CORAL SNAKE ANTIVENOM PRODUCED IN CHICKENS (*Gallus domesticus*)

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SUMMARY

The production of anti-snake venom from large mammal's blood has been found to be low-yielding and arduous, consequently, antivenom immunoglobulins for treatment are achieved regularly as polyvalent serum. We have standardized an undemanding technique for making purified immunoglobulin IgY antivenom consisting of polyclonal antibodies against coral snake venom in the egg yolk of immunized hens. We have adapted a reported process of antibody purification from egg yolks, and achieved 90% antibody purity. The customized technique consisted of the removal of lipids from distilled water-diluted egg yolks by a freeze–thaw sequence. The specific immunoglobulins were present in the egg yolk for up to 180 days postimmunization. Therefore, by means of small venom quantities, a significant amount of immunoglobulins were found in an adequately purified state (The obtained material contained about 90% pure IgY). The antigen binding of the immunoglobulins was detected by a double immunodiffusion test. Titers of antibodies in the yolk were estimated with a serum protection assay (Median effective dose = ED_{50}) (ED_{50}= 477 mg/kg). Given that breeding hens is economically feasible, egg gathering is noninvasive and the purification of IgY antibodies is quick and easy, chicken immunization is an excellent alternative for the production of polyclonal antibodies. To the best of our knowledge, this is the first coral snake anti-venom prepared in birds.

KEYWORDS: Coral antivenom; Elapidae; IgY immunoglobulins; *Micrurus*; venom.

INTRODUCTION

Snake envenomation produces tissue effects such as local swelling and necrosis, neurotoxicity and hemostatic disorders. Considerable advancement has been made in understanding the pathophysiology of ophidic accidents, motivating transformations in treatment procedures. Latest advances, including the production of new antivenoms using new processes[1-4,19], have encouraged developing coral snake antivenoms with attractive protocols.

Nearly two hundred species from the Elapidae family are dispersed across the Southeastern and Southwestern United States, as well as all of Mexico, Central America and South America, and are also established in the African, Asian and Oceanic continents[3]. On the American continent, more than 120 species and subspecies have been described, separated into three genera: *Leptomicrurus* with three species, *Micruroides*, with one, and *Micrurus*, with approximately 70 species[21,22].

The production of safe, efficient and reasonably priced antivenoms is a priority. Alternative progress in the therapeutics of coral snake bite victims in Venezuela requires an answer to the production of new animal models, logistical, financial, marketing, delivery and storage difficulties related to the supply of antivenom. Furthermore, the norms and regulations for physicians to improve antivenom use must also be addressed. Even though coral snake envenomations could be handled using medications that act on presynaptic and postsynaptic receptors, since some patients may only survive under intensive therapy treatment such as respiratory support[23], specific treatment with antivenoms continues to be the elected method for treating these incidents, which can efficiently deactivate all systemic activities of the venom. Nevertheless, there are some collateral effects of antivenom such as anaphylaxis and serum sickness[5]. The majority of these alterations seem to be caused by the action of high concentrated proteins, which are not immunoglobulins, but contaminating polyvalent antivenoms. However, the benefits of antivenom treatment may be more important than its risks.

The specific therapeutics for coral snake envenomations is the use of heterologous antivenom, and to date, this type of antivenom is not available in Venezuela. In light of the information that coral snake venom can reveal a multiplicity of composition and toxic activities, we have included the most important venom species occurring in Venezuela and United States in the immunization protocol. At this time, we present a study on the production of a specific coral snake (*Micrurus*) antivenom and its purification of immunoglobulins from the egg yolk of immunized hens, with the purpose of providing a more efficient antivenom for therapeutic treatments.

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MATERIALS AND METHODS

Ethical statement: All the experimental events concerning the use of live animals were carried out by specialized staff. The relevant regulations of Venezuela as well as institutional guidelines, according to protocols approved by the Institute of Anatomy of the Universidad Central de Venezuela, and the norms obtained from the guidelines for the care and use of laboratory animals[13] were followed.

Coral snakes *(Micrurus)* venom: Venom from a collection (twenty specimens) of different *Micrurus* species from Venezuela and the United States were used in the immunization protocol. The venom of Venezuelan coral snakes consisted of (*Micrurus isozonus* (Calabozo, Guárico State), *Micrurus isozonus* (Caracas, D.C), *Micrurus isozonus* (La Boyera, Miranda State), and *Micrurus isozonus* (Maracay, Aragua State) which were supplied by the Serpentarium of the Tropical Medicine Institute of the Universidad Central de Venezuela, Caracas, Venezuela. The venom from the U.S. consisted of *Micrurus fulvius fulvius* (Eastern, USA), and *Micrurus tener tener* (Western, USA), purchased from the National Natural Toxins Research Center, Texas A&M University-Kingsville, Kingsville, Texas, USA.

Fifteen days prior to venom extraction, the coral snakes were fed and made to fast to guarantee enough venom in their glands. The venom was collected through a 50-mL plastic centrifuge tube transversely cut and covered on the top with Parafilm® (Millipore Corp, USA). The snake was forced to bite the Parafilm. Venom was collected by glass capillaries through the excretory conduit in the base of the fangs, centrifuged, and supernatants were placed in Eppendorf® (Eppendorf Int, USA) tubes and stored at -30 °C until use. Stock solutions were prepared in phosphate-buffered saline (PBS) (10 Mm sodium phosphate containing 150 Mm NaCl, Ph 7.2) at 1.0 mg/mL.

Mice: Female mice (INH strain) weighing 18-20 g were obtained from the Instituto Nacional de Higiene "Rafael Rangel", Caracas, Venezuela. The colony of mice was kept in plastic boxes (Tecniplast, Italy) at six mice per cage, in a room maintained at 23 °C on a 12/12-hr light/dark cycle.

Hens: Six egg-laying, red hens *(Gallus domesticus)* of Rhode Island strain, approximately 16 weeks old, obtained from a poultry farm from Lagunita town, Miranda State, Venezuela, were located in individual henhouses (Centenosuplidores C.A, Venezuela)(one hen per henhouse) prior to the commencement of the production of eggs. Hens were maintained on 12/12-hr light/dark cycle at 23 °C with food and water *ad libitum.*

Determination of protein concentration: Protein determination was established by the method of LOWRY *et al.*[15].

***Micrurus'* venom lethality:** Lethality of crude venom was determined by intravenous injections into mice and the LD_{50} value calculated according to the Spearman-Karber method[31]. The venom was diluted in a phosphate-buffered saline solution (PBS). The endpoint of lethality of the mice was determined after 48h. All solutions during the experiments were stored at 4 ℃ and warmed to 37 ℃ prior to being injected into mice. The lethal toxicity was determined in five groups containing five mice. A total of 0.2 mL of venom (dosages from 0.05 to 0.8 mg/kg) was injected into the tail vein of 18-20 g female BALB/c mice. A comparable volume of PBS was injected as a negative control group.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of coral snake's crude venom: Pools of different coral snakes' crude venom under non-reduced conditions were electrophoresed using a MINIPROTEAN II (BioRad, USA) chamber. SDS-PAGE was performed according to the Laemmli method (1970)[14] using 15% gels. Wide range molecular weight markers (Bio-Rad) were run in parallel and gels were stained with Comassie blue (National Diagnostic, USA).

Immunization: A pool was made with concentrations of venom, corresponding to the LD_{50} median. A sub-lethal dose was used for immunizations. Four-month-old egg-laying hens, weighing ~1 kg were maintained pathogen-free and immunized with the pool of coral snakes' venom. Venom (0.24 mg/kg in 0.1 mL) was taken into an Eppendorf tube and then mixed with an equal volume of Freund's complete adjuvant, whereas the second doses consisted of venom emulsified with Freund's incomplete adjuvant (GIBCO, USA). The third venom doses were mixed with a saline solution. All doses were administered subcutaneously, via the deltoid region in four different places, alternating right and left every two weeks for eight weeks. One week after the last dose, the hens' blood was obtained for the detection of immunoglobulins that could recognize and precipitate the coral snake venom.

Isolation of immunoglobulin: The modified method of SVENDSON *et al.* (1995)[30] using the freeze and thaw principle to remove lipids was used. For a brief period, the egg yolk was diluted 10 times with distilled water, and the diluted egg yolk was frozen at -80 ℃ overnight, and permitted to thaw at an increased temperature rate of ~ 2 ℃ (six min) by maintaining it at 2-4 ℃. The egg yolk was then centrifuged at 18,000 g for 1h at 4 ℃ and the supernatant gathered was cleared by filtration on Whatman (N°. 1) filter paper (Whatman, USA). To precipitate the immunoglobulins, the filtered supernatant was then precipitated by 40% ammonium sulfate at 4 °C. Afterwards, the pellet was re-suspended in 0.01M Tris-HCl (pH 8.0) to a volume equal to half of the supernatant. Following centrifugation for 15 min at 13 000 g, the pellet was washed twice with 40% ammonium sulfate (ReAgent, UK)**.** The solution was dialyzed carefully (three buffer changes, at least 150 times volume) against 10 mM phosphate buffer, pH 7.0, for 24 to 48h in a dialyzing tube with molecular cut off weight of 14 KDa to remove the ammonium sulfate[5,19].

Antibody activity and purity were determined using a SDS-PAGE[14], double gel diffusion test[18] and a serum protection test (ED_{50}), respectively.

Gel diffusion assay using specific IgY against coral snake venom: To show the specific IgY immunoglobulin activity, a 1% agarose (Sigma-Aldrich Co. USA) double gel diffusion test was used[18]. The immunizing pool of coral snake crude venom at 10 mg/mL was placed in the central well, while different coral snake venoms and PBS were placed in the outer wells and incubated at 37 °C.

Serum protection test (Median effective dose = ED_{50}): For antivenom effectiveness, five groups of eight mice were challenged with a mixture of varying concentrations of IgY antivenom containing three LD_{50} of venom. The antivenom/venom mixture was prepared at 0 °C and incubated for 30 min at 37 °C prior to injection. Each mouse was injected with 0.2 mL of venom/antivenom mixture into the tail vein. The mice were observed

for 48 h and the survival percentage and ED_{50} were calculated according to Spearman and Karber[31]. Saline controls and antivenom controls were used. Neutralizing capability was expressed as the 50% effective dose (the amount of antivenom that protects 50% of the population) (Table 2).

Specificity of coral snake antivenom (IgY) to various snake venom via Western blot: To explore the specificity of the immunoglobulins against coral snake venom, the antibodies were also assayed with *Crotalus* and *Bothrops* venom. A total of nine venom (22 **μg/each**) consisting of *M. isozonus*, *M. surinamensis*, *M. f. fulvius*, *M. t. tener*, *Naja kaouthia*, *Naja pallida*, *Bothrops colombiensis*, *Crotalus durissus cumanensis* and *C. vegrandis* were electrophoresed on a 10-20% Tricine SDS gel using a XCell *SureLock*™ system at 150V (Bio-Rad PowerPac Basic) for one hour. The proteins were transferred onto a 0.2 μm nitrocellulose membrane (Millipore) using a Trans Blot SD system (BioRad) at 100 mA for one hour. The primary antibody (chicken anti-coral snake venom IgY) was diluted to 1/200, the secondary antibody (rabbit anti-chicken IgY-alkaline phosphatase) (Sigma-Aldrich Co. USA) was diluted to 1/50,000. SIGMA *FAST*™ BCIP/NBT tablets were used to visualize the bands on the blot and SimplyBlue (Life Technologies, USA) was used to visualize the bands on the gel. SeeBluePlus2 (Life Technologies, USA) markers were used as standards.

Antivenom: The antivenom passed the standard tests for neutralizing potency, miocrobiological purity, lack of pyrogenicity, appropriate protein concentration, lack of abnormal toxicity, sterility, and pH (6.9)[11.]

RESULTS

Lethality assay: The LD_{50} calculated as a median from the mixture of coral snake venom used for immunization was 0.58 mg/kg. The LD_{50} values were different among *Micrurus* venoms tested, with LD_{50} of 0.32 mg/kg (*Micrurus fulvius*), 0.5 mg/kg *(Micrurus isozonus)* and 0.78 mg/kg (*Micrurus tener*) (Table 1).

Table 1
LD_{50}s of Venezuelan and United States coral snake venoms. Lethality of crude venom was determined by intravenous injections into mice and the LD_{50} value calculated according to Spearman-Karber method[31]

Species	Pool	$LD_{50}{}^{a}$ ± SD
M. isozonus	La Boyera, Miranda State (Venezuela)	0.5 ± 0.012
M. isozonus	Caracas, Capital District (Venezuela)	0.5 ± 0.012
M. isozonus	Calabozo, Guárico State (Venezuela)	0.8 ± 0.016
M. isozonus	Maracay, Aragua State (Venezuela)	0.6 ± 0.02
M. tener tener	Kingsville, Texas State (United States)	0.78 ± 0.14
M. fulvius	Tampa, Florida State (United States)	0.32 ± 0.12

[a] The LD_{50} is the concentration of venom required to kill 50% of a mouse population after 48 h. Results are expressed in mg venom/kg body weight. Mean LD_{50} = 0.58(mg/kg).

Immunization: The LD_{50}s for the venoms were carried out according to the SÁNCHEZ *et al.*[23] method. A sub-lethal dose of the pool of venoms made with concentrations corresponding to the LD_{50} median of 0.3 mg/kg, was used to immunize the hens (Table 1).

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of coral snake crude venom: The SDS-PAGE (15%) protein profiles of *Micrurus isozonus* venoms were analyzed. The individual venom differed in composition, quantity and intensity of bands (Fig. 1). The *M. isozonus* venom from Calabozo contained more protein bands above 31.kDa (Fig. 1; lane 2) than all other *M. isozonus* venom. The *M. isozonus* venom from Caracas and Maracay both contained a similar protein band between 66.2 and 97.4 kDa (Fig. 1; lanes 3 and 5, respectively). The sample from La Boyera (Fig. 1; lane 4) contained all protein bands below 21.5 kDa.
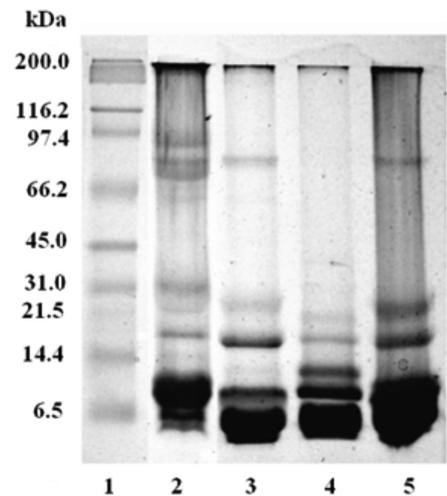

Fig. 1 - SDS-polyacrylamide gel electrophoresis (15%) of individual *M. isozonus* venoms from Venezuela. A total of 20 μg of venom sample was applied to the gel. Lanes: 1) Markers; 2) *M. isozonus* (Calabozo); 3) *M. isozonus* (Caracas); 4) *M. isozonus* (La Boyera); 5) *M. isozonus* (Maracay). The gel was stained with Comassie blue.

Isolation of immunoglobulin: This method[30] involved a gently synchronized -70 °C freeze and 4 °C thaw cycle, consequently giving a clear egg yolk solution. The total protein concentration in one egg yolk was around 1.3 ± 0.5 g. In this method, a larger component of the additional proteins was excluded through lipid elimination of egg yolk by the freeze and thaw cycle. IgY was reduced with β-Mercaptoethanol (Sigma-Aldrich Co. USA) showing a heavy chain fragment of 68 kDa and light chain of 27 kDa (Fig. 3). The material, after precipitation, contained about 90% pure IgY.

Gel diffusion assay using specific IgY against coral snake venom: The existence of antivenom IgY in egg yolk was tested by Ouchterlony's immunodiffusion assay[18] using crude venom as the antigen (Fig. 2). The protein concentration in the wells (1-12) was 1 mg/ mL, and 20 μL was added to each well. Antivenom added to the middle well was 20 μL, containing a concentration of 1 mg/mL Lowry's protein. Several precipitin lines at each location pointed out the polyvalent character of the antibodies. The antibody titer augmented after the booster dose, and antibodies were present 180 days after the first injection.
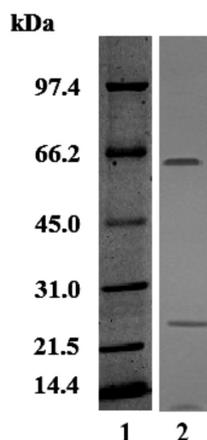
Fig. 2 - SDS-PAGE analysis (15% gel concentration, under reducing conditions) of IgY Antibodies after precipitation with ammonium sulfate and dialysis. 1) Molecular weight markers. 2) IgY.

Serum protection test (Median effective dose = ED$_{50}$): Coral snake antivenom (IgY) was able to effectively neutralize the pool of all *Micrurus isozonus* coral snake venom used in the immunization with a mean ED$_{50}$ of 477.8 mg/kg (Table 2).

Specificity of coral snake antivenom (IgY) to various snake venom via Western blot: The coral snake antivenom was able to recognize, to some degree, all the coral snake venoms used in this study in addition to two cobra snake venoms and three Venezuelan pit vipers. The antivenom was most specific to *M. f. fulvius* followed by *M. isozonus*, *M. t. tener* and *M. surinamensis*. The two *Naja* spp. had similar reactions to each other, and the two *Crotalus* spp. also showed similarities. *Bothrops colombiensis* venom had a different reactivity pattern than the other Venezuelan venom. *Micrurus surinamensis* venom had the least reaction with the antivenom compared to all the venom tested. No protein bands below 12 kDa were detected for any venom.

DISCUSSION

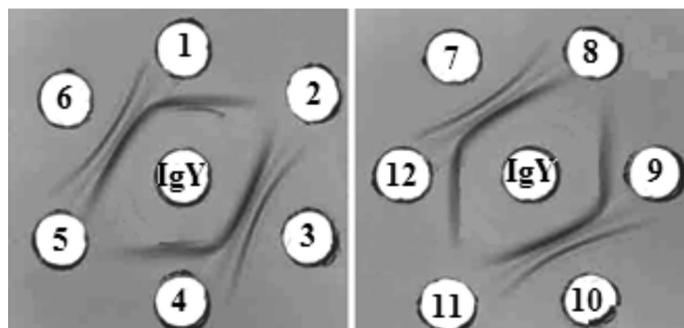Despite the significant pressure due to death and grave consequences

Fig. 3 - Gel diffusion assay using specific IgY against coral snake venom. 1. *Micrurus isozonus* (Calabozo, Guárico State); 2. PBS; 3. *Micrurus isozonus* (Caracas, D.C.); 4. *Micrurus isozonus* (La Boyera, Miranda State); 5. PBS; 6. *Micrurus isozonus* (Maracay, Aragua State); 7. *Micrurus surinamensis* (Amazonas State); 8. PBS; 9. *Micrurus dissoleucus dissoleucus* (Paraguaná, Falcón State); 10. *Micrurus tener* (Western, USA); 11 PBS; *Micrurus fulvius* (Eastern, USA).

resulting from accidents with coral snakes, the research and investment of funds for the production of antivenoms is considered insufficient. On the other hand, biochemical studies relating to *Micrurus* venoms are limited, due to the complexity of accurately identifying the species, the difficulties in maintaining them in captivity, and the difficulty in obtaining a desirable amount of venom; in addition, venom varies between intra and interspecies in composition, associated with their age, gender, geographic distribution, and diet[4,10].

In this study, the competence of hens to make antibodies against small quantities of antigens was studied. In some conditions, there may be problems with getting adequate amounts of antigen to make immunizations. In this experiment the hens were immunized with 0.1-100 μg of antigen with booster immunizations. Every two weeks for eight weeks, the IgY response in the yolk demonstrated a similar picture as that in the serum. The study confirmed that it is possible to obtain a good immune response with less amounts of antigen than is usually recommended to immunize sheep or horses. Studies by some groups have established that antibody responses to foreign antigens are genetically controlled. It has been feasible to breed hens that are high and low antibody responders intramuscularly[20] or by intravenous[9,16] injections.

Table 2
Effective dose fifty (ED$_{50}$=477 mg/kg/3 LD$_{50}$) assay of yielded antibodies (IgY coral snake antivenom neutralizing lethal toxic activity of coral snake venom

Antivenom concentration (mg/mL)	Total protein of antivenom (mg/mouse)	Number of mice			Percentage of deaths (%)
		Died	Lived	Total	
86	17.2	0	8	8	0
43	8.6	4	4	8	50
21.5	4.3	8	0	8	100
10.7	21.5	8	0	8	100
5.3	5.3	8	0	8	100
	ED$_{50}$ = 477 mg/kg[a]				

The ED$_{50}$ was calculated according to Spearman and Karber [31]: $\log ED_{50} = \log X_{100} - \dfrac{\log FD}{n}(\Sigma t - n/2)$

ED$_{50}$ = the 50% effective dose. $\log X_{100}$ = log dose giving 100% survival and having 100% survival for all higher doses. log Fd = the log dilution factor. N = # mice used at each dose level. T = #mice alive at each dose level. Σ = the sum of mice surviving at every dose level. [a] The ED$_{50}$ is the effective dose of IgY that will protect 50% of the mouse population when injected with 3LD$_{50}$s.

The production of IgY from small amounts of antigen required for immunization, the ease in collecting the eggs, and the uncomplicated purification techniques to enhance the function of immunological assays, make the use of IgY attractive and profitable.

Normally, antivenoms are achieved by immunizing horses with increasing doses of venom to obtain a high-quality antibody titer[8]. A range of side effects exists in the administration of antivenom; for instance, anaphylaxis and serum sickness[27]. The elevated concentrations of proteins, which are not antibodies, existing in many common antivenom probably produce most of these symptoms. With the aim of eliminating the unpleasant effects of the antivenom treatment, it is necessary to achieve antivenom immunoglobulins in a reasonable purification state. Investigators[24,25,30] have proposed that birds are a suitable and economical supply of IgY immunoglobulins. Chicken egg yolks have IgY, which is a species-specific immunoglobulin with a molecular weight of 190kDa[26]. Our laboratory has produced hen antibodies against *Scolopendra gigantea* toxins with high titers[19]. Others have reported the production of immunoglobulins against different types of protein antigens, synthetic peptides, etc.[29]. We have improved the production of polyclonal antibodies by means of crude coral snake venom injected in hens with an alternative protocol, from that of the classical method used for horse immunoglobulin production. In our current work, we were capable of obtaining a good antibody titer with immunized hens.

Due to the high incidences of coral snake envenomations by *M. isozonus* in Venezuela, this particular snake venom was used to carry out the *in vivo* efficacy test of coral snake IgY antivenom, which resulted in ED_{50} of 477 mg/kg body weight (Table 2). This value falls within acceptable ED_{50} ranges of other antivenoms tested on different snake venoms[7]. A Western blot was done to determine the specificity of the IgY antivenom to different coral snake venoms as well as other Venezuelan venomous species. It appears that the IgY coral snake venom antivenom was able to recognize all four coral snake venoms used in the immunization protocol with *M. isozonus, M. surinamensis, M. f. fulvius* and *M. t. tener* having the highest recognition. The venom of *M. surinamensis* was the least reactive (Fig. 4). In addition to those venoms used to produce the IgY antivenom, two cobra venoms (*N. kaouthia* and *N. pallida*), and three Venezuelan Viperidae species (*B. colombiensis, C. d. cumanensis* and *C. vegrandis*) were also detected by the antivenom. This cross reactivity suggests there are similar venom toxins found in species of snakes located in distinct geographical locations. The fact that M. *surinamensis* had the least reactivity of all venoms, including those not used in the immunization protocol, indicates the uniqueness of the proteins found in that venom. The possible use of IgY in therapy and/or diagnostic assays either in humans or animals provides an invaluable field of interesting and useful applications[12,17,28].

RESUMEN

Antiveneno de serpiente coral producido en gallinas *(Gallus domesticus)*

La producción de antiveneno de serpiente usando sangre de grandes mamíferos se ha encontrado que es de bajo rendimiento y de trabajo arduo, en consecuencia, las inmunoglobulinas antiveneno para el tratamiento se obtienen generalmente, como suero polivalente. Hemos estandarizado una técnica poco exigente para la fabricación de inmunoglobulina purificada IgY, que consistió en generar anticuerpos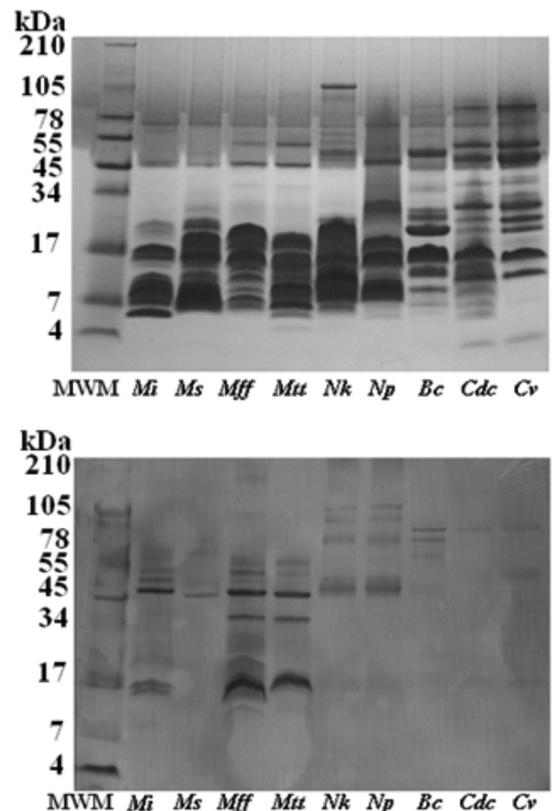 policlonales contra el veneno de la serpiente coral en huevos de gallinas inmunizadas. La técnica consistió en la eliminación de lípidos de las yemas del huevo, diluidas en agua y en una secuencia de congelación-descongelación. Las inmunoglobulinas específicas estuvieron presentes en la yema de huevo hasta 180 días después de la inmunización. La unión del antígeno a las inmunoglobulinas se detectó mediante un ensayo de inmunodifusión doble. Los títulos de anticuerpos en la yema fueron estimados con un ensayo de protección (dosis efectiva media = ED50). Dado que las gallinas reproductoras son económicamente viables, la recolección de huevos es no invasiva y la purificación de anticuerpos IgY es rápida y fácil, la inmunización de la gallina es una excelente alternativa para la producción de anticuerpos policlonales. A nuestro entender, esta es el primer anti-veneno contra serpiente de coral preparado en aves.

Fig. 4 - Gel electrophoresis and Western blot analysis of various snake venoms with IgY antibodies. A total of nine venoms (22 µg/each) consisting of *M. isozonus* (Mi), *M. surinamensis* (Ms), *M. f. fulvius* (Mff), *M. t. tener* (Mtt), *Naja kaouthia* (Nk), *Naja pallida* (Np), *Bothrops colombiensis* (Bs), *Crotalus durissus cumanensis* (Cdc) and *C. vegrandis* (Cv) were electrophoresed on a 10-20% Tricine SDS gel using a XCell *SureLock*™ system at 150V (Bio-Rad PowerPac Basic) for 1h. The proteins were transferred onto a 0.2 µm nitrocellulose membrane (Millipore) using a Trans Blot SD system (BioRad) at 100 mA for 1 h. SIGMA *FAST*™ BCIP/NBT tablets were used to visualize the bands on the blot and SimplyBlue (Life Techonologies) was used to visualize the bands on the gel. SeeBluePlus2 (Life Techonologies) markers were used as standards.

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AUTHOR CONTRIBUTIONS

Irma Aguilar is a Ph.D student who worked on the immunization of animals and purified the IgYs. She was involved in all the experiments done. Elda E. Sánchez developed the LD_{50} and ED_{50} assays, the gel electrophoresis and Western blot of the various snake venoms with the I_gG antibodies as well as collaborated with the writing of the manuscript. María E. Girón and Amalid Estrella are Research Assistants who did the general experiments. Belsy Guerrero carried out the electrophoresis and Western blot as well as collaborated with the writing of the manuscript. Alexis Rodriguez-Acosta designed and supervised the project and wrote the manuscript. He is the thesis tutor of MSc. Aguilar.

CONFLICT OF INTEREST STATEMENT

We declare that we have no conflict of interest. Authors transfer the copyright of the article to the publisher.

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