Clinical Toxicology (2014), **52**, 594–603

ISSN: 1556-3650 print / 1556-9519 online
DOI: 10.3109/15563650.2014.925561

informa
healthcare

RESEARCH ARTICLE

Protein content of antivenoms and relationship with their immunochemical reactivity and neutralization assays

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Context. Therapy for snakebites relies on the application of antivenoms, which may be produced with different immunogenic mixtures of venom and possess different pharmaceutical characteristics. For these reasons, immunological cross-reactivity and heterologous neutralization were analyzed relative to the protein content of three antivenoms used in the Americas. *Methods.* The antivenoms studied were composed of equine $F(ab')_2$ fragments from animals immunized with Crotalinae venoms. The antivenoms were tested against venoms of seven pit viper species from Argentina, seven from Mexico, one from Costa Rica, and one from Colombia. *Results*. Immunoblotting showed high cross-reactivity of all major protein bands with all the antivenoms tested. ELISA results also showed high cross-reactivity among the different venoms and antivenoms, and a high heterologous neutralization was observed. The results can be interpreted in different ways depending on whether the reactivity is considered in terms of the volume of antivenom used or by the amount of protein contained in this volume of antivenom. The antivenoms with high immunochemical reactivity and neutralizing capacity were those with higher protein content per vial; but when doses were adjusted by protein content, antivenoms of apparently lower neutralizing capacity and immunochemical reactivity showed at least similar potency and reactivity although volumetrically at higher doses. *Conclusion.* Protein content relative to neutralization potency of different products must be taken into account when antivenoms are compared, in addition to the volume required for therapeutic effect. These results show the importance of obtaining high-affinity and high-avidity antibodies to achieve good neutralization using low protein concentration and low-volume antivenoms.

Keywords Snakes; Toxinology; Antivenins

Introduction

One of the most controversial subjects in the treatment of snakebites is the use of heterologous antivenoms (antivenoms produced by immunizing animals with venoms from snakes different from, but related to, those intended to treat), and the principal controversies involve the neutralizing capacity and the dose of antivenom required for neutralization. Relative to neutralizing capacity, several studies have indicated that homologous antivenoms (those produced by immunization of animals with the venoms of the snakes whose venom must be neutralized) are the best choice for the treatment of snakebites.[1–5] International organizations strongly recommend the use of homologous antivenoms, and their use as the first choice to treat envenomations is unquestioned. Despite this, the utility of heterologous antivenoms has been recognized since the beginnings of serum therapy.[6–9] A high degree of immunochemical cross-reactivity among different antivenoms and snake venoms has been experimentally and clinically established using antivenoms not only in which the venom immunogens used in production are from taxonomically related snakes, but also when these are from distantly related snakes.[9–19]

One of the most serious safety concerns in antivenom use is the amount of protein contained in the pharmaceutical preparation, because this is directly related to the eventual dose of foreign protein to which patients will be exposed.[8] However, the effectiveness of different immunogenic mixtures used for antivenom production is commonly compared between products with different pharmaceutical formulations, including protein content, grade of purification, whole versus digested immunoglobulins, and so on.[20,21] If these parameters are not fully taken into consideration, misconceptions can arise regarding the true effectiveness of different immunizing

Received 18 January 2014; accepted 13 May 2014.

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mixtures in rendering good neutralizing antibodies, as well as about the neutralizing potency of an antivenom. This may result in the rejection of potentially useful products against related venoms in different geographic regions, and it discourages their production even where homologous venoms are unavailable. These are not trivial considerations, as the only quasi-specific treatment of snakebites available in some regions of the world is heterologous antivenom.[8,22]

Immunogenicity and antigenicity of venoms of Crotalinae snakes have been compared among North American species, Central American species, and South American species.[10,11,23–33] All of these studies have concluded that there is an extensive immunological cross-reactivity among the analyzed venoms and that high cross-neutralization is achieved with heterologous antivenoms. Nevertheless, neutralization can be interpreted very differently when the physical characteristics of the antivenoms such as protein content are considered.[20] In this work, we extend these observations for other venoms and antivenoms of the Americas. We compare the immunochemical reactivity and neutralizing capacity of three antivenoms for therapeutic use, and interpret these results with respect to the volume of antivenom required to achieve neutralization and the amount of protein required for this purpose.

Materials and methods

Venoms

Venoms were obtained from adult specimens by manual extraction, vacuum-dried, and storage at −20°C. Venoms used were of specimens of *Bothrops (B.) alternatus, B. ammodytoides, B. neuwiedi, B. moojeni, B. jararaca, B. jararacussu*, and *Crotalus (C.) durissus (d.) terrificus* (Argentina), *B. atrox asper* (Colombia), *B. asper, B. undulatus, Athropoides (Ath.) nummifer, C. basiliscus, C. d. durissus, C. scutulatus*, and *Agkistrodron (Agk.) bilineatus (b.) bilineatus* (Mexico) and *Lachesis (L.) muta stenophry* (Costa Rica). Additional details are provided in the Supplementary Appendix available online at http://informahealthcare.com/doi/abs/10.3109/15563650.2014.925561.

Antivenoms

The antivenoms used in this study are routinely used for the treatment of snakebite in South America and Mexico. These antivenoms are $F(ab')_2$ fragments of equine immunoglobulins. They include the antibothropic Botrópico Bivalente (produced in Argentina, henceforth Both-2), the antibothropic Soro Antibotrópico (produced in Brazil, henceforth Both-5), and the antibothropic—crotalic Antivipmyn™ (produced in Mexico, henceforth Both-Crot). All experiments were conducted during the validity periods of the antivenoms. Snake venom species used for immunization and other details are provided in the Supplementary Appendix Supplementary available online at http://informahealthcare.com/doi/abs/10.3109/15563650.2014.925561.

Animals for bioassays

For the determination of toxic activities and neutralization test, in mice, techniques suggested by the WHO[7,8,38] were used. For care and management of animals, the recommendations of the National Research Council were followed.[39] The Institute of Biotechnology (Cuernavaca, Morelos, Mexico) Committee for Animal Welfare approved the experimental protocol for animal management.

Methods

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blot

These were carried out as described by Laemmli[34] and Towbin et al.[35] For details, see the Supplementary Appendix available online at http://informahealthcare.com/doi/abs/10.3109/15563650.2014.925561.

Solid-phase enzyme immunoassay (ELISA)

This was carried out according to published procedures with some modifications.[36] For details, see the Supplementary Appendix available online at http://informahealthcare.com/doi/abs/10.3109/15563650.2014.925561.

Competitive solid-phase enzyme immunoassay

Inhibition of binding of Both-Crot or Both-2 to solid-phase-bound venoms by competing with antigens in solution was carried out according to the strategy described by King et al.[37] For details, see the Supplementary Appendix available online at http://informahealthcare.com/doi/abs/10.3109/15563650.2014.925561.

Toxicity and Neutralization assays

Lethal potency

For determination of lethal doses, Molinengo's method modified by Meier and Theakston[40] was used. For details, see the Supplementary Appendix available online at http://informahealthcare.com/doi/abs/10.3109/15563650.2014.925561.

Determination of hemorrhagic activity

This was determined as described by Theakston and Reid, and modified by Ferreira et al.[38,41] For details, see the Supplementary Appendix available online at http://informahealthcare.com/doi/abs/10.3109/15563650.2014.925561.

Determination of procoagulant activity

This was determined on human normal plasma as described by Theakston and Reid.[38] For details, see the Supplementary Appendix available online at http://informahealthcare.com/doi/abs/10.3109/15563650.2014.925561.

Neutralization of lethal activity

This was determined in mice as the dose of antivenom that protected 50% of mice challenged with a dose of 5.0 LD_{50} of venom. For details, see the Supplementary Appendix available online at http://informahealthcare.com/doi/abs/10.3109/15563650.2014.925561.

Neutralization of hemorrhagic and coagulant activities

These were studied as suggested by the WHO.[7,8,38] For details, see the Supplementary Appendix available online

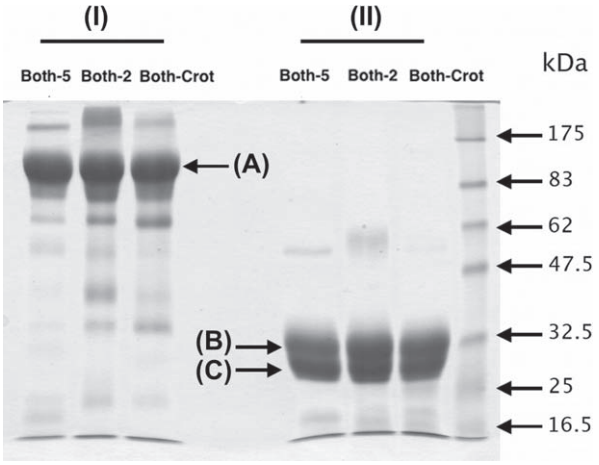

Fig. 1. SDS–PAGE of Both-2, Both-5, and Both-Crot antivenoms. 25 µg of protein was loaded per lane under non-reducing (I) and reducing (II) conditions. The position of molecular weight mass markers is indicated. (A), (B), and (C) point to $F(ab')_2$ fragments, pepsin-digested heavy chains, and light chains of immunoglobulins, respectively.

at http://informahealthcare.com/doi/abs/10.3109/15563650.2014.925561.

Protein determination

Proteins concentration of venoms and antivenoms was determined using the Biuret method. For details, see the Supplementary Appendix available online at http://informahealthcare.com/doi/abs/10.3109/15563650.2014.925561.

Statistics

For toxicity assays and for neutralizing assays, linear (Lethal dose) or nonlinear regressions by sigmoidal dose–response of variable slope were used (for neutralization assays). When possible, 95% confidence intervals were calculated. For these determinations as well as for means and standard deviations, when applicable, Prism 3.0 (GraphPad, Inc. CA) software was used.

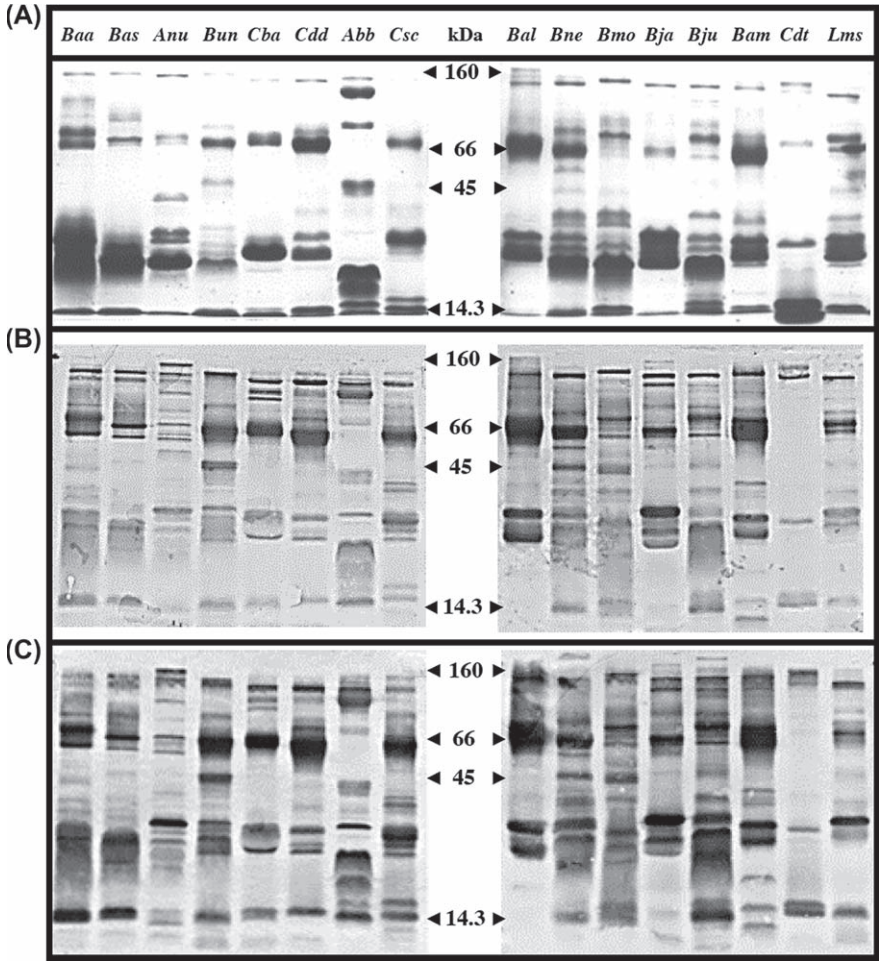

Fig. 2. (A) SDS–PAGE and (B and C) Western blots of venoms. Venoms were separated under non-reducing conditions. Both-2 and Both-Crot antivenoms were used as the probes in B and C, respectively. Whole immunoglobulin, bovine serum albumin, ovalbumin, and lysozyme were used as the molecular mass standards, and their positions are indicated by arrowheads. Abbreviations for venoms are as follows: Baa, *B. atrox asper*; Bas, *B. asper*; Anu, *Ath. nummifer*; Bun, *B. undulatus*; Cba, *C. basiliscus*; Cdd, *C. durissus durissus*; Abb, *Agk. bilineatus bilineatus*; Csc, *C. scutulatus*; Bal, *B. alternatus*; Bne, *B. neuwiedi*; Bmo, *B. moojeni*; Bja, *B. jararaca*; Bju, *B. jararacussu*; Bam, *B. ammodytoides*; Cdt, *C. durissus terrificus*; Lms, *L. muta stenophrys*. An important cross-reactivity was observed in all the cases with the components of high, medium, and low molecular weight.

Table 1. ELISA immunoreactivity of Both-2 and Both-Crot against North and South American snake venoms.

	Conventional Titers (μl)		Specific Titer Units (mg)	
Venoms	Both-2	Both-Crot	Both-2	Both-Crot
Bal	**58177 ± 5789**	15821 ± 8520	**617 ± 61**	1774 ± 955
Bne	**96270 ± 25194**	27227 ± 9239	**1021 ± 180**	3052 ± 1036
Bmo	78093 ± 11401	19890 ± 9580	828 ± 21	2230 ± 1074
Bja	61000 ± 21863	22943 ± 3931	647 ± 157	2572 ± 441
Bju	57260 ± 7848	26007 ± 14344	607 ± 111	2916 ± 1608
Bam	42957 ± 10728	12828 ± 3002	456 ± 174	1438 ± 337
Cdt	3401 ± 1349	9273 ± 4674	36 ± 62	1040 ± 524
Lms	35867 ± 10283	19470 ± 6114	380 ± 46	2183 ± 686
Baa	101503 ± 16517	19420 ± 812	1076 ± 175	2177 ± 91
Bas	96333 ± 17007	**25353 ± 5346**	1022 ± 180	**2842 ± 599**
Anu	24080 ± 1951	7935 ± 2188	255 ± 21	890 ± 245
Bun	78160 ± 14784	16077 ± 1682	829 ± 157	1802 ± 189
Cba	66203 ± 10476	27410 ± 11388	702 ± 111	3073 ± 1277
Cdd	46497 ± 16369	**10933 ± 1575**	493 ± 174	**1226 ± 177**
Abb	21770 ± 5818	9989 ± 5323	231 ± 62	1120 ± 597
Csc	78007 ± 4319	25727 ± 3634	827 ± 46	2884 ± 407

Conventional titers were calculated from the midpoint of the curve and correspond to half of maximal binding, expressed in microliters. Immunoreactivity was also calculated as specific titer units (TU) where one TU was defined as the amount of protein in the antivenom dilution resulting in half of maximal binding. Data are mean ± 1 standard deviation from triplicate experiments. Titers against venoms used as immunogens (homologous venoms) are given in boldface. Abbreviations for venoms are as in Fig. 2.

Results

Physicochemical characterization of the antivenoms

The amount of protein per vial of Both-2, Both-5, and Both-Crot was 943, 490, and 89 mg, respectively. When analyzed using gel electrophoresis under non-reducing conditions, a major strongly stained band at about 110 kDa, representing $F(ab')_2$ fragments, was found in all cases; also a protein band of higher molecular mass, possibly $F(ab')_2$ aggregates, and several minor bands of lower molecular mass, possibly digestion products, were detected (Lanes 1 to 3 in Fig. 1). Under reducing conditions, two highly conspicuous stained bands were observed: the one with lower molecular mass is the light chain and the other is the digested heavy chain of horse immunoglobulins (Lanes 4 to 6 in Fig. 1). By this technique, in no instance were strongly stained bands of mobility compatible with important presence of undigested immunoglobulins or serum albumin noted. However, to assure the absence of non-digested material (heavy chains of IgG), additional studies would need to be carried out as HPLC analysis or the Western blot using anti-equine serum, since as recently was published, non-digested material in traces can be observed in some preparations of $F(ab')_2$ antivenoms.[43]

In vitro cross-reactivity analysis using Western blot

As can be seen in Fig. 2B and C, a strong recognition of various protein bands of high, medium, and low molecular weight was apparent using Western blot, not only against the venoms used for immunization to produce the antivenoms (homologous recognition), but also against the ones not used for immunization (heterologous recognition). No bands were detected when the blot was developed with the control antivenom (Alacramyn™) specific to *Centruroides* venoms (results not shown).

Table 2. Inhibition of binding of antibodies to solid-phase venom proteins by homologous or heterologous venoms.

Source of antibodies	Venom on solid phase	Competitor venom	Percent inhibition*
Both-Crot	***B. asper***	***B. asper***	96.1
		C. d. durissus	56.0
		B. jararaca	74.4
		B. jararacussu	69.7
		B. alternatus	60.3
		B. neuwiedi	81.4
Both-Crot	***C. d. durissus***	***B. asper***	44.3
		C. d. durissus	95.3
		B. jararaca	48.9
		B. jararacussu	36.2
		B. alternatus	47.2
		B. neuwiedi	49.4
Both-2	*B. jararaca*	*B. asper*	52.5
		C. d. durissus	12.5
		B. jararaca	95.4
		B. jararacussu	61.8
Both-2	*B. jararacussu*	*B. asper*	32.8
		C. d. durissus	19.7
		B. jararaca	79.1
		B. jararacussu	93.9
Both-2	***B. neuwiedi***	*B. asper*	67.0
		C. d. durissus	58.5
		B. alternatus	32.9
		B. neuwiedi	97.0

*Values represent the highest inhibition of antibody binding to solid-phase venoms when high concentrations of competitor venoms in the liquid phase were used. Venoms used as immunogens (**homologous venoms**) are given in boldface.

Table 3. Neutralization of lethality of antivenoms, expressed in different ways.

	Median Effective Doses (ED_{50})[a]						Potency[b]					
	Both-2		Both-5		Both-Crot		Both-2		Both-5		Both-Crot	
Venoms	µl	mg	µl	mg	µl	mg	mg/ml	µg/mg	mg/ml	µg/mg	mg/ml	µg/mg
B. alternatus	**40***	**3.8***	**91**	**4.5**	659	5.9	**7.0***	**74.3***	**3.1**	**62.8**	0.4	47.6
	(36–44)	**(3.4–4.2)**	**(85–97)**	**(4.2–4.8)**	(196–2160)	(1.8–19.8)	**(6.4–7.8)**	**(67–82)**	**(2.9–3.3)**	**(58–67)**	(0.13–1.3)	(14–155)
B. neuwiedi	**41***	**3.9**	**82**	**4.0**	182	1.6*	**7.2***	**76.5**	**3.6**	**73.6**	1.6	182.7*
	(36–47)	**(3.4–4.4)**	**(75–88)**	**(3.7–4.3)**	(158–221)	(1.4–1.9)	**(6.3–8.2)**	**(67–87)**	**(3.4–3.9)**	**(69–80)**	(1.3–1.9)	(156–211)
B. jararaca	77	7.3	**18***	**0.9***	236	2.1	3.2	34.2	**13.8***	**275.6***	1.05	118.7
	(74–80)	(7.0–7.5)	**(16–20)**	**(0,8–1,0)**	(219–254)	(2.0–2.3)	(3.1–3.4)	(33–35)	**(12.4–15.5)**	**(275–310)**	(0.98–1.13)	(108–124)
B. moojeni	23*	2.2	**59**	**2.9**	106	1.0*	12.9*	136.4	**5.0**	**102.4**	2.8	296.0*
	(20–25)	(1.9–2.4)	**(48–72)**	**(2.4–3.5)**	(94–119)	(0.8–1.1)	(11.8–14.8)	(123–156)	**(4.1–6.2)**	**(85–123)**	(2.49–3.15)	(269–370)
B. jararacussu	41*	3.9	**74**	**4.5**	280	2.5*	2.0*	20.7	**1.1**	**17.8**	0.3	32.0*
	(38–44)	(3.6–4.2)	(63–87)	**(3,1–4,3)**	(257–300)	(2.3–2.7)	(1.8–2.1)	(19–22)	**(0.9–1.3)**	**(19–26)**	(0.27–0.31)	(30–35)
Agk. bilineatus	311	29.3	>350 µl	>17.2	492	4.4*	1.5*	15.8	<1.3	<2.7	0.9	106.0*
	(264–366)	(24.9–34.5)			(457–529)	(4.1–4.7)	(1.3–1.8)	(19–29)			(0.87–1.02)	(99–113)
B. asper	75	7.1	151	7.4	**68***	**0.6***	3.1	32.8	1.5	31.4	**3.4***	**387.0***
	(66–85)	(6.2–8.0)	(113–199)	(5.5–9.8)	**(55–85)**	**(0.5–0.8)**	(2.7–3.5)	(29–37)	(1.2–2.1)	(24–42)	**(2.7–4.2)**	**(290–464)**
C. d. durissus	41*	3.9	122	6.0	**194**	**1.7***	11.1*	117.0	3.7	76.3	**2.4**	**268.0***
	(39–42)	(3.7–4.0)	(115–129)	(5.6–6.3)	**(175–215)**	**(1.6–1.9)**	(10.9–11.7)	(65–114)	(3.5–4.0)	(72–81)	(2.1–2.6)	(240–285)
Ath. nummifer	341*	32.2	N. D.	N. D.	790	7.1*	1.6*	17.4	ND	ND	0.7	79.0*
	(293–396)	(27.6–37.3)			(752–830)	(6.7–7.4)	(1.4–1.9)	(15–20)			(0.67–0.74)	(76–84)

95% confidence intervals are given in parentheses. Venoms used as immunogens for the production of each antivenom are in boldface.
N.D., not determined
Asterisks (*) indicate the antivenom with best neutralization characteristic based on how neutralization is expressed.
[a]ED_{50} of the three antivenoms as microliters (µl) or milligrams (mg) of antivenom required for protecting mice against 5 LD_{50} of indicated venom.
[b]Potency indicates the mg of venom neutralized by 1 ml of antivenom (mg/ml) or the µg of venom neutralized by 1 mg of antivenom (µg/mg).

Table 4. Neutralization of hemorrhagic and coagulant activities by Both-2 and Both-Crot.

	Antivenoms							
	Both-2				Both-Crot			
Venoms	Coagulation		Hemorrhage		Coagulation		Hemorrhage	
	µl	µg	µl	µg	µl	µg	µl	µg
B. alternatus	25	2.36	9.8 ± 3.0	0.92 ± 0.28	200	1.78	30 ± 6	0.23 ± 0.05
B. neuwiedii	62	5.8	5.6 ± 4.4	0.52 ± 0.41	300	2.67	13 ± 2.7	0.12 ± 0.02
B. asper	100	9.43	> 15	> 1.41	200	1.78	10 ± 3.1	0.09 ± 0.03

Results are expressed in microliters or micrograms of antivenom required for the neutralization of these toxic activities.

Immunoreactivity of two antivenoms against the various venoms determined using ELISA titers

Both conventional titers (volumetric) and specific titer units (mg of protein) are reported in Table 1. Differences in reactivity can be observed as expressed volumetrically or as protein. Inhibition of binding of antibodies to solid-phase venom proteins by homologous or heterologous venoms is summarized in Table 2.

Determination of lethal and toxic activities

The values of lethal potency expressed as LD_{50} (ug of venom by g of weight) were as follows (mean lethal dose and 95% confidence intervals): 3.5 (2.8–4.1) for *B. alternatus*, 3.7 (3.2–5.5) for *B. neuwiedi*, 3.1 (2.1–4.3) for *B. jararaca*, 1.0 (0.5–1.3) for *B. jararacussu*, 3.7 (2.9–4.3) for *B. moojeni*, 5.7 (3.0–8.4) for *C. simus*, 2.9 (2.4–3.4) for *B. asper*, 5.8 (2.8–7.8) for *A. b. bilineatus*, and 7.0 (1.6–9.7) for *Ath. nummifer*. The minimal hemorrhagic dose (MHD), dose of venom that produced an hemorrhagic spot of 1 c m of diameter (see Supplementary Appendix available online at http://informahealthcare.com/doi/abs/10.3109/15563650.2014.925561), for the venoms studied were 14 ± 3 ug for *B. alternatus*, 11 ± 4 ug for *B. neuwiedi*, and 6 ± 2 ug for *B. asper*. The minimal coagulant dose on plasma (MCD-P), the dose of venom that clot plasma in 60 s (see Supplementary Appendix available online at http://informahealthcare.com/doi/abs/10.3109/15563650.2014.925561), was 4.3 ± 2 ug for *B. alternatus* venom, 6.1 ± 2.7 for *B. neuwiedi* venom, and 10.2 ± 3.8 for *B. asper* venom.

Seroneutralization tests

We found very high levels of cross-neutralization between each of the three antivenoms and all of the venoms tested. Lethality neutralization assays of the three antivenoms are summarized in Table 3. On a per-volume basis, Both-2 neutralized the lethal potency of the different venoms more efficiently than Both-5 and Both-Crot, since it had significant differences in ED_{50} values in several cases when compared with Both-5 and in all cases when compared with Both-Crot, except in the neutralization of *B. asper* venom (Table 3). However, since the difference in protein content of the antivenoms was very high, we estimated the ED_{50} not only as microliter of antivenom used but also as milligram of protein required to protect half of challenged mice. These results showed that, correcting for protein content, the differences between Both-2 and Both-5 are reduced, and that Both-Crot was significantly more effective in neutralization of the majority of the venoms tested, with the exception of *B. alternatus* (Table 3).

In addition, an important neutralization of the procoagulant and hemorrhagic activities of *B. alternatus, B. asper,* and *B. neuwiedi* venoms by Both-2 and Both-Crot was observed. Similar to what was observed with lethality neutralization assays, when protein content was adjusted, the differences in potency change drastically (Table 4).

When the antivenom necessary to neutralize 100 mg of venom was estimated, doses volumetrically ranged from 9 to 67 ml (Both-2), 7 to 91 ml (Both-5), and 36 to 333 ml (Both-Crot) of antivenom. When the protein content was estimated, the doses were 0.73–6.3 g (Both-2), 0.36–5.63 g (Both-5), and 0.26–3.12 g (Both-Crot) of antivenom (Table 4).

Discussion

The pharmaceutical composition of all three antivenoms was proved to be $F(ab')_2$ fragments with a good grade of purity as observed in the studies using SDS–PAGE (Fig. 1). The Western blot analysis showed that Both-2 and Both-Crot antivenoms cross-reacted with high, medium, and low molecular weight proteins in almost all the studied venoms (Fig. 2).

Antivenoms reacted strongly with their own immunogens but in addition also with venoms of snakes (not used as immunogens) of the same genera from different geographic regions, as well as with venoms of snakes belonging to other genera (e.g., *Athropoides*, *Lachesis,* and *Agkistrodon*), although this reactivity was lower.

The lowest immunochemical reactivity of both antibothropic antivenoms was found with the venom of *C. d. terrificus.* This venom is biochemically different from the other American Crotalinae venoms, as 30–50% of the venom mass is crotoxin (β-neurotoxin). Both-Crot showed good recognition of this venom, presumably because one of the immunogens is *C. simus (ex-Crotalus d. durissus*), which may share several components with *C. d. terrificus*, as they are so closely related that they were once considered the same species. Both-Crot was unable, however, to protect against the lethality of venom of *C. d. terrificus* (data not shown), in agreement with previous data indicating that the differences

in the venoms of *Crotalus* vipers are very important in both hemispheres.[4,44]

Despite the high cross-reactivity observed using direct ELISA, the ELISA competition assays showed that the maximal inhibition of binding occurred when an antivenom was incubated with its homologous venom. Nevertheless, competition with some heterologous venoms was also high (Table 2).

Although other assays have been suggested to test the effectiveness of antivenoms, neutralization of lethality is the one required by most Pharmacopoeias to test antivenoms, for which reason this assay was chosen.[8,22,45] The two South American antivenoms had higher neutralizing potency against all tested venoms, with the exception of *B.asper* venom based on the volume of antivenom required for neutralization. In this regard, Both-5 showed the highest potency against their principal homologous venom and the Both-2 was superior to Both-5 by twofold or more in the neutralization of several venoms (Table 3). Nevertheless, when dose as a function of protein content is considered, neutralization by Both-5 and Both-Crot increased notably (Tables 3 and 4).

The dose of antivenom necessary to neutralize 100 mg of each venom varied across antivenoms. Again, volumes necessary to neutralize the specific (homologous) venoms were not always lower than those required to neutralize nonspecific (heterologous) venoms. Volumes required for Both-2 and Both-5 were lower than those required for Both-Crot with the exception of *B. asper* (Table 5). Volume required for Both-Crot was on occasion over tenfold greater than that required for the other antivenoms: for example, over 17-fold to neutralize *B. alternatus* venom relative to Both-2 or over 13-fold to neutralize *B. jararaca* venom relative to Both-5. On the other hand, the neutralizing dose of protein was higher for Both-Crot only in the cases of *B. alternatus* and *B. jararaca* relative to those required for Both-2 and Both-5, while neutralization of the non-homologous venom by Both-2 and Both-5 antivenoms required protein doses ranging from over twofold (*C. durissus, now C. simus*) to over 10 fold (*B. asper*) greater than the dose required using the homologous Both-Crot (Table 5).

Immunological cross-reactivity among viper venoms has been well documented, clinically and experimentally.[46–52] Surprisingly, however, the use of antivenoms that developed with venoms of snakes of different species and/or of snakes from distant regions and from the one whose venoms have to be neutralized remains controversial.

Immunochemical cross-reactivity and cross-neutralization of viper venoms are related to the similarity in primary, secondary, and tertiary structures of some venom proteins (phospholipases A_2, metalloproteinases, serine proteases and possibly other components not so deeply studied).[53–60] In this regards, the conserved structure of these groups of snake venom toxins confers extensive immunological cross-reactivity, among venoms, to toxin-specific antibodies.[61]

In most cases, we observed higher reactivity of antivenoms with homologous venoms in the study (Table 1); however, in several occasions, the antivenoms tested had a substantial neutralizing capacity against venoms of unrelated snakes (Tables 3 and 4). It must be taken into account that the reactivity by ELISA is due to the recognition of all the components of the venom, the toxic components as well as those without toxicity.

Regarding the immunological analysis, some comments are warranted. Often, the usefulness of an immunogenic mixture for the production of an antivenom is judged using neutralization assays, considering only the volume of antivenom necessary to neutralize a determined challenge dose of a venom. Judged this way, as seen in Tables 3–5, homologous and heterologous neutralization test conferred by Both-2 or Both-5 were higher when compared with that by Both-Crot.

However, considering the amount of protein in each antivenom, Both-Crot showed a higher potency than the other antivenoms in neutralizing all venoms tested, excluding *B. alternatus* and *B. jararaca* venoms. In addition, when the neutralizing potencies of Both-2 and Both-5 are compared after adjusting for protein content, the ratio between the two is close to one excepting with the homologous *B. jararaca* venom (Table 3). This clearly illustrates the importance of the purity of the product relative to its protein content, for judging the immunogenic mixture used in production or the

Table 5. Doses of antivenoms required to neutralize 100 mg of venom.

	Both-2		Both-5		Both-Crot	
	ml/100 mg	g/100 mg	ml/100 mg	g/100 mg	ml/100 mg	g/100 mg
B. alternatus	**14**	**1.35**	**32**	**1.59**	250	2.10
B. neuwiedi	**14**	**1.31**	**28**	**1.36**	63	0.55
B. jararaca	31	2.93	**7**	**0.36**	95	0.84
B. moojeni	8	0.73	**20**	**0.98**	36	0.34
B. jararacussu	50	4.84	**91**	**5.63**	333	3.13
Agk. bilineatus	67	6.32	77	>2.7	111	0.94
B. asper	32	3.05	67	3.19	**29**	**0.26**
C. d. durissus	9	0.85	27	1.31	**42**	**0.37**
Ath. nummifer	63	5.74	N.D.	N.D.	143	1.27

Values of neutralization are indicated as ml or g of antivenom required for the neutralization of 100 mg of different venoms.
Venoms used as immunogens for the production of antivenoms are given in boldface.
N.D., not determined

resultant quality of antibodies as recently noted by Da Silva and Tambourgi.[20] In our case, the comparison was valid since all the products showed a similar grade of purity (Fig. 1).

The therapeutic potency of an antivenom is generally considered to be the amount of venom neutralized per vial, and this is a useful clinical measure for dosing of individual products. Nevertheless, we observed a high immunochemical reactivity and neutralizing capacity of the $F(ab')_2$ fragments of the antivenom tested with lower amount of protein per vial (Both-Crot) or similar neutralizing capacity of two South American anti-*Bothrops* antivenoms when the protein content was considered (Both-2 and Both-5). Thus, it can be reasonably concluded that the lower protein content per vial in products with similar biochemical and immunological characteristics could be a cause of a differential neutralization by vial. In this way, a product with high immunochemical reactivity and with antibodies with good neutralizing potency would paradoxically appear to be less useful (and consequently the immunogenic mixture of venoms used for their production) if the amount of available antibodies packaged per vial is not high enough when compared with that of the other related products.[20] Conversely, a product with a high protein content might be less useful if the reactivity of its antibodies is low, facilitating adverse reactions to occur. At similar grades of purity, the equilibrium between the quality of the neutralizing antibodies and their concentration should define the true quality of antivenom.

An additional observation is related to the volume of antivenom to be applied. If 20 vials of 10 ml of antivenom (or more) are necessary to treat Mexican rattlesnake envenomations,[62–66] this implies the application of 200 ml or more volume of antivenom intravenously, plus the necessary parenteral solution to be applied, which can complicate the injection of the full dose, particularly in children or people with renal impairment. Another important consideration is that a large volume of antivenom represents a proportionally high dose of preservatives, with the negative connotations for the patient's health that it potentially could represent. The same could be considered for other antivenoms to treat different snake envenomations requiring high volumetric doses in various circumstances.[63–69]

Taken together, our results indicate that it is necessary to take into account several factors to assure the real usefulness of an immunogenic mixture for the production of an antivenom. It is beyond the scope of this discussion that therapeutic use is given by the neutralizing potency per vial. However, in order to state that an immunizing mixture does or does not provide good neutralizing antibodies, it is absolutely necessary to consider the pharmaceutical quality and protein content of the antivenom tested when neutralization experiments are interpreted. The low protein dose of an antivenom is a predictor of product safety. The ideal antivenom should provide the greatest possible neutralizing potency per the minimal milligram of protein, in an adequate volume, regardless of the nominal dose, in vials, required to accomplish that neutralization. Snakebites around the world routinely require more than one vial to treat, and physicians should be aware of the dose of protein to which their patients are exposed, when determining the total dose for the management of any particular case and a rational volume to be applied. Finally, these results show the importance of obtaining high affinity and avidity antibodies to achieve a good neutralization using low protein concentration and adequate volumes for the easy application of antivenoms.

Acknowledgments

The authors thank Mr. Felipe Olvera for technical assistance. The authors are grateful to the anonymous reviewers and the editor for their comments, which helped to improve the quality of the manuscript.

Declaration of interest

The authors report no declarations of interest. The authors alone are responsible for the content and writing of the paper.

This work was partially supported by CONACYT (México), Programa de Biotecnología 2000 (No. Z-002).

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Supplementary material available online

Supplementary Appendix.