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In-depth immunorecognition and neutralization analyses of *Micrurus mipartitus* and *M. dumerilii* venoms and toxins by a commercial antivenom

Juan Diego Piedrahita [a], Ana Cardona-Ruda [b], Jaime Andrés Pereañez [b], Paola Rey-Suárez [b, c, *]

[a] Departamento de Toxicología, Facultad de Medicina, Universidad de Antioquia, Medellín, Colombia
[b] Grupo de Investigación en Toxinología, Alternativas Terapéuticas y Alimentarias, Facultad de Ciencias Farmacéuticas y Alimentarias, Universidad de Antioquia, Medellín, Colombia
[c] Centro de Investigación en Recursos Naturales y Sustentabilidad, Universidad Bernardo O'Higgins, Santiago, Chile

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ABSTRACT

In Colombia, the *Micrurus* genus comprises 30 species, including *M. mipar*titus and *M. dumerilii*, which are of major clinical relevance due to their wide geographical distribution and the number of snakebites inflicted by them. These neurotoxic envenomations are characterized by neuromuscular paralysis attributed to venom components such as three-finger toxins (3FTx) and phospholipases (PLA_2). Additionally, there is limited information available on the neutralizing coverage of commercially available antivenoms, underscoring the need to perform studies to assess the cross-neutralizing ability of these life-saving products. Therefore, we present an in-depth immunorecognition analysis by the anticoral-INS antivenom from Colombia on the *M. mipartitus* and *M. dumerilii* venoms. The antivenom cross-recognized the whole venoms and their components with different intensities. For instance, the antivenom showed better recognition on PLA_2s than on 3FTxs in both venoms. Moreover, at doses tested, the antivenom totally neutralized the lethal effect of *M. dumerilii* venom; however, it did not neutralize this effect induced by *M. mipartitus* venom and its main toxic components from the southwestern region of the department of Antioquia. Furthermore, the anticoral-INS antivenom displayed better cross-immunorecognition of PLA_2-predominant *Micrurus* venoms than of 3FTx-predominant *Micrurus* venoms. This highlights the need to include venoms from both types of venom patterns in the immunization mixture to produce antivenoms against coral snakes. Finally, our results suggest the need for further research to optimize the composition of immunizing mixtures for antivenom production and improve their efficacy against coral snake envenomation in Colombia and the Americas.

1. Introduction

Snakebite is considered by the World Health Organization (WHO) a Neglected Tropical Disease (NTD) with 5.4 million cases per year, of which 1.8 to 2.7 million result in envenomation, causing approximately 81,400 to 137,000 deaths and about triple cases of amputations [1,2]. It has been described as an event linked to rural communities in countries with low socioeconomic resources and with health systems where supplies and medical staffs are scarce [3].

In Colombia, 5421 snakebites were registered in 2022 [4]. They mainly affect male farmers of productive age, residents of rural areas, and belonging to the subsidized regime of the health system [4,5]. Historically, about 90–95 % of total cases are attributed to viperids, while *Micrurus* snakes (commonly called coral snakes) accounted for 1–2% of the cases [5,6]. Due to their wide geographical distribution and the number of snakebites caused, *Micrurus mipartitus* and *Micrurus dumerilii* are considered coral snakes of medical importance in Colombia [6,7]. The venom from these snakes can cause envenoming, resulting in death or prolonged hospitalization in intensive care units due to the neurotoxic

* Corresponding author. Grupo de Investigación en Toxinología, Alternativas Terapéuticas y Alimentarias, Facultad de Ciencias Farmacéuticas y Alimentarias, Universidad de Antioquia, Medellín, Colombia.
E-mail address: jessica.rey@udea.edu.co (P. Rey-Suárez).

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effects of the venom [6,7]. The main complication is generalized neuromuscular paralysis, which requires management with invasive mechanical ventilation [7–9]. Additionally, this approach represents a significant expense in health systems. Therefore, developing effective strategies for preventing and treating snakebite envenoming is crucial to minimizing the burden on healthcare systems.

The treatment approved for *Micrurus* snakebite envenoming is the intravenous administration of specific immunotherapy called anticoral antivenom [9,10]. In America, several countries have developed anticoral antivenom, including the National Institute for the Production of Biologics (ANPB) in Argentina, the Clodomiro Picado Institute (ICP) in Costa Rica, the Butantan Institute in Brazil, the Bioclon Institute in Mexico, as well as PROBIOL Laboratories (not currently available) and the National Institute of Health (INS) in Colombia [10,11].

The anticoral antivenom produced by PROBIOL laboratories recognized *Micrurus mipartitus and M. dumerilii venoms*, however, this antivenom neutralized the lethal effect of *M. dumerilii* but not that caused by *M. mipartitus* venom [12]. In contrast, the antivenom produced by the INS from Colombia has shown neutralization abilities against the venoms of *M. dumerilii, M. mipartitus, M. isozonus, M. surinamensis, M. lemniscatus, M. spixii*, and *M. medemi* [10]. However, the immunorecognition and specific neutralization of the venom and its lethal toxins from *M. mipartitus* and *M. dumerilii* by the anticoral-INS antivenom have not yet been explored.

Therefore, in this study, we aimed to assess the in-depth immunorecognition and capacity for neutralizing of the lethality by the commercial anticoral antivenom produced by INS from Colombia against the venom and toxins from the two most significant coral snakes in our country *M. mipartitus* and *M. dumerilii.*

2. Materials and methods

2.1. Venoms and antivenoms

The Serpentarium at the University of Antioquia (Medellín, Antioquia) provided the lyophilized venoms of five specimens of *M. mipartitus* and *M. dumerilii* from the southwestern region of the department of Antioquia. The venoms were stored at −20 °C until use. The study used the polyvalent anticoral antivenom (Anticoral-INS; lot No. 18AMP03; expiration date April 2022) produced by INS. The manufacturer declares that the anticoral antivenom is produced by immunization with *M. dumerilii, M. mipartitus, M. isozonus*, and *M. surinamensis* venoms.

2.2. Animals

Swiss-Webster mice of both sexes (18–20 g body weight) were used. The experiment followed the protocols approved by the institutional Committee for the Use and Care of Research Animals at the Universidad de Antioquia (License No. 110 of 2017).

2.3. Enzyme-linked immunosorbent assay (ELISA)

To evaluate the antigenic recognition of the anticoral-INS antivenom, a sandwich enzyme-linked immunosorbent assay (ELISA) was performed following the methodology proposed by Lomonte [13]. Initially, the antibody titers of the antivenom were assessed against the complete *M. mipartitus* and *M. dumerilii* venoms. For this, each well of the microplates (Falcon ref 35072) was coated with 0.1 μg of the complete venom diluted in 100 μL of coating buffer (0.1 M Tris, 0.15 M NaCl, pH 9.0) and incubated overnight at room temperature. The wells were then blocked with 100 μL of 1 % bovine serum albumin in phosphate buffer (0.04 M phosphates, 0.12 M NaCl, pH 7.2) for 90 min. Serial dilutions of the antivenom (1:500 to 1:256,000) were added and incubated for 90 min at room temperature. The plate was washed and a peroxidase labeled anti-horse IgG conjugate (Sigma-Aldrich) was added as a second antibody and incubated for 1 h and 30 min at room temperature. After washing, 100 μL of peroxidase substrate (2 mg/mL OPD diluted in 0.1 M sodium citrate, pH 5.0; 4 μL of 30 % H_2O_2 per 10 mL of final solution) was added. Finally, the absorbance was measured at 492 nm using a Multiskan sky spectrophotometer from Thermo Scientific (Waltham, MA, USA).

To evaluate the antigenic recognition of the anticoral antivenom (Anticoral-INS), a second experiment was conducted, wherein the components separated by RP-HPLC of each venom were used. To achieve this, 2 mg of each venom (*M. mipartitus* and *M. dumerilii*) were fractionated using a C18 RESTEK column (250 mm × 4.6 mm, particle size 5 μm; RESTEK) with protein detection at 215 nm on a Shimadzu 20A chromatograph. Elution was performed using a linear gradient of 0.1 % trifluoroacetic acid (solution A) and 99 % acetonitrile (solution B) following the methodology proposed by Lomonte and Calvete [14]. The gradient conditions were as follows: 0 % B isocratically for 5 min, 0–15 % B for 10 min, 15–45 % B for 60 min, 45–70 % B for 10 min, and 70 % B for 5 min at a flow rate of 1 mL/min. To identify the protein family present in the fractions of the resulting chromatograms, the elution times were compared and associated with those obtained and identified by mass spectrometry in previous studies [15,16]. 0.1 μg of each fraction was coated onto the plate and evaluated using a 1:1000 dilution of the antivenom, following the same protocol as described previously.

To analyze the antibody titers present in the anticoral-INS antivenom against the toxins responsible for lethality in each venom, specific fractions were obtained for RP-HPLC using the method proposed by Lomonte and Calvete [14]. For *M. mipartitus*, fractions Mm-8 and Mm-20 were used, which were previously reported as lethal proteins [15], while for *M. dumerilii*, fractions Md-7 and Md-21 were used [16]. The fractions were tested coated with 0.1 μg of each toxin, with different doses of anticoral-INS antivenom (1:500–1:4000) to assess their recognition and neutralizing capacity, following the same protocol.

In addition, the ability of the anticoral-INS antivenom to cross-recognize venoms from other *M. dumerilii, M. nigrocinctus, M. mosquitensis, M. clarki, M. mipartitus, M. alleni, and M. ancoralis* (These venoms from Central America were donated by the Clodomiro Picado Institute), venom was evaluated by using a 1:1000 dilution of the antivenom and following the same protocol.

2.4. Lethality neutralization assays

The ability of Anticoral-INS to neutralize the lethality of *M. mipartitus* and *M. dumerilii* venoms was evaluated by pre-incubation experiments (30 min at 37 °C). Groups of three mice per venom and dose level were used. The animals were injected intraperitoneally with 500 μl of a mixture of 27 μg of *M. mipartitus* venom or 67 μg of *M. dumerilii* venom (amount of venom equivalent to 3 LD_{50}; The LD_{50} reported in the literature was used) [16,17] and anticoral-INS antivenom in a proportion of 0.1 mg or 0.2 mg of venom per mL of antivenom. The control group received the same venom dose and was incubated only with PBS. Surviving mice were recorded after 48 h of observation. Finally, the neutralizing capacity of Anticoral-INS on the lethal toxins of *M. mipartitus* venom was evaluated, for this, 17.4 μg of Mm-8 and 2.5 μg of Mm-20 (equivalent to 3 LD_{50}) [18] were tested against the same proportion of anticoral-INS antivenom as previously described.

2.5. Statistical analysis

In all cases, the results were expressed as mean ± SD, in addition, the normality was evaluated using a Shapiro–Wilk test and analysis was performed with two-way ANOVA followed by a Bonferroni test for multiple comparisons. They were statistically significant when $p < 0.05$.

3. Results

3.1. Cross-recognition of M. dumerilii and M. mipartitus whole venoms and its lethal toxins by the anticoral-INS antivenom

Between dilutions from 1:500 and 1:2000 the anticoral-INS antivenom showed better recognition of *M. dumerilii* venom than *M. mipartitus* venom ($p < 0.05$). At other doses tested, the recognition was similar for both venoms, and it was maintained up to 1:128,000. The background signal level of the normal serum control (Non-immunized horse serum) was low (Fig. 1). Furthermore, the immunorecognition of anticoral-INS antivenom was performed on the lethal toxins previously identified in the *M. dumerilii* and *M. mipartitus* venoms. The Mm-8 and Md-7 toxins belong to 3FTx protein family (light gray) and Mm-20 and Md-21 for PLA_2 protein family (dark gray bars). The antivenom recognized all four mentioned proteins, but lower titers were detected against 3FTxs in both venoms (Fig. 2A and B).

3.2. Cross-recognition of M. dumerilii and M. mipartitus toxins by anticoral-INS antivenom

The toxins of *M. dumerilii* and *M. mipartitus* venoms were separated under the same conditions performed in the proteomic studies on the venoms of these species [15,16]. Moreover, the identity of each fraction was determined by comparing the retention times with those previously reported [15,16]. For the *M. mipartitus* venom, recognition of all the fractions tested was observed at a similar level for fractions 9, 10, 11, 12, and 15. Fraction 16 showed a slightly higher recognition than the others, while fractions 2, 3, 4, 5, 13, and 14 presented moderate signals, and finally, fraction 7 exhibited the lowest signal (Fig. 3). In contrast, the anticoral-INS displayed better recognition of all fractions of the *M. dumerilii* venom, only a marginally lower response was shown for fraction 12 (Fig. 3).

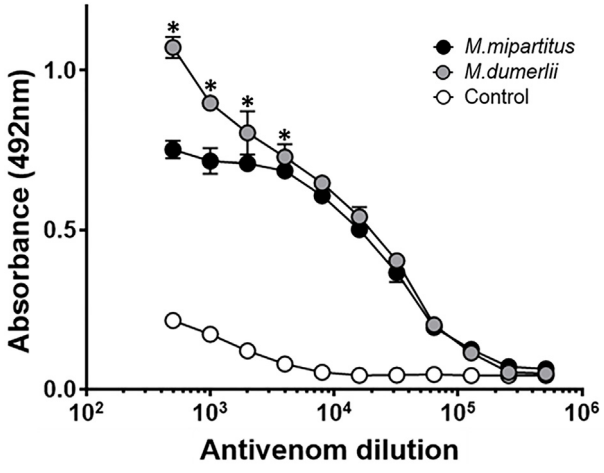

Fig. 1. Recognition of the anticoral-INS antivenom on the whole venoms of *M. mipartitus* and *M. dumerilii*. Venoms were coated onto ELISA microplates, and antibody binding was detected with peroxidase-labeled anti-horse IgG conjugate, as described in Materials and Methods section. Serum from a non-immunized horse was used as a negative control. Each point on the graph represents the mean ± SD of the triplicate. The significant difference between both venoms is shown with a * ($p < 0.05$).

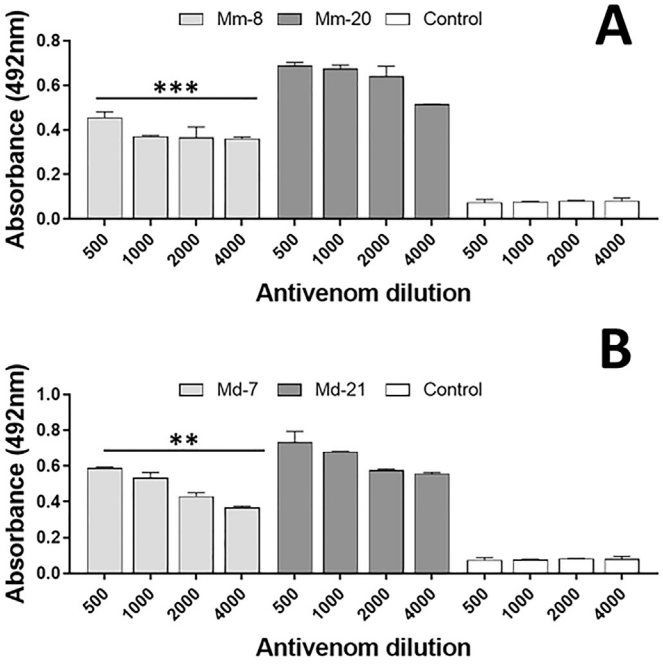

Fig. 2. Recognition of the anticoral-INS antivenom on the lethal fractions. (A) M. mipartitus venom (B) *M. dumerilii* venom. The lethal fractions were coated onto ELISA microplates, and antibody binding was detected with peroxidase-labeled anti-horse IgG conjugate, as described in Materials and Methods section. Each bar on the graph represents the mean ± SD of the triplicate. The significant difference between protein families is shown with a ** ($p < 0.001$) or *** ($p < 0.0001$). Bars in white color are negative controls (serum from a non-immunized horse), bars in light gray color for 3FTx family and bars in dark gray color for PLA_2 family.

3.3. Cross-recognition of different Micrurus venoms by anticoral-INS antivenom

In the same way, the cross-reactivity of the anticoral-INS antivenom on different *Micrurus* venoms was explored by ELISA. All included venoms were recognized by antivenom, showing significant statistical differences with non-immunized horse serum ($p < 0.05$). *M. dumerilii, M. nigrocinctus, M. mosquitensis* and *M. clarki,* venoms showed higher recognition than *M. mipartitus, M. alleni, and M. ancoralis* venoms (Fig. 4).

3.4. Neutralization of M dumerilii and M mipartitus and their toxins by anticoral-INS antivenom

The anticoral-INS antivenom neutralized the lethal effect of *M. dumerilii* venom in a proportion of 0.1 mg/mL and 0.2 mg/mL and, it was not able to protect mice against the *M. mipartitus* venom in any of the ratios evaluated. In addition, the antivenom did not neutralize the toxins responsible for the lethality of the venom of *M. mipartitus* (Table 1). The lethal fractions of the venom of *M. dumerilii* were not evaluated since the whole venom was neutralized by the antivenom.

4. Discussion

The neurotoxic effects observed in coral snakebites are potentially life-threatening; however, some geographical areas of the Americas have limited availability of coral antivenoms. The most

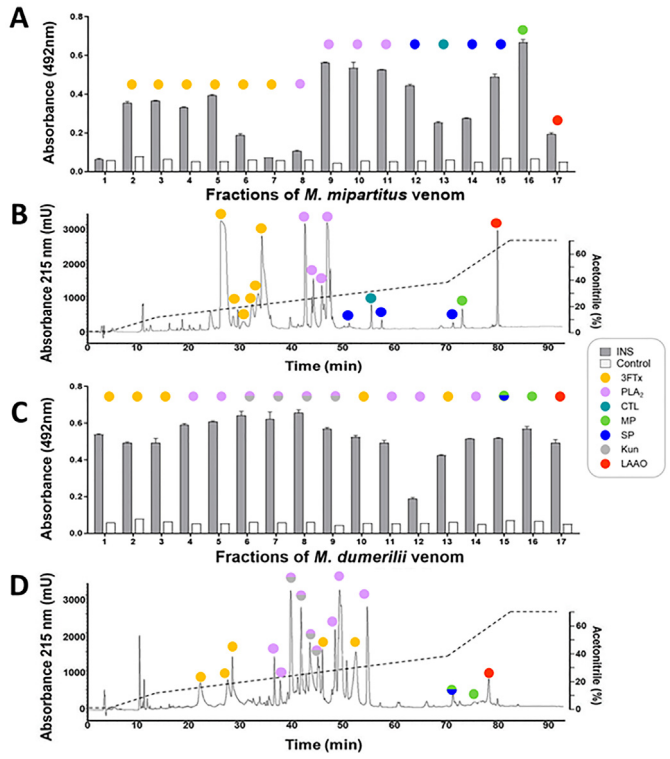

Fig. 3. Immunorecognition of the RP-HPLC fractions by the anticoral-INS antivenom by ELISA. A) Fraction of *Micrurus mipartitus* venom C) Fraction of *Micrurus dumerilii* venom. 96-well plates were coated with fractions of each venom as described in materials and methods; a 1:1000 dilution of the antivenom, peroxidase-labeled anti-equine immunoglobulins conjugate and OPD were used as substrates. Non-immunized horse plasma was used as a negative control. Each bar represents the mean ± SD of the triplicate. The position of venom fractions in the RP-HPLC profiles are shown for *M. mipartitus* (B) and *M. dumerilii* (D). The colored circles on the bars indicate the protein family to which it belongs, according to proteomic studies [15,16].

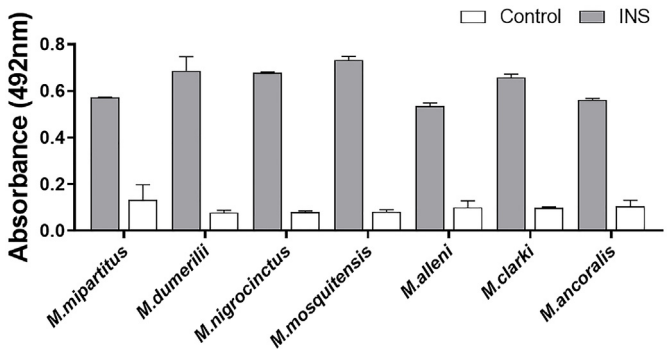

Fig. 4. Cross-recognition of different *Micrurus* venoms by the anticoral-INS antivenom. 96-well plates were covered with fractions of each venom as described in materials and methods; a 1:1000 dilution of the antivenom, peroxidase-labeled anti-equine immunoglobulins conjugate and OPD were used. Non-immunized horse plasma was used as a negative control. Each bar represents the mean ± SD of the triplicate.

important cause of the lack of availability is the scarce amounts of venom that *Micrurus* produce; nevertheless, it should also be considered the low survival in captivity and the difficulty to find them in the field, since they have semifossorial habits and some of them are small [19,20]. Despite the mentioned above, in recent years, the INS from Colombia developed an anti-coral antivenom, which considered the immunization process with *M. dumerilii, M. mipartitus, M. isozonus*, and *M. surinamensis* venoms [10]. Additionally, some authors have performed studies with continental commercial antivenoms to increase the knowledge on the immunological cross-recognition and neutralization of these products and suggest alternatives when specific antivenom is not available [12,21–23].

Table 1
Neutralizing ability of anticoral-INS antivenom on lethal effect of *M. mipartitus* and *M. dumerilii* venoms in mice.

GROUP	VENOM/ANTIVENOM	DEAD/INJECTED
M. mipartitus venom[a]	0	3/3
M. mipartitus + Anticoral-INS	0.1 mg/mL	3/3
M. mipartitus + Anticoral-INS	0.2 mg/mL	3/3
Mm-8[c]+ Anticoral-INS	0.1 mg/mL	3/3
Mm-20[c] + Anticoral-INS	0.1 mg/mL	3/3
M. dumerilii venom[b]	0	3/3
M. dumerilii venom + Anticoral-INS	0.1 mg/mL	0/3
M. dumerilii venom + Anticoral-INS	0.2 mg/mL	1/3

The lethality neutralizing ability of antivenom was evaluated by preincubating at 37 °C for 30 min, and then injecting the mixture in mice (18–20 g of body weight) by i.p. route. Deaths were recorded after 48 h.
[a] Venom challenge dose was 3 x LD_{50} (27 μg).
[b] Venom challenge dose was 3 x LD_{50} (67 μg).
[c] Toxins challenge dose were 17.4 μg of Mm-8 and 2.5 μg of Mm-20 (equivalent to 3 LD_{50}).

In this study, we explored the specific immunorecognition of RP-HPLC venom fractions of the *M. mipartitus* and *M. dumerilii* venoms by anticoral-INS antivenom, using an ELISA-based profiling for antibody binding. Despite the cross-recognition of the complete venom and major fractions of *M. mipartitus*, the antibody titers from anticoral-INS antivenom were comparatively lower in both whole venom ELISA and immunoprofiling-ELISA analyses compared to *M. dumerilii* venom. These results could be due to lower antibody concentrations or lower antibody affinities. Similar findings were reported by Rey-Suárez & Lomonte [12] when they tested PROBIOL antivenom from Colombia (available on that date) and SAC-ICP antivenom from Costa Rica on these two species. In addition, the anticoral-INS antivenom showed lower recognition against fractions containing 3FTxs (marked with yellow circles in Fig. 3), confirmed when antivenom immunorecognition on isolated 3FTxs and PLA_2s were assayed (Fig. 2A and 1B). The antivenom showed a lower signal on Mm-8 and Md-7, 3FTxs from *M. mipartitus* and *M. dumerilii*, respectively, when comparing the absorbances obtained with PLA_2s from both venoms. Several authors obtained similar results when they tested commercial antivenoms from Colombia and Costa Rica on *M. mipartitus*, *M. dumerilii*, *M. ruatanus,* and *M. yatesi* venoms [12,24,25].

The anticoral-INS antivenom neutralized the lethal effect of *M. dumerilii* venom in mice, However, at the doses tested, it did not neutralize the lethality induced by *M. mipartitus* venom. This result is similar to that reported with PROBIOL and SAC-ICP antivenoms [12,16]. For this reason, we decided to test the ability of the anticoral-INS antivenom to neutralize the previously reported lethal fractions Mm-8 and Mm-20 from *M. mipartitus* venom [15]. The anticoral-INS antivenom did not neutralize the lethal activity of the mentioned fractions, which are the main components that contribute to the lethal effect of the whole venom, as described by Cardona-Ruda et al. [18], who reported that the lethal effect of whole venom was only completely neutralized when a mixture of antibodies raised against the mentioned toxins is used. It is important to note that Mm-8, is a 3FTx named Mipartoxin-I, lethal, and the most abundant toxin of the *M. mipartitus* venom, accounting for nearly 28 % of its total protein content [26]. Therefore, antivenoms must ensure the neutralization of such components; otherwise, it will not be possible to neutralize the lethal effect of

the antivenom, as reported for the most abundant 3FTxs of *Naja samarensis*, which was weakly cross-neutralized by the Philippine cobra antivenom [27]. In addition, it has been reported that a significant divergence within 3FTxs occurs in *Micrurus* venoms, further contributing to low cross-recognition [28].

Proteomic analyses of *Micrurus* venoms have revealed a dichotomic compositional pattern, with some species containing more PLA_2s than 3FTxs, while in others 3FTx predominate [29,30]. *Micrurus* species inhabiting South America tend to express the 3FTx-predominant venom phenotype, while the PLA_2-rich pattern is observed in species inhabiting North America. Species found in Central America and northern South America present either of the two venom patterns [29,30]. This divergence in venom composition has serious implications for the efficacy of antivenoms, as venoms within the same dichotomic group display high cross-neutralization, unlike venoms across the two groups [29]. The anticoral-INS antivenom cross-recognized the *Micrurus* venoms tested. However, better results were observed with PLA_2-predominant venoms, such as *M. dumerilii, M. nigrocinctus* and *M. mosquitensis* [16,31,32]. In contrast, the antivenom showed lower recognition capacity against 3FTxs-predominant venoms, such as *M. mipartitus* and *M. alleni* [15,31]. Which agrees with the low activity against 3FTxs mentioned above. This highlights the need to include venoms from both types of venom patterns in the immunization mixture to produce antivenoms against coral snakes. The venom of *M. clarki* is considered a more equilibrated venom in the dichotomy compositional pattern [33]; however, the anticoral-INS antivenom recognized it in a similar way to PLA_2-predominant venoms. On the other hand, the proteome from the venom of *M. ancoralis* has not been published. Therefore, there is no information about which group it belongs.

The manufacturer declares that for anticoral-INS antivenom production, *M. mipartitus* venom is considered in the immunization process. Moreover, Castillo-Beltrán et al. [10] reported that this antivenom neutralized the lethal effect induced by *M. mipartitus* venom. In contrast, we informed herein that the mentioned antivenom did not neutralize the lethal activity of *M. mipartitus* venom and its main toxic components. These discrepancies may be explained by venom differences derived from different geographical origins of the venoms tested [34]. For instance, Castillo-Beltrán et al. [10], reported *M. mipartitus* from the Middle Magdalena Valley, while we used *M. mipartitus* venom from the southwestern region of the Antioquia department. These differences in geographical origins lead to the classification of two distinct subspecies: *M. mipartitus mipartitus* and *M. mipartitus decussatus*, as indicated by Campbell and Lamar [35]. However, to date, no studies have evaluated whether venom variation exists at the intra-specific level for this species. Further investigation into the venom composition of these sub-species could contribute to the development of more targeted and effective antivenoms. Also, but less likely, it is the influence of the strain of mice used for neutralization assays, Castillo-Beltrán et al. [10] used CD-1 ICR strain, while we used Swiss-Webster strain. Additionally, the liquid presentation of the antivenom did not allow for assays with higher antivenom concentrations, considering that the recommended maximum intraperitoneal administration limit in mice is 500 μL [36]. A experimentally lyophilized presentation of this antivenom would allow for an increase in the challenge doses and the ability to determine the EC_{50} of the antivenom for these venoms. As observed in the immunorecognition assays, the antivenom exhibits antibody titers against this venom. The differences noted here could likely be mitigated through an increase in the quantity of challenged antivenom.

In conclusion, we present an in-depth analysis of immunorecognition by the anticoral-INS antivenom from Colombia on the *M. mipartitus* and *M. dumerilii* venoms. The antivenom cross-recognized the whole venoms and their components with different intensities, exhibiting superior recognition of PLA_2 compared to 3FTxs in both venoms. Furthermore, the antivenom neutralized the lethal effect of *M. dumerilii* venom at the tested doses, but it did not neutralize this effect induced by *M. mipartitus* venom or its main toxic components from the southwestern region of the department of Antioquia at the doses evaluated. Moreover, the anticoral-INS antivenom displayed better cross-immunorecognition on PLA_2-predominant *Micrurus* venoms than on 3FTx-predominant *Micrurus* venoms. Further research is necessary to optimize the composition of antivenom mixtures and improve their efficacy against coral snake envenomation in Colombia and the Americas.

Author contributions

Conceptualization, P.R.S.; methodology, J.D.P., A.C.R., P.R.S.; formal analysis, P.R.S, and J.A.P.; investigation, J.D.P., A.C.R., P.R.S.; resources, P.R.S and J.A.P.; data curation, J.D.P., and P.R.S.; writing—original draft preparation, J.D.P., P.R.S.; writing—review and editing, P.R.S, and J.A.P.; funding acquisition, J.A.P.; All authors have read and agreed to the published version of the manuscript.".

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:Jaime Perenaez reports financial support was provided by Colombia Ministry of Science Technology and Innovation.

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