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Research Article
Comparison of Neutralization of Two Experimental Monovalent Antivenoms of Colombia's *Bothrops asper* from Different Localities

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Abstract

Background and Objectives: Colombia does not produce fabotherapic antivenoms, the aim of current study was comparison of the neutralization of *Bothrops asper* venom from Atlantic and Andean region with 2 experimental monovalent antivenoms. **Materials and Methods:** The protein of the venom and antivenoms was quantified by absorbance at 280 nm and characterized by SDS-PAGE. The protein profile venoms were performed by RT-HPLC. It was calculated LD_{50} for the venoms and ED_{50} for the antivenoms, using Prism-statMate. An immunization scheme of 3 months was conducted in equine using Andean venom. The complete IgG antivenom was produced with caprylic acid, while the fabotherapic F(ab')2 antivenom with pepsin. Both antivenoms were tested with Andean venom and Atlantic venom. Electrophoresis was conducted to verify the antivenoms by weight. **Results:** The protein was 11.5 mg/mL to Andean venom and 13.4 mg/mL to Atlantic venom. The SDS-PAGE showed bands of 15-20 kDa for both venoms. The LD_{50} to Andean venom was 2.8 and 2.3 mg/kg to Atlantic venom. The RT-HPLC showed similar protein fractions at first 40 min in both venoms. The protein for IgG was 9.8 mg/mL, while for F(ab')2 it was 11.2 mg/mL. The SDS-PAGE showed bands of 130-200 kDa for IgG whyle of 94-150 kDa for F(ab')2. The ED_{50} with Atlantic venom was 1.4 mg/mL using IgG and 1.75 mg/mL using F(ab')2. The ED_{50} with Andean venom was 1.6 mg/mL using IgG and 1.9 mg/mL using F(ab')2. **Conclusion:** Both antivenoms were efficient to neutralize the venom of Andean and Atlantic venom, without significant differences.

Key words: *Bothrops*, snake venom, antivenoms, Colombia, *Viperidae*, toxicity, lethal dose 50, neutralization test, immunoglobulin fab fragments

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

In Colombia for the year 2017 around 5,000 cases of snakebites were registered, 96 cases on average/epidemiologic week[1] with 1% mortality and 5.4% disability[2,3]. The snakes of the genus *Bothrops* are highest morbidity and mortality due to snakebite in Colombia and *B. asper* is considered responsible for about 80% of deaths[1], for which studies of the composition of the venom are constantly carried out to also to improve the quality of antivenoms[4].

The treatment in Colombia to counteract the snakebites poisoning includes purified complete immunoglobulins of equine origin, however, given their molecular size they may produce adverse reactions[5]. Fabotherapic antivenoms are comprised by ab fragments known as Fab[6,7] or by united ab fragments known[8] as F(ab')2 which possess a lower molecular weight than the complete immunoglobulins, reducing thus the probability of producing adverse reactions[9]. The venom is the first link to look for the specificity in the production of immunoglobulins, given the fact that the answer of the immune system depends on the epitopes to which the animal assigned for the production of antibodies is exposed[10]. These may have 4 direct and indirect action mechanisms to neutralize the venom depending on the procedure used in their preparation: (a) Acknowledgment of epitopes of the toxin by the paratopes of the antibodies, (b) Neutralization through steric hindrance when the epitope is located surrounding the toxin, (c) Reduction of the capacity of the toxin and (d) Formation of immune complexes with the venom toxins[11]. For this reason, the purpose of this study was considered to generate 2 experimental monovalent antivenoms as of immunization with *Bothrops asper* venom of Andina ecoregion (ANDIBV) and demonstrate its efficiency to neutralization of ANDIBV and *Bothrops asper* venom of Atlantic ecoregion (ATLBV).

MATERIALS AND METHODS

Study area: This project was carried during 24 months 2016-2018 at Pontificia Universidad Javeriana, Colombia after approval by committees from different institutions.

Murines: The toxicological tests corresponding to median lethal dose (LD_{50}) and median effective dose (ED_{50}) were conducted in strain Balb-c mice with weight between 18-20 g, male and female, arising from and maintained in the comparative biology unit (UBC) of the Pontificia Universidad Javeriana (PUJ). With temperature control at 23±3°C, *ad libitum* food and water, according to the WHO production guide[12] and to the Manual of Immunological Methods of the Clodomiro Picado Institute (ICP)[13]. The animals were maintained in the UBC in stainless steel boxes with shavings bed, in groups of 3 and a notch on the right ear identified them. The boxes were marked according to the treatment established for each group of animals. The format of use of animals (FUA) and formats of assessment of animal welfare were handled. The necrosis of the tail in <24 h was established as final point criterion.

Equine: A local horse was obtained by agreement with the Fundación Universitaria Agraria de Colombia (Uniagraria) and it was kept at the Center of Technological Research and Development (CIDT), Pinares de Tenjo, which belongs to Uniagraria. The equine weighted 380 kg, it was 4 years of age, 3.8 of body composition, complete vaccination scheme according to the Colombian Agricultural Institute (ICA), in adequate health condition certified by a veterinarian specialized in equines. A 3 week quarantine was conducted for adaptation and removal of parasites of the equine. The standardized care indicated by the CIDT was applied, under complementary pasture land, 2 kg feed concentrate/day, in controlled health conditions according to the recommendations of the guide for the production, control and regulation of immunoglobulins and antivenoms against snakes[11]. The horse was observed after all the inoculations with ANDIBV during the immunization scheme. A veterinarian belonging to Uniagraria conducted the care of the animal, the processes involved in the production of immunoglobulins and a veterinarian specialized in equines.

Venoms: A venom pool was obtained from 2 adult samples of *B. asper* from Caldas, Colombia's Andean eco-region. In addition, a venom pool was obtained from two adult samples of *B. asper* from Bolivar, Colombia's Atlantic ecoregion. Both venoms were lyophilized and stored at-20°C according to the international norm[12]. The total concentration of protein was mean calculated determined by absorbance at 280 nm by direct measurement by Nanodrop®, using 2 µL of each venom of diluted venom in HPLC water. The protein profile venom was performed by reverse HPLC, using a Lichosper 100 RP C18 column of dimensions 250×4 and 5 mm pore. The HPLC was run for 180 min in a linear gradient of 5-75% solvent B (95% acetonitrile concentraining 0.1% trifluoroacetic acid (TFA) with solvent A (5% acetonitrile 0.1% TFA) as starting and equilibration eluent[14]. The flow rate of column eluates was set at 1 mL/min and monitored[15,16] at UV215 nm. The venom was analyzed by electrophoresis SDS-PAGE (polyacrylamide gels 15%) under reducing conditions[16] and staining with Coomassie blue R-250.

Table 1: Immunization scheme and components used in each dose

Days	Venom (mg)	Inactivation	Adjuvant	Vehicle	Sample gathering post inoculation
0					Hemogram, hepatic profile
1	0.5	EDTA 10 nM	Complete Freund		Hemogram, hepatic profile
15	1.0	EDTA 10 nM	Incomplete Freund		Hemogram, hepatic profile
30	2.0	EDTA 10 nM	Gel $Al(OH)_3$		Hemogram, hepatic profile, proteins
45	3.0		Gel $Al(OH)_3$		Hemogram, hepatic profile
55	4.0			NaCl	Hemogram, hepatic profile
65	4.0			NaCl	Hemogram, proteins, hepatic profile
90					Bloodletting

EDTA: Ethylenediaminetetraacetic acid, Complete Freund: Consists of an aqueous solution with Ag, together with a mineral oil and a dispersing agent, but incorporates a heat-dead mycobacterium suspension, complete induces granulomas equally better, Incomplete Freund: Consists of an aqueous solution with Ag, together with a mineral oil and a dispersing agent, Gel $Al(OH)_3$: Aluminum hydroxide, NaCl: Sodium chloride

LD_{50}: The LD_{50} was determined for the ANDIBV and ATLBV using the technique described by the WHO[12] for the norms of security, asepsis and antisepsis for handling laboratory animals. The toxicological tests were conducted in 2 phases to satisfy the principle of reduction in the use of laboratory animals. In first phase, only 1 mouse was used/dose, with a sequential factor with 5 different doses including a positive control, considering the theoretical LD_{50} reported for said gender in Colombia. Second phase was conducted using 3 animals/group of treatment, with 5 different doses including the positive control. It was not necessary to make repetitions. The inoculation was conducted in all cases intravenously in the tail's lateral vein, with an approximate final volume of 0.1 mL adjusted with 0.9% saline solution. After the inoculation of the dilution, the animals were observed during 24 h, with special attention to the behavior and final point criterion and the mortality was indicated at the end of the 24 h period. The results were analyzed with the Prism StatMate (GraphPad Inc., San Diego, CA) combined software.

ED_{50}: The neutralizing power of the IgG and F(ab')2 antivenoms was determined applying the ED_{50} technique under the norms of security, asepsis and antisepsis for handling laboratory animals[14]. The ED_{50} was conducted in both antivenoms with different doses, regarding 3 LD_{50} of ATLBV and 3 LD_{50} of ANDIBV in different test.

The ED_{50} was conducted in 2 phases to satisfy the principle of reduction in the use of laboratory animals. First phase 1 mouse was used/dose, with a sequential factor with 4 different antivenom doses including the negative control. Maximum three repetitions were conducted. The 2nd phase was conducted using 3 animals/group of treatment, with 5 different doses including the negative control. No repetitions were necessary. The inoculation was conducted in all the cases intravenously in the tail's lateral vein of the animal, with an approximate final volume of 0.1 mL adjusted with 0.9% saline solution.

After the inoculation of the dilution, the animals were observed for 24 h, with special attention to the final point criterion and the survival rate after the 24 h period was indicated. The results were analyzed with the Prism StatMate (GraphPad Inc., San Diego, CA) combined software and finally expressed in mg of neutralized venom/1 mL of antivenom.

Antivenoms

Immunization scheme to obtain the antivenoms: The immunization scheme described by De Roodt *et al.*[17], with the collaboration and supervision of a veterinarian specialized in equines was used. During the process, no adverse reaction was observed in the equine. Subcutaneous inoculation was conducted in the neck table in cranial-caudal sections, with venom doses established according to the LD_{50}. The amounts of ANDIBV and adjuvant, the days of inoculation and sample gathering are summarized in Table 1.

Sample gathering: With the aim of determining the response of the horse, samples of 10 mL of plasma were gathered through the jugular vein of the animal, as of day 0 and blood cells and hepatic profile were measured. In addition, on days 30 and 65 the production of proteins and the immune response were evaluated using electrophoresis in polyacrylamide gels in the presence of sodium dodecyl sulfate (SDS-PAGE). On day 90 the extraction of approximately 3 L of complete blood was conducted.

Bloodletting: Bloodletting was conducted under the direction of the veterinarians with the technique described by the WHO[12] and Núñez *et al.*[14]. For this procedure the animal was introduced into a restriction stable to avoid physical injury during the process. The conditions that could increase stress were reduced. After bloodletting feed concentrate and water were administered *ad libitum* and the animal was observed during the following 6 h period without finding any hemodynamic alteration signs. About 3 L of blood were obtained and refrigerated at 2-8°C, during the centrifugation procedure to separate plasma from erythrocytes.

Purification of immunoglobulins: The technique for the purification of the complete immunoglobulins was based on the standardized protocols available in research[13,18,19]. The complete IgG were obtained by fractioning 5% caprylic acid at pH 5.5. The fabotherapic antivenom was produced by digesting the plasma during 50 min with 1% pepsin at pH 3.0 (p/v)[13,20,21]. Then, the pH was adjusted at 7.0 and the purification was conducted with caprylic acid at a concentration of 7.0% (v/v). Both antivenoms were subject to diafiltration, formulated with sodium chloride and phenol. The pH was adjusted and the antivenoms were strained through sterile filters of 0.22 µ.

Specificity and purity of the fragments: The characterization of the protein profiles and the estimate of molecular weights of the antivenom proteins was conducted by the electrophoresis SDS-PAGE at 12.5% acrylamide/bisacrylamide. (polyacrylamide gels 12.5%)[17]. Each column 15 µg of protein were applied and staining with Coomassie blue R 250, according to the Otero *et al.*[22] protocol.

Statistical analysis: All data were calculated and analyzed through the Prism StatMate (GraphPad Inc., San Diego, CA) combined software.

RESULTS

The mean protein quantification was 11.5±0.5 mg/mL in ANDIBV and 13.4±0.4 mg/mL in ATLBV. The SDS-PAGE showed electrophoretic profile of 15-20 kDa bands under reducing conditions for both venoms (Fig. 1), however, components between 20-25 and 50 kDa were detected in both venoms. The ANDIBV showed one band of 220 kDa, while ATLBV one band of 160 kDa and both presented between 50-100 kDa bands were weakly stained indicating a lower abundance of these toxins in these venoms.

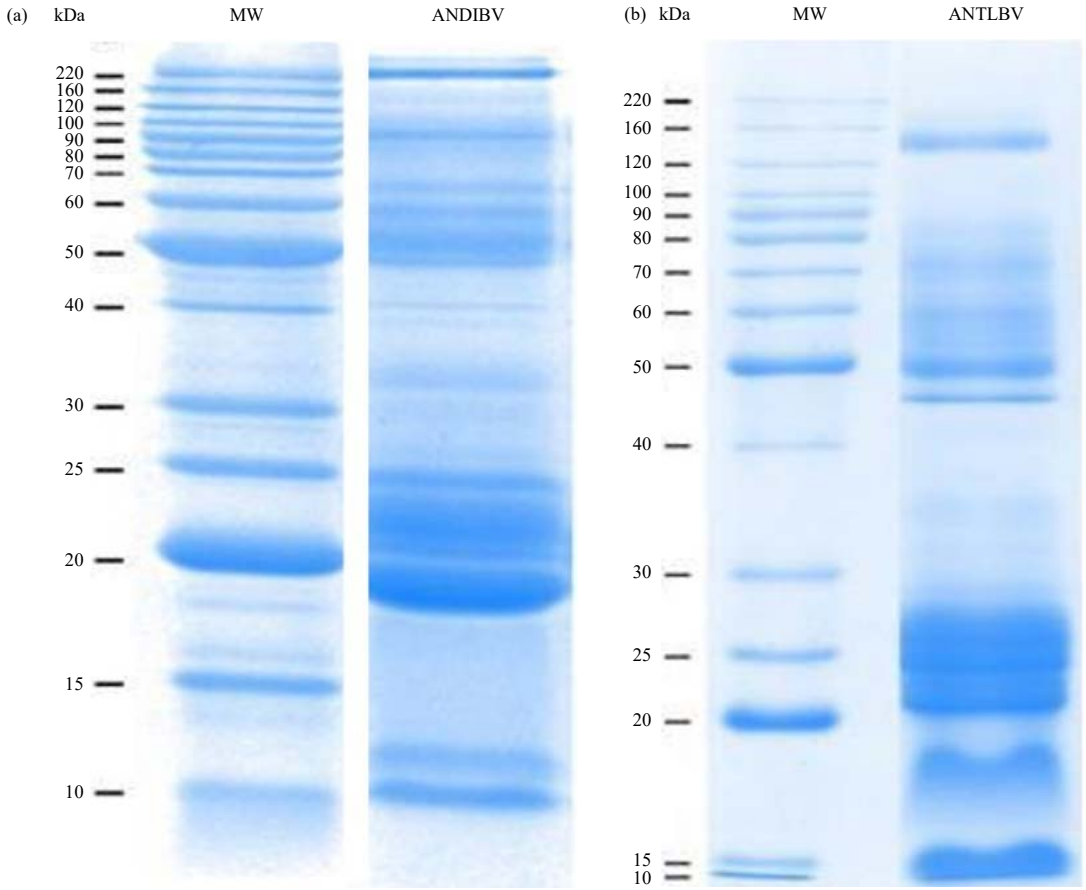

Fig. 1(a-b): SDS-PAGE, profiles of *B. asper* from different ecoregion, (a) Andean ecoregion and (b) Atlantic ecoregion
SDS-PAGE (polyacrylamide gels 15%) under reducing conditions with coomassie blue R-250, MW: Mobility of molecular mass markers, ANDIBV: Andean ecoregion *Bothops asper* venom, ATLBV: Atlantic ecoregion *Bothops asper* venom

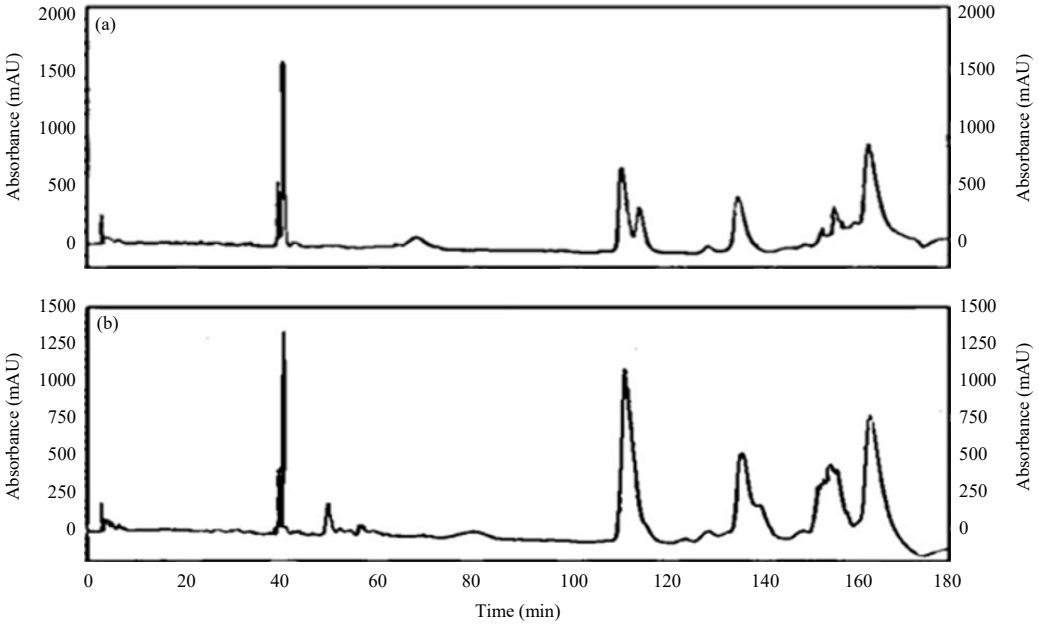

Fig. 2(a-b): RT-HPLC, profiles of *B. asper* from different eco-region, (a) Andean ecoregion and (b) Atlantic ecoregion

HPLC was run for 180 min in a linear gradient of 5-75% solvent B (95% acetonitrile concentraining 0.1% trifluoroacetic acid (TFA) with solvent A (5% acetonitrile 0.1% TFA) as starting and equilibration eluent, flow rate of column eluated was set at 1 mL/min and monitored at UV215 nm

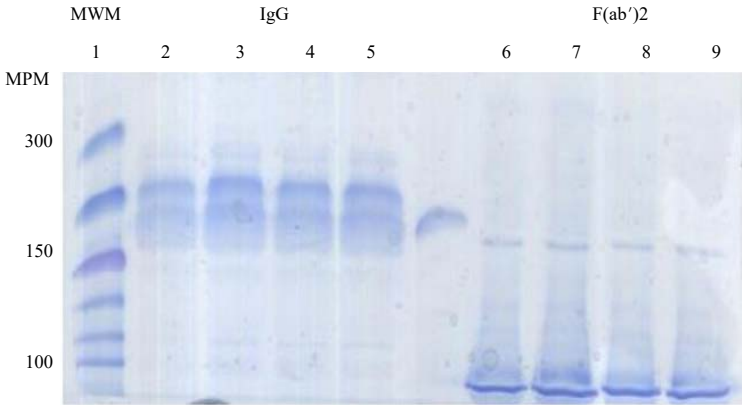

Fig. 3: SDS-PAGE, profiles of IgG and F(ab')2 antivenoms

SDS-PAGE at 12.5% acrylamide/bisacrylamide, each column 15 µg of protein were applied, column 1: MWM: Molecular weight markers, columns 2-5: Replicas IgG antivenom, columns 6-9: Replicas F(ab')2 antivenom, MPM: Migration of molecular weight

The LD_{50} to ANDIBV was found in 57.5 mg/mouse or 2.8 mg/kg and 45.7 mg/mouse or 2.3 mg/kg to ATLBV. It was observed similar protein profile between both venoms from *B. asper* by RT-HPLC between at same time to recollection. Figure 2 showed similar protein fractions and abundance at first 40 min, however, peaks of 110, 140 and 150 min showed little high abundance in ATLBV than ANDIBV.

About antivenoms, the quantification of total proteins for IgG antivenom was 9.8±1.5 mg/mL, while for F(ab')2 it was 11.2±0.9 mg/mL. The SDS-PAGE showed bands between 130-200 kDa in IgG while 94-110 kDa bands was evidenced in the F(ab')2 (Fig. 3).

The ED_{50} with ATLBV was calculated in 1.4 mg/mL with IgG and 1.75 mg/mL with F(ab')2. The ED_{50} with ANDIBV was calculated in 1.6 mg/mL with IgG and 1.9 mg/mL with F(ab')2. The neutralization was without statistically difference between these 2 antivenoms (Table 2).

Table 2: Summarizes the data found for the venoms and the antivenoms

Parameters	Units	ANDIBV	ATLBV	IgG	F(ab')2
Quantification of protein	mg/mL	11.5±0.5	13.4±0.4	9.8±1.5	11.2±0.9
Toxicity of the venom LD_{50}	mg/kg	2.8	2.3		
ED_{50} using ATLBV	mg/mL			1.4	1.75
ED_{50} using ANDIBV	mg/mL			1.6	1.90

ANDIBV: Andean ecoregion *Bothops asper* venom, ATLBV: Atlantic ecoregion *Bothops asper* venom

DISCUSSION

Regarding the power of the ANDIBV used in this study obtained was LD_{50} of 57.5 µg/mouse equivalent to 2.8 mg/kg and of the ATLBV 45.7 µg/mouse, or 2.3 mg/kg were found. These data are similar to those found by Otero *et al.*[22], who found 66.2 µg/mouse (equivalent to 3.48 mg/kg) for LD_{50} *B. asper*. García *et al.*[23] founded a range of LD_{50} between 50.2-79.2 µg/mouse (equivalent to 2.79 and 3.96 mg/kg) for *B. atrox*. Rojas *et al.*[24] obtained a LD_{50} between 5.12-7.12 mg/kg for *B. atrox*. Galindez *et al.*[10] founded a LD_{50} of 3.61 mg/kg for *B. atrox* and Segura *et al.*[25] founded the LD_{50} of 2.08 mg/kg for *B. asper*. This is consistent with previous findings in venom arising from samples of the *Bothrops* sp. gender and indicates that the venom was adequately obtained, transported and stored, capable of inducing the expected production of antibodies in the immunized animal.

About chromatographic profile of *B. asper* venom from the Atlantic and Andean regions was observed similar fractions of the venom proteins and in their relative abundance. Our results are similar with Scovino[15] and Pérez [4] where all 3 had low molecular weight proteins until approximately 80 min of run with height around 100 mAU, with the exception of two peaks, 1 and 2 in the venom from the Andean region (500 and 1,700 mAU), peaks 3 and 4 of the Atlantic region (500 and 1,300 mAU) and peaks 2 and 3 of the Pacific region (800 and 1,900 mAU), respectively. However, there are also differences with respect to relative abundance. Apparently, the location of these species does not affect the protein content of the venoms in Colombia, but if it could affect their abundances, at first glance, the unique and specific presence of a family of proteins for single venom is not noted, on the contrary, the profiles are similar in terms of the presence of proteins.

The neutralizing capacity of the antivenoms is one of the critical points in the quality control, expressed as average ED_{50}, which is defined as the volume of antivenom with which 50% of the mice injected with a specific venom dose survives. It is expressed in terms of mg of venom or number of average LD_{50} neutralized by mL of antivenom[26]. For the present study, the neutralizing capacity of the antivenoms was 1.4 mg/mL and of 1.75 mg/mL for IgG and F(ab')2, respectively using ATLBV, while, was 1.6 and 1.9 mg/mL for IgG and F(ab')2, respectively using ANDIBV. This is consistent with the results obtained by De Roodt *et al.*[17], who found theoretical power of the antivenoms against *B. alternatus* of 1.3 and 1.69 mg/mL for IgG and F(ab')2, respectively; against *B. diporus* of 1.81 and 1.28 mg/mL for IgG and F(ab')2, respectively and against *B. jararacussu* of 2.9 and 2.8 mg/mL for IgG and F(ab')2, respectively. The above indicates that the experimental results of this study are comparable with other works and that the methodology for the production and purification of the immunoglobulins was adequate[27,28].

In spite of the similarity of the results of this study with the findings of De Roodt *et al.*[17], we have also found great differences between the ED_{50} obtained by García *et al.*[23], who found a ED_{50} of 7.12 mg/mL for a commercial polyvalent Bothropic antivenom when challenged with *B. atrox* venom. The above indicates an adequate specificity of the antivenom used with a possible crossed reactivity in the neutralization of the venom. The same can be concluded when comparing the results of our work with those obtained by Patino *et al.*[5] obtained an ED_{50} of 6.1 and 3.7 mg/mL for a commercial polyvalent Both ropic antivenom (Antivipmyn-Tri® Batch B-2G-02) against *B. asper* and *B. atrox* venoms, respectively.

According to the findings of Gutiérrez *et al.*[28] the similarity between the neutralizing capacity of the IgG and F(ab')2 antivenoms in this study is possibly the result of the preparation process of the 2 antivenoms. On the other hand, the average life of the IgG and F(ab')2 antivenoms in the bloodstream can affect the antigen-antibody union rate, however, in the present study it was not possible to corroborate given the fact that no pharmacokinetics studies were conducted.

CONCLUSION

These results showed adequate neutralization for *B. asper* venom from two regions of Colombia, maybe because there is similarity of the protein profile of the venoms, independent of the use of IgG or F(ab')2 monovalent antivenoms, also the development with *B. asper* from one of these regions. It is relevant issue for the development of fabotherapic antivenoms and continuing research in Colombia.

SIGNIFICANCE STATEMENT

This study showed possible manufacture fabotherapic antivenoms against the venom of one of the most important species that cause high morbidity and mortality by snakebites in Colombia and there is evidence the F(ab')2 can reduce side effect due to their low molecular weight. It also showed there are not many differences in the composition of *B. asper* venom from different regions of this country. This study will help to provide evidence to improve the production of fabotherapic antivenoms as vital medicine not available in Colombia.

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