecular weight estimation of venom proteins, a kit of molecular weight markers (BioRad Broad Range) was run in the same gel. Gels were stained with Coomasie Brilliant Blue R (Sigma).

Double Immunodiffusion

Double immunodiffusions were performed in Petri dishes (10 cm) containing 1% Agarose (Sigma) in PBS pH 7.4 as described by Siles Villarroel (29). Wells (0.3 cm) were punched and filled with 10 μl of the different Micrurus venoms (concentration 1 mg/ml). The venoms were confronted against serial dilutions of the different antivenoms. After 48 h, Petri dishes were washed with 0.15 M NaCl, dried at 37°C, and immunocomplexes were stained with Amido Black (Sigma).

Antivenoms

The antivenoms used were Suero Anti-Micrurus, from the Instituto Nacional de Producción de Biológicos—A.N.L.I.S. ‘Dr. Carlos G. Malbrán,’’ Buenos Aires, Argentina (batch 111, expiration date June 20, 1999) with a protein content of 55 ± 2.5 mg/ml; and Coralmyn, from Instituto Biocloín, Mexico DF, Mexico (batch B-2D-06, expiration date October 16, 2004). The pharmaceutical presentation is lyophilized to be reconstituted in 5 ml of diluent, with a final protein content of 40 ± 1.5 mg/ml. Both antivenoms were F(ab')2 fragments of equine immunoglobulins with similar degree of purity.

Neutralization Assay

This assay was performed as suggested by the World Health Organization (26,27). CF-1 mice (18–20 g) were injected i.p. with 3.0 LD50 of each venom preincubated for 30 min at 37°C with different doses of each antivenom (six animals per dose) in a final volume of 0.5 ml. After 48 h deaths were recorded and the data
analyzed by nonlinear regression using the software Prism (GraphPad Inc., CA). The neutralizing capacity was expressed in microliters as the effective dose 50% (ED$_{50}$), that is, the antivenom dose which protects half of the injected mice (26,27) or as the milligram of venom neutralized per vial of antivenom (30).

**RESULTS**

The electrophoretic profile showed differences between the venoms. The venom from *M. surinamensis* showed few proteins over 20 kDa with strong stained bands under 14 kDa whereas the venoms from *M. nigrocinctus* and *M. fulvius* showed strong stained bands around 6.4–50 kDa with differences in intensity and mobility between both venoms (Fig. 1).

Double immunodiffusion showed that *Coralmyn* strongly recognized the venom from *M. nigrocinctus* and *M. fulvius* and did not recognize the venom of *M. surinamensis* (Fig. 2) or *M. pyrrhocryptus* (data not shown) venoms. On the other hand, Anti-*Micrurus* antivenom recognized the venom of *M. surinamensis* and weakly the venoms of *M. nigrocinctus* and *M. fulvius* (Fig. 2).

The lethal doses found for the different venoms were 0.85 mg/kg (confidence interval, CI, 0.75 to 1.11 mg) for *M. nigrocinctus* venom, 0.48 mg/kg (CI 0.45 to

![Figure 1. SDS-PAGE of venoms from different species of *Micrurus*. It was performed in a 12.5% Acrylamide/Bisacrylamide gel in not reducing conditions. (1) *M. surinamensis* venom (from left to right: 10, 20 and 30 μg); (2) *M. nigrocinctus* venom (20, 35 and 50 μg) and (3) *M. fulvius* (same as in *M. nigrocinctus*). The migration of the molecular weight markers is expressed in kDa in the right (Molecular weight markers BioRad Broad Range).](image1)

![Figure 2. Double immunoprecipitation (Ouchterlony method) in 1% agarose of the venoms of *M. fulvius*, *M. nigrocinctus* and *M. surinamensis* against Coralmyn (left side of the gels) and Anti-*Micrurus* (right side of the gels) antivenoms. The central wells were filled with 10 μl of a solution of 1 mg/ml of a) *M. fuvius* (*M.f.*), b) *M. surinamensis* (*M.s.*), or c) *M. nigrocinctus* (*M.n.*). The peripheral wells were filled with 10 μl of dilutions of the antivenoms (1/1, 1/2, 1/4 and 1/8 in NaCl 0.15 M). After 48 h the gels were dried and stained with Amido Schwarz.](image2)
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**Table 1.** Neutralizing capacity of the antivenoms on the lethal potency of the different *Micrurus* venoms.

| Antivenoms | Coralmy | Anti-Micrurus |
|------------|---------|---------------|
| Venoms     | ED<sub>50</sub> | Potency per vial (5 ml) | ED<sub>50</sub> | Potency per vial (5 ml) |
| *M. fulvius* | 39 μl (25–58) | 2.46 mg | 385 μl (360–412) | 0.25 mg |
| *M. nigrocinctus* | 63 μl (50–78) | 2.69 mg | 123 μl (114–131) | 1.4 mg |
| *M. surinamensis* | > 500 μl | < 0.15 mg | 36 μl (29–44) | 2.11 mg |
| *M. pyrrhocryptus* | > 500 μl | < 0.52 mg | 88 μl (82–95) | 2.95 mg |

The ED<sub>50</sub> (Effective Dose 50%) indicate the amount of antivenom required to protect 50% of mice challenged with 3.0 i.p. doses of each venom and it is expressed in microliters. The 95% intervals of confidence are indicated into brackets. The potency per vial expresses the amount of venom (in milligrams) neutralized by one vial of antivenom.

0.50) for *M. fulvius* venom, 0.38 mg/kg (CI 0.25 to 0.95) for *M. surinamensis* and 1.3 mg/kg (CI 0.6 to 1.9) for *M. pyrrhocryptus* venom.

The antivenoms showed different neutralizing capacity against the different venoms (Table 1). *Coralmy* was effective against venoms from *M. fulvius* and *M. nigrocinctus* (ED<sub>50</sub>s of 39 μl and 63 μl, respectively) but did not neutralize the venom from the South American *Micrurus* since the ED<sub>50</sub>s were over 500 μl of antivenom. On the other hand, Anti-Micrurus antivenom was effective in the neutralization of *M. surinamensis* and *M. pyrrhocryptus* venom (ED<sub>50</sub>s of 36 μl and 88 μl, respectively), but it showed lower neutralizing capacity against *M. fulvius* venom (385 μl) and *M. nigrocinctus* venom (123 μl), being necessary to duplicate the dose to neutralize *M. nigrocinctus* venom or to use tenfold the dose to neutralize *M. fulvius* venom when compared with the ED<sub>50</sub> determined for these venoms with *Coralmy* (Table 1).

The calculated neutralizing potency per vial of antivenom (30) for the Anti-Micrurus were 2.95 mg for *M. pyrrhocryptus*, 2.11 mg for *M. surinamensis*, 0.25 mg for *M. fulvius*, and 1.4 mg for *M. nigrocinctus* venoms. For *Coralmy* the potency per vial was 2.46 mg for *M. fulvius* venom and 2.69 mg for *M. nigrocinctus* venom.

**DISCUSSION**

The venoms showed differences in lethal potency and in electrophoretic pattern. A differential immunochemical reactivity of the venoms with the different antivenoms was also observed, which was consistent with the data obtained from the experiments of seroneutralization of lethality.

The lethal potencies of the venoms found in this study are close to those reported for other *Micrurus* species (31), and the electrophoretic profiles of these venoms in general have the major characteristics described for *Micrurus* venoms (31–34) with most of the Coomassie-stained material under 20 kDa.

The immunochemical assay showed that the antivenoms show greater reactivity toward venoms from geographically related snakes. The neutralization assays showed similar results. *Coralmy*, an antivenom widely used in North America and Central America, showed a good neutralization capacity against the venom of *M. nigrocinctus* (the venom used as immunogen) from Costa Rica and *M. fulvius* from La Florida (United States) but was not effective against venom of *M. surinamensis* (Colombia) or *M. pyrrhocryptus* (Argentina). On the other hand, Anti-Micrurus, an antivenin used in Argentina, was effective against the venoms of *M. surinamensis* and *M. pyrrhocryptus* (the latter used as immunogen), but it confers very low protection against venom from *M. nigrocinctus* and *M. fulvius*.

With the exception of *M. corallinus* venom, that possesses presynaptic neurotoxins in addition to the alpha neurotoxins (10,35,36), the principal toxic components in *Micrurus* venoms are the latter (10,24,37–40). For this reason, in Brazil an antivenom raised against venom of *M. frontalis* and *M. corallinus* is used (6,23,41). However, *M. corallinus* venom seems to be effectively neutralized by antivenoms raised against other venoms (22).

An important cross-reactivity among *Micrurus* venoms has been well described (22–24,43–46). Bolaños (22) proposed four antigenically related groups: 1) *M. fulvius*, *M. nigrocinctus*, and *M. carinicauda*, 2) *M. corallinus*, *M. frontalis*, and *M. spixii*, 3) *M. halleni* and *M. mipartitus* and 4) *M. surinamensis* venom as a separate group. In addition, Alape-Girón (42) described, by means of monoclonal antibodies, three antigenically related groups: 1) *M. nigrocinctus*, *M. fulvius*, *M. dumerili*, and *M. albicinctus*, 2) *M. frontalis* and *M. brasiiliensis*, 3) *M. alleni* and *M. spixii* that present features of groups 1) and 2) but with particular characteristics. Venoms from *M. surinamensis*, *M. corallinus*,...
M. ibicoca, M. hempritchi, M. lemniscatus, and M. mipartitus have characteristics that preclude their inclusion in any of the groups.

Owing to the fact that these venoms have important cross-reactivity, a PanAmerican anti-Micrurus antivenom raised with the venoms from M. frontalis, M. nigrocinctus, and M. fulvius was prepared in the past (22). This antivenom was effective for neutralization of venoms from several Micrurus species but was not useful for the treatment of M. surinamensis envenomation.

Several studies made in Brazil with venom from various species of Micrurus showed that they display significant cross-reactivity (23,24) but that the best neutralization is conferred by antivenoms generated against homologous venoms (23). In Brazil, as has been mentioned, an antivenom raised against venom of M. frontalis and M. corallinus is used. However, this antivenom seems to have a low neutralizing ability against the venom of M. altirostris (25), which is surprising considering that this species was considered years ago a subspecies of M. frontalis. In a past study, the antivenin used in Argentina displayed a good neutralizing capacity against 5.0 LD50 of the venom from M. pyrrhocryptus (ED50 around 80 μl) but had a lower neutralizing capacity on the venom of M. corallinus and M. balyocoriphus (formerly, M. mesopotamicus) since doses of 400 μl could not protect 50% of mice challenged with M. corallinus venom and the ED50 for M. balyocoriphus was around 350 μl (de Roodt, unpublished results). This is also surprising, considering that both snakes were considered few years ago to be subspecies of M. frontalis (5).

In this study it is shown that both antivenoms could neutralize venom of Micrurus species not used as immunogen. For example, Coralmylin and Anti-Micrurus antivenoms neutralized the heterologous M. fulvius and M. surinamensis venoms, respectively, with near half of the dose required to neutralize the homologous venom (see Table 1). However, neutralization of the venoms from the other hemisphere was very low. Two recent papers also found good neutralization of M. fulvius venom with antivenoms obtained by immunization of horses with the venom of M. nigrocinctus (45,46).

Unfortunately the scarcity of Micrurus venoms make study on the neutralizing potency of their specific and non-specific antivenoms difficult. However, special attention must be paid to the improvement of the available antivenoms to treat the Micrurus envenoming in several regions, as has been shown in this study and by the Brazilian experience (25).

Taking into account the technical difficulties involved in the production of antivenom to treat coral snake bites, and until a good polyvalent antivenom is available in the regions where it could be useful, special attention must be paid to the evaluation of the antivenoms used for treatment of a particular coral snake bite. In addition, complementary measures for treatment, such as the use of inhibitors of acetylcholinesterases and atropin, or the use of artificial respiration, have to be systematically evaluated (6,10,11,47–50).

At present, we are working on the development of a polyvalent experimental anti-Micrurus antivenom to be used therapeutically in the neutralization of venoms from Micrurus species of North, Central, and South America (51).

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