A new approach of immunotherapy against *Crotalus* snakes envenoming: ostrich *(Struthio camelus)* egg yolk antibodies (IgY-technology).

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Abstract. Crotalid envenomation is a neglected collective health problem involving many countries in America, which need secure and inexpensive snake anti-venom treatments. Here, high antibody titers (IgY) were raised in the Ostrich (*Struthio camelus*) egg yolk by immunizing with the venom of Venezuelan venomous *Crotalus* snakes. Ostriches were immunized with a pool of venoms from common rattlesnake (*Crotalus durissus cumanensis*), Uracoan rattlesnake (*Crotalus vegrandis*), Guayana rattlesnake (*Crotalus durissus ruruima*) and black rattlesnake (*Crotalus pifanorum*). The anti-snake venom antibodies were prepared from egg yolk by the water dilution method, enriched by the addition of caprylic acid (CA) and precipitation with ammonium sulfate at 30% (W/V). The purity and molecular mass of the final product was satisfactory, yielding a single ~ 175 kDa band in SDS-PAGE gels ran under non-reducing conditions. In the immunoblot analysis, specific binding of the antivenom was observed with most venom proteins. The LD_{50} was 16.5 g/mouse (825 μg/kg body weight). High titers of IgY against Crot/pool venom were shown by ELISA. The median effective dose (ED_{50}) was 19.66 mg/2LD_{50}. IgY antibodies neutralized efficiently the *Crot*/pool venom lethality. As far as we know, this is the first anti-snake venom produced in ostriches, which could make this technology an affordable alternative for low-income countries, since it is likely to produce

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about 2-4 g of IgY per ostrich egg. Hence, almost 400 g of IgY can be purified from only one ostrich during a year. In addition, there are enormous differences in the cost of investment in the maintenance of horses, from the points of view of infrastructure, feeding and veterinary care, in which the cost can reach USD 100 per animal per day, compared to a maintenance cost of USD 146 per month per producing bird. These results are encouraging and could easily be extrapolated to the manufacturing of other antivenoms and antitoxins as well, as they could be applied to the manufacturing of potential diagnostic tools.

Un nuevo enfoque de inmunoterapia contra el envenenamiento de serpientes *Crotalus:* anticuerpos de yema de huevo de avestruz *(Struthio camelus)* (tecnología IgY).

Resumen. El envenenamiento por crotálidos es un problema de salud colectiva desatendido, que involucra a muchos países del continente americano, los cuales necesitan tratamientos seguros y económicos. En este trabajo, se obtuvieron títulos altos de anticuerpos (IgY) producidos en yema de huevo de avestruz (*Struthio camelus*) mediante la inmunización con el veneno de serpientes venezolanas del genero *Crotalus*. Se inmunizaron avestruces con una colección de veneno de serpientes de cascabel común (*Crotalus durissus cumanensis*), cascabel de Uracoa (*Crotalus vegrandis*), cascabel de Guayana (*Crotalus durissus ruruima*) y cascabel negra (*Crotalus pifanorum*). Los anticuerpos anti-veneno de serpiente se prepararon a partir de yema de huevo por el método de dilución en agua, enriquecidos mediante la adición de ácido caprílico (CA), seguido de una precipitación con sulfato de amonio al 30% (P/V). La pureza y masa molecular de los anticuerpos (IgY) se definieron mediante ensayos de SDS-PAGE nativos y las masas moleculares se establecieron electroforéticamente, obteniéndose una única banda de IgY de $\sim$ 175 kDa. El análisis de inmunotransferencia mostró la unión específica del antiveneno con la mayoría de las proteínas del veneno. La DL_{50} fue de 16,5 μg/ratón (825 μg / kg de peso corporal); Se mostraron títulos altos de IgY contra el veneno de Crot / pool mediante ELISA. La dosis mediana efectiva (DE_{50}) fue de 19,66 mg/2 LD_{50}. Los anticuerpos IgY neutralizaron eficazmente la letalidad del veneno de Crot / pool. Hasta donde sabemos, se trata del primer antídoto de serpiente producido en avestruces, lo que podría abaratar la producción de este tratamiento en países del tercer mundo. Ya que es probable que se obtengan alrededor de 2-4 g de IgY por huevo de avestruz. Por lo tanto, se podrían purificar casi 400 g de IgY de un solo avestruz durante un año. Asimismo, debido a las enormes diferencias en el costo de inversión en el manteni-

miento de los caballos desde el punto de vista de infraestructura, alimentación y atención veterinaria, en los que el costo puede llegar a los 100 USD por día, frente a los 146 USD por mes de mantenimiento de la producción de aves. Estos resultados abren un campo terapéutico, para la fabricación de otros antivenenos contra un amplio espectro de toxinas y también como probables herramientas de diagnóstico.

INTRODUCTION

Venomous snakebite is a worldwide problem, especially in tropical and subtropical geographical regions. There are more than five million snakebite accidents annually worldwide; the members of the Viperidae family [1] produce the most common snakebites that take place in the American continent (~ 98%).

Traditional antivenom production is based on purified antibodies extracted from hyperimmunized horses' plasma [2]. In recent years, several authors report that bird's (mainly chicken) antibodies produced against snake venoms, presented venom effective neutralization activities [2,3]. However, despite their potential considering their body size and egg-laying advantages, ostriches (*Struthio camelus*) have not been previously tested for snake anti-venom production. Clinical assays will be need to assess their security as an antidote to human victims of ophitoxemia and further experimentation addressing IgY-based antivenoms safety and effectiveness is required.

MATERIALS AND METHODS

Reagents

Polyvalent anti-ophidic serum (PAOS) (Biotecfar C.A, Universidad Central de Venezuela, Pharmacy Faculty, Caracas, República Bolivariana de Venezuela). Sodium chloride, sodium citrate, sodium azide, tris, hydrochloric acid, sodium hydroxide, Coomassie blue, acrylamide, bis-acrylamide, ammonium per-sulphate, glacial acetic acid, temed, glycerol, sodium bicarbonate, caprylic acid (Sigma-Aldrich, Missouri, USA). Saran wrap (S.C. Johnson & Son, Inc, USA). Complete Freund's and incomplete Freund's adjuvants (GIBCO, USA). Antibody goat anti-avian (included anti-ostrich) (Laboratories ABCAM, USA). Peroxidase substrate (TMB) (Vector Lab, USA). 30 kDa cassette filtration unit (Vivaflow 50 R, Sartorius, Germany). Filter (Sartorious Laboratories, Germany). Microtitration plates (Corning® ELISA microplates, USA). Automatic ELISA reader (Bio-Tek Laboratories, USA). Mini-Protean II system (Bio-Rad Laboratories, USA). Trans Blot SD system (Bio-Rad Laboratories, USA). Molecular mass standards for SDS-PAGE were from Bio-Rad Laboratories Ltd (California, USA).

Ostriches

Two female healthy adult ostriches (*Struthio camelus*) were obtained from a local ostrich farm (Villa de Cura town, Aragua state, República Bolivariana de Venezuela). They were housed under standard environment (humidity, lighting and temperature) and fed *ad libitum* with standard ostrich diet and potable water (Fig. 1).

Venom

A pool of venoms from common rattlesnake (*Crotalus durissus cumanensis*) (0.15 mg/mL), Uracoan rattlesnake (*Crotalus vegrandis*) (0.03 mg/mL), Guayana rat-
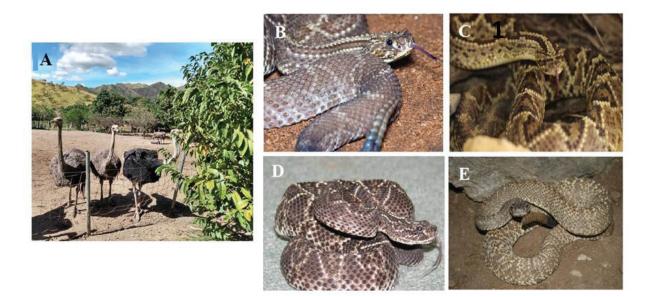
Fig. 1. (A) Ostriches at bird's farm, Villa de Cura, Aragua state, Venezuela. (B) *Crotalus durissus cumanensis.* (C) *Crotalus ruruima.* (D) *Crotalus pifanorum.* (E) *Crotalus vegrandis* (1).

tlesnake (*Crotalus durissus ruruima*) (0.02 mg/mL) and black rattlesnake (*Crotalus pifanorum*) (0.012mg/mL) were obtained by milking the snakes and then crystallized under vacuum in a desiccator containing $CaCl_2$ as a desiccant and maintained at 4°C until use (Fig. 1).

Ethical statement

Skilled staff prepared all the experimental methods relating to the use of live animals. These methods were permitted by the Institute of Anatomy Ethical Committee of the Universidad Central de Venezuela under assurance number (Protocol N° 190619) and followed the norms obtained from the Guidelines for the Care and Use of Laboratory Animals, published by the USA National Institute of Health [4].

Immunization procedure

Two female ostriches were injected intramuscularly intro the bird's anterior thigh muscles on day 0 with the *Crot*/pool of the Venezuelan rattlesnake venoms (1μg/kg body weight) emulsified with an equivalent volume of complete Freund's adjuvant. The primary booster dose was administered two weeks later in an incomplete Freund's adjuvant. Successively, the second and third boosters emulsified with an equivalent volume of incomplete Freund's adjuvant were administered at 45-day intervals in order to sustain high antibody titers. Blood was collected through the wing vein, arranged to obtain the serum. Sera were separated by centrifugation (1500 G for 15 min). Preimmune serum and egg yolk replicas collected from ostriches were used as negative controls. They were stored in a freezer at −20°C, until used.

Purification of antibodies from egg yolk

Isolation of IgY from egg yolk was performed with a modified method [5]. Briefly, once the egg shell was opened, the yolk was softly detached from the white of the egg, washing it with abundant distilled water, until all the white egg disappeared. Then, the yolk was punctured with a syringe and all its contents were extracted and mixed completely with a five-fold dilution with PBS pH 7.4 and slow addition of caprylic acid (CA), then the pH was adjusted to 5.0 with 10 M

hydrochloric acid. Concisely, the CA was slowly added dropwise at an approximate rate of 0.6 mL/min, until a final concentration of 6% (v/v) was obtained.

After the first step of the process, the sample was filtered through a 15 μm and then 0.45 μm filter.

The preparation was kept to room temperature, and afterward ammonium sulfate at 30% (W/V) concentration was added, keeping it under constant stirring for one hour at room temperature and then filtered through a 2-μm filter. In order to eliminate the ammonium sulfate, a tangential filtration process was applied using a cassette of 30 kDa. The sample was resuspended in saline solution and kept under stirring for one hour- Subsequently, filtered through a 2-μm filter to remove remaining solids and a diafiltration process was started until eight diafiltration volumes were completed. When the ammonium sulfate was removed, the washed sediment was dissolved in 200 mL saline pH 6.3.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of IgY

Purified IgY under non-reduced and reduced conditions were electrophoresed with a MINIPROTEAN II (BioRad, USA) chamber. SDS-PAGE was performed using 12% gels. Wide range molecular weight markers (Bio-Rad) were run in parallel and gels were stained with Comassie blue (National Diagnostic, USA).

Determination of antibody titers by an indirect ELISA

The immunoglobulins titers in serum and egg yolk of immunized ostriches were tested by enzyme-linked immunosorbent assay (ELISA). Titer was considered as the concentration of an antibody, as determined by finding the highest dilution at which it was still able to cause recognition of the antigen. The uncomplicated way to do this is as follows: selecting the highest responding serum and another that shows low re-

sponse. Do readings for serial dilutions as we have done (Fig. 3). Looking for one dilution were both sera are in the steepest part of the curve, are clearly different (generally a 1:1000 – 1:4000 dilutions will do it) and the OD450nm of the most responding serum is nearly 1.0. Then it is possible to define the titer as the OD450nm for each serum at the dilution obtained before.

All ELISA incubations were carried out at 24-26°C. Briefly, aliquots (100 μL) of the *Crot*/pool venom (1 μg/mL PBS) were pipetted into the wells of the micro-titration plates in overlay phosphate buffer saline (PBS) pH 7.4 that were protected with saran wrap and stored overnight at 4°C. The wells content was aspirated, and washed three times with washing buffer (PBS, pH 7.4 containing 0.05% Tween-20), the wells were then overflowed with blocking buffer (skimmed milk 2% in PBS-T) and left for one hour. After the aspiration of blocking buffer, the ostrich sera or purified IgY immunoglobulins samples were diluted properly in blocking buffer and 100 μL added to the wells before incubating at 37°C for one hour. Later, the plates were washed three times with washing buffer and incubated with goat anti-avian (included anti-ostrich) IgY peroxidase (1:10.000) at 37°C for one hour. The contents of the wells were subsequently aspirated, the wells washed three times with PBS-0.05% Tween 20 (PBS-TW), and 100 μL of peroxidase substrate (TMB)(Vector Lab, USA) was added to each well. The plates were maintained in the dark, at room temperature for 20 min for the progress of dye. The reaction was stopped with 50 μL of sulfuric acid 1 M. The absorbance of solutions at X = 450 nm was determined after the addition of substrate by an automatic ELISA reader.

Immunoblot analysis

Recognition of IgY antibodies was performed by western blot according to the modified method [6] (n=3). Briefly, to determine the specificity of the immunoglobulins against *Crot*/pool snake venoms, the anti-

bodies were tested with a total of six venoms *Crotalus durissus cumanensis, Crotalus vegrandis, Crotalus durissus ruruima,* and *Crotalus pifanorum* venoms.

These venoms were electrophoresed on a 10% SDS gel using a Mini-Protean II system at 150V (Bio-Rad PowerPac Basic) for one hour. Then, were transferred onto a 0.2 μm nitrocellulose membrane (Millipore) using a Trans Blot SD system at 100 mA for one hour. After blocking the membrane for one hour at room temperature with 5% skimmed dry milk in PBS buffer pH 7.4 containing 0.05% (w/v) Tween 20, the nitrocellulose membrane was incubated under stirring for one hour with anti-*Crot*/pool ostrich IgY antivenom diluted to 1:1000 in PBS-Tween 20. After the rinses, the secondary antibody goat anti-avian IgY (coupled to horseradish peroxidase) diluted 1:20000 in PBS-Tween 20 was complemented. Finally, blots electrophoretic bands identified by *Crot*/pool ostrich IgY antivenom were colorimetric visualized using the peroxidase substrate (TMB), and the image was analyzed.

Lethality Dose (LD_{50})

Five groups of five NIH female mice (*Mus musculus*) for *Crot*/pool venom were maintained in plastic boxes (Tecniplast, Italy) and observed throughout the quarantine period and experiments. The endpoint of lethality of the mice was established after 48 hr. The venom was suspended in 0.85% saline at the maximum test dose per mouse. Serial dilutions of 2-fold using saline solution were prepared to obtain four extra concentrations. All solutions throughout the experiment were kept at 0°C and warmed to 37°C before being injected into mice. The lethal toxicity was determined by injecting 0.2 mL of venom (containing dosages ranging between 38.0 to 11.6 μg/mouse) into the peritoneum of 18–20 g female NIH mice. The injections were dispensed using a 1-mL syringe fitted with a 25-gauge, 0.5-inch needle. Saline as normal controls were used. The lethal dose fifty (LD_{50}) was calculated following the

Spearman-Kärber (n = 3 ± SD) method [7]. The estimated LD_{50} was then used for testing median effective dose fifty ($ED_{50}/2\ LD_{50}$).

Antivenom neutralization test: median effective dose fifty ($ED_{50}/2\ LD_{50}$) assays of yielded antibodies (anti-Crot/pool ostrich IgY antivenom neutralizing lethal toxic activity of Crot/pool venom)

The median effective dose value (ED_{50}) of the *anti-Crot/pool* ostrich IgY antivenom from ostrich egg yolk was measured for analysis of quantitative and categorical data, with the aid of the Prism 8 program (Graphpad, USA). Five groups of five female NIH mice (18–20 g) were confronted with a combination of serial dilutions of a certain amount of anti-*Crot*/pool venoms (1.488, 1.395, 1.321, 1.145, 0.843), containing constant concentration of *Crot*/pool venom (33 µg). $2LD_{50}$: (LD_{50}=16.5 μg/mouse x 2 = 33 μg venom/20 g mouse). The Ostrich IgY anti-*Crot*/pool venom/ *Crot*/pool venom combinations were pre-incubated for 30 min at 37°C, then was intravenously injected into mice for each dose. Negative control mice were injected with two LD_{50} of venom alone. The neutralizing potency reproduces the ratio of mL of anti-*Crot*/pool venom /mg of *Crot*/pool venom or mg of antivenom/mg of venom. The control group was also injected with venom pre-incubated with normal ostrich serum.

RESULTS

Immune response

Two female ostriches were immunized as described in Materials and Methods. Detectable specific IgY anti-venom responses were not observed in sera until 15 days from the initial dose, by the time of the first booster-dose. This secondary response was sustained thereafter by successive booster injections given at 45-days pauses.

IgY purification

At a pH of 5-6 most of the immunoglobulins were recovered and the caprylic acid

precipitation leads to a maximum recovery of IgY, with minimum contaminating proteins. After ammonium sulfate purification, the obtained sample under reduction conditions gave two mains electrophoretic IgY bands of ~65 and ~20 kDa. Otherwise, under non-reducing conditions a IgY single band of ~175 kDa was observed (Fig. 2). The average recovery of IgY from a single egg yolk was ~ 3800 mg (N=5).

Immune-specific recognition of crotalic venom pool by IgY

The anti- *Crot*/pool venom-specific activity of IgY in partially purified preparations was assayed by a home-designed indirect ELISA as described in Materials and Methods. The purification procedure resulted in venom-specific IgY recognition of *Crot*/pool venoms (Fig. 3). Microtiter plates with pre-immune serum showed no binding.
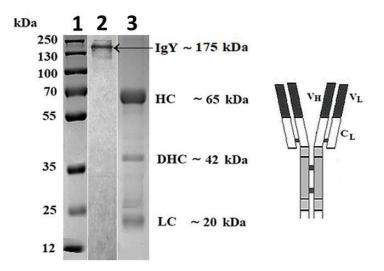

Fig. 2. IgY tested under native and reduction conditions. (**1**) Molecular mass markers. (**2**) IgY (native conditions); (**3**) Under reducing conditions HC: Heavy chain; DHC: Detritus heavy chain; LC: Light chain; VH: Variable fraction of heavy chain; VL: Portion variable light chain; CL: Constant fraction of light chain.
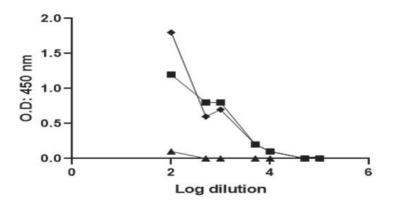

Fig. 3. ELISA immuno-specific recognition of IgY. The anti- *Crot*/pool venom-specific activity of IgY showed that final product (rhombus) and final product dialyzed (square) resulted in the venom-specific IgY recognition of *Crot*/pool venoms. The pre-immune serum (triangle) was negative.

Specificity of Crot/pool antivenom (IgY) to *Crotalus* venoms via Western blot

The identity of the *Crot*/pool venom proteins was confirmed with the *Crot*/pool antivenom (IgY) by Western-blot, identifying most of the fraction venoms used in the present work (Fig. 4). As a reference, the polyvalent antivenom of equine origin produced by BIOTECFAR C.A (Caracas, Venezuela) was used.

Antivenom effective dose (ED$_{50}$)

In order to estimate the ED$_{50}$ of the final *Crot*/pool antivenom (IgY), different amounts of this antivenom were preincubated with 2LD$_{50}$ (33 µg) of a *Crot*/venom pool, as described in Materials and Methods. An ED$_{50}$ of 19.66 mg was calculated for the *Crot*/pool antivenom, as the minimum amount of purified IgY preparation able to protect 50% of the mice population. There were no survivals in the control group (Table 1, Fig. 5).

DISCUSSION

Snakebite accidents, categorized as a neglected tropical disease by the World Health Organization (WHO) is responsible for nearly 50.000 deaths annually, mostly in Third World countries [8].

In the current work specific anti-*Crotalus* snake venoms antibodies were obtained by an immunization schedule in female ostriches *(Struthio camelus)*. The eggs from bird species have demonstrated being a desirable basis for the production of antibodies, without invasive methods, which present a predominant class of IgY immunoglobulin [3, 9,10]. This IgY has been used in diagnosis, research, and immunotherapy [11-13]. Furthermore, bird's immunoglobulins production poses several benefits over mammalian antibodies with respect to the antigenic specificity and low manufacturing expenses [3,12]. It was possible to obtain about 2-4 g of IgY per ostrich egg. Hence, almost 400 g of IgY can be purified from only one ostrich per year. For that reason, ostrich eggs could represent an exceptional source of immunoglobulins for antivenom production.

A great amount of cross-reactive and neutralizing antibodies (IgY) were produced in the egg yolk of ostriches (*Struthio camelus*) by immunizing with the venom of four *Crotalus* (*Crot*/pool) using a simple and
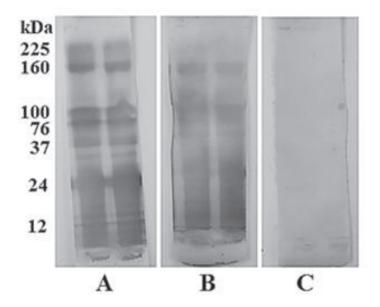
Fig. 4. Western blot. (A) *Crot*/pool venom+ PAOS (Biotecfar C.A, Venezuela); (B) *Crot*/pool venom+ immunized ostrich IgY; (C) Negative control (Non-immunized ostrich IgY+PBS).

Table 1

Determination of the survive percentage of mice after the injection of $2LD_{50}$ of *Crot*/pool venom with different concentrations of ostrich IgY. Evaluation of the neutralizing capacity of anti-erotalic IgY antibodies.

LD50 16.5 (μg/mouse)

Concentration (mg/mL)	Vol. (mL) Mx	Amount of proteins (mg)	Vol. (mL) Venom	Amount of injected venom (μg)	Vol. (mL) Saline solution	Final Vol. (mL)	Inoculated animals	Dead	Alive	Accumulated Deaths	Accumulated Alive	% survival
	1.655	30.78	0.330		0.015	2	5	0	5	0	13	100.00
	1.335	24.83			0	1.5	5	1	4	1	8	88.89
18.6	1.125	20.93		33	0.375	1.5	5	2	3	3	4	57.14
	0.750	13.95	0.165		0.750	1.5	5	4	1	7	1	12.50
	0.375	6.98			1.125	1.5	5	5	0	12	0	0.00
	0.190	3.53			1.310	1.5	5	5	0			

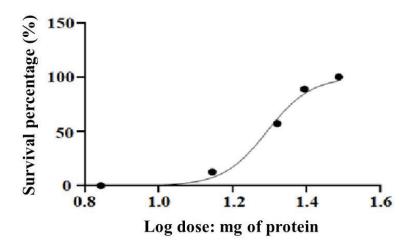LD_{50}: Lethal dose fifty; Vol: Volume.

Fig.5. Curve of Log doses of mg of protein with the percentage of survival animals. An ED_{50} of 19.66 mg *Crot/* pool antivenom (IgY) was calculated from the represented data as the mass of IgY that was able to protect at least 50% of the mice population against a $2LD_{50}$ (33 μg) challenge of *Crot*/pool venom. In the control group there were no survivals. (%): percentage. (Log): logarithm.

inexpensive method. Knowledge of venom variations permits the selection of suitable specimens for production of more effective antivenoms and biological substances.

The anti- *Crot*/pool venom immunoglobulins were carried out from egg yolk by the McLaren RD et *al.* [5] method and given a single pure electrophoretic IgY band of 174 kDa (native conditions) and 65/20 kDa (under reduced conditions) in the SDS-PAGE.

Neutralization experiments demonstrated a high neutralization capacity of the anti- *Crot*/pool preparation, as a preincubated mixture of both purified antivenom (19.66 mg) with two LD_{50} dose of *Crot*/pool venom (33 μg) protected 50% of the mice.

It has been showed since the early nineties (1990) that chicken egg yolk and their IgY immunoglobulins were able to neutralize scorpion and rattlesnake venoms, confirmed by *in vivo* experiments performed in rodents [13]. Comparing hens with ostriches, the ostrich is one of the most primitive living avian suggesting that the diverse features of the bird Ig genes appeared very early during the divergence of the avian species and are thus common by most, if not all, bird's species [14].

Antibodies are competent of explicitly recognizing a wide diversity of antigens with different affinities. Affinity, simultaneously with avidity is closely related to sensitivity, which is an experimentally measurable value in terms of antibody titer. Affinity is the concentration of the antigen that is needed to occupy the binding sites, of half of the antibody molecules present in an antibody solution, while avidity is the universal summation of the affinities of various antibodies (bivalent, multivalent and of different isotypes) fixed to all places to all available epitopes, considering conformational and valence aspects of the antibody [15]. Authors [3] have developed successful anti-coral snake venom IgY antibodies, which were carried out in chicken egg yolks and their neutralizing action was similarly presented in mice by *in vivo* counteraction experiments. These antivenom immunoglobulins neutralized the toxic and lethal consequences of venom and, accordingly, could function to treat coral snake envenomed victims. Similarly, authors [16] have also produced hen antibodies against *Scolopendra gigantea* toxins with high neutralization titers and antivenom for the treatment of scolopendrism [17].

The present experimental work refers to the production of anti-(*Crot*/pool) antivenom in ostrich egg yolk and its ability in deactivating the lethal consequences of the above mentioned *Crotalus* venoms. Immunization of ostriches with *Crot*/pool venom provoked a characteristic primary humoral response of low antibody titer in the ostrich sera and the egg yolks at 15 days, followed for a higher secondary response. The immunoglobulins titres amplified after the second booster and the intensities were sustained by continuing boosters. Ostrich eggs kept at 5°C during a year exhibited no substantial reduction in the antibody titers.

The caprylic acid [18, 19] method showed that under controlled pH conditions, at a constricted pH range of 5.3–6.3 and low ionic strength of acidified water simplified the separation of IgY, after the yolk lipids aggregation, producing a clear IgY enriched supernatant, which was confirmed by SDS-PAGE analysis. This technique has been used for experimental purposes [18], but actually is used in the production of horse antivenoms [19], with novel fractionation approaches for antivenom production with the purpose of achieving antivenoms of higher purity, which would stimulate less allergic reactions in snake bitten patients treated with this product.

In the current work, it has been established by an immunoblot and/or ELISA assays that the anti-(*Crot*/pool) antivenom IgY recognized and responded to *Crot*/pool venom proteins. Immunoblot analysis revealed not only the specific binding of the antivenom but also dose-dependent blocking of antivenom by venom proteins.

The outcomes of inhibition studies show a specific neutralizing capacity of venom activity in the experimental anti-(*Crot*/pool) IgY antivenom. This ending activity was regularly dose dependent, presenting ample inhibition at a concentration of $2LD_{50}$ ($33\mu g$) of *Crot*/pool venom, being indicative of specific binding of the IgY antibodies to venom proteins to which they were developed and their capacity to block the lethal effects of *Crot*/pool venoms. These types of immuno-neutralization help reverse toxicity and define the kinetics of toxins and antibodies.

Until now, traditional treatment of snakebite accidents is based on the use of antivenoms from horse's origin. Nevertheless, equine serum in theory could activate complement cascade and initiate acute hypersensitivity reactions in patients formerly sensitized to horse serum proteins [20]. The immunoglobulin IgY has the capacity of eliminating this possible side effect since it does not react as the mammalian IgG performs and it does not activate the mammalian complement factors [9]. For that reason, the horse serum mammalian IgG activating mammalian complement factors [20] does not permit physicians to give out much larger doses of horse antivenom.

Regarding recent developments of chicken antivenoms, Latin American authors [17] have characterized IgY antivenoms capable of neutralizing the lethal activity of *B. alternatus* snake venom, at a preclinical level. An antivenom, as an alternative to the conventional antivenom production with egg yolk antibodies (IgY-technology) was proposed. Similarly, to the results presented in the current work, antivenom efficacy assays were carried out by them and after successive immunizations, levels of specific IgY reached a maximum that was maintained throughout the observation period; IgY antivenoms obtained after several immunizations neutralized $35.65~\mu g$ of *B. alternatus* venom per mg of antivenom. Other authors [21] proposed that birds were excellent hosts for the production of neutralization antibodies at low cost. These antibodies could be applied in the development of diagnostic kits or as an alternative for snakebite envenomation handling in the immediate future.

In conclusion, the specificity and specific activity of the antibody were scrutinized by western blotting and confirmed the presence of highly specific antibodies to *Crot*/

pool venoms in the treated ostrich egg yolk. Ostrich's antivenom can represent an excellent alternative for producing high amounts of antivenoms at very low costs [22]. Thus, they could be a very good option to treat these accidents in countries with low economic resources where the ophitoxemia is a collective health problem. Therefore, the cleanliness, efficiency, and simplicity of producing antivenoms in ostriches, and the inability of these antibodies (IgY) to bind to the human complement formulates an interesting alternative to other antivenoms produced in mammals. These findings point out that ostrich egg antibody can be helpful as a therapeutic instrument to treat snakebites in humans, cattle and domestic animals.

In addition, these results open a therapeutic field, for the manufacturing of other antivenoms against the broad spectrum of toxins and also as a probable diagnostic tool.

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Declaration of conflict of interest

The authors pronounce that they have no known competing financial interests or personal associations that could give the idea to influence the work described in this work.

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Authors' contribution

MAL, FT and ARA envisioned and planned research; CB, JCJ, MVC and ARA carried out experiments; CB, FT, JCJ and ARA analysed data; CB, FT, JCJ and ARA elucidated results of experiments; CB and ARA drafted manuscript; MAL, CB, FT, JCJ and ARA edited and revised manuscript; MAL, CB, FT, JCJ, MVC and ARA accepted final version of manuscript.

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