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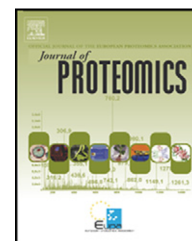

Review

Snake venomics and antivenomics: Proteomic tools in the design and control of antivenoms for the treatment of snakebite envenoming

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A B S T R A C T

Snakebite envenoming represents a neglected tropical disease that has a heavy public health impact, particularly in Asia, Africa and Latin America. A global initiative, aimed at increasing antivenom production and accessibility, is being promoted by the World Health Organization and others. This work discusses several aspects of antivenom manufacture and control in which the proteomic analysis of snake venoms, for which the term 'snake venomics' has been coined, might play a relevant supporting role. Snake venomics has already shown its usefulness for generating knowledge at different levels (ontogenetic, individual, and geographic) on inter- and intraspecies venom variability. This information has applications for the quality control of venom preparations used in antivenom manufacture. Moreover, the design of the best venom mixtures for immunization, aimed at increasing the effectiveness of antivenoms, may also be guided by venom proteome analysis, including molecular studies of the cross-reactivity of antivenoms and heterologous venoms through a recently developed methodological approach termed 'antivenomics'. Results generated by proteomic protocols should be complemented by preclinical testing of antivenom efficacy using functional neutralization assays. Snake venomics might be also helpful in designing alternative *in vitro* tests for the assessment of antivenom efficacy that would eventually substitute current *in vivo* tests.
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1. The global issue of snakebite envenoming

Snakebite envenoming constitutes a highly relevant public health issue on a global basis, although it has been systematically neglected by health authorities in many parts of the world [1–3]. Being a pathology mainly affecting young agricultural workers living in villages far from health care centers in low-income countries of Africa, Asia and Latin America, it must be regarded as a 'neglected tropical disease' [1]. The actual incidence of snakebite envenoming world wide and its associated mortality are difficult to estimate, since there are many countries where this disease is not appropriately reported, and since epidemiological data are often fragmentary. Nevertheless, a recently published study estimated at least 421,000 cases of envenoming and 20,000 deaths yearly, though these largely hospital-based figures may be as high as 1,841,000 envenomings resulting in 94,000 deaths [4], and a previous report had estimated a total of 2.5 million envenomings and over 125,000 deaths [5]. Even realizing that the actual impact of this pathology is likely to be underestimated, because many snakebite victims seek traditional treatment and may die at home unrecorded, it is evident that snakebite envenoming occupies a prominent position as a public health issue in many regions of the world. Moreover, an unknown percentage of snakebite victims end up with permanent physical disability, due to local necrosis, and with psychological sequelae, both of which greatly jeopardize the quality of their lives. Therefore, if this pathology is analyzed in terms of DALYs ('disability-adjusted life years') lost, its impact is even greater [6].

As in many other neglected tropical diseases [7], the task of confronting the snakebite envenoming problem as a public health issue demands concerted efforts at various levels:

(a) It is necessary to know the actual incidence and mortality of this disease in the affected areas.
(b) The snake species responsible for the majority of envenomings in different parts of the world have to be identified.
(c) Safe and effective antivenoms should be produced in sufficient quantities, and controlled and effectively deployed to the regions where they are needed.
(d) The access to health services has to be extended to the remote rural locations where most snakebite accidents occur, thus ensuring that victims can get appropriate medical care within the next hours after the bite.
(e) A rational and effective system for antivenom distribution and storage has to be designed in each affected country.
(f) Health system staff in charge of treating snakebite cases, mostly physicians and nurses, should be trained in the basic aspects of the therapy.

The growing concern of the relevance of the snakebite pathology has prompted an international initiative, under the leadership of the World Health Organization [6] and involving many participants, aimed at coordinating actions at a global level to confront this neglected, though treatable, health problem. One of the key aspects of this initiative is the improvement of the quality, quantity and access of antivenoms. Here, we review the application of proteomic methodologies, together with biological tests of toxicity, for designing, manufacturing and controlling antivenoms, in the context of the efforts to improve the production and access to safe and effective antivenoms.

2. The challenge of generating effective antivenoms

The parenteral administration of animal-derived antivenoms is the cornerstone in the therapy of snakebite envenoming world wide [6,8,9]. The demonstration that sera from hyperimmunized animals effectively neutralized venom-induced toxic effects was first performed in 1894, simultaneously by Calmette and by Phisalix and Bertrand [10]. Soon thereafter, antivenoms were produced and successfully used for snakebite envenoming treatment in various regions of the world [10,11]. First generation antivenoms, produced over 100 years ago, comprised unpurified serum from animals hyperimmunized with venom. Since then, the technologies for antivenom production have been greatly improved and their therapeutic effectiveness has been widely demonstrated, particularly for controlling the systemic manifestations of these envenomings [6,8,9]. Current antivenoms, mainly produced in horses but in some cases also in donkeys and sheep, consist of purified immunoglobulins [12] or antibody fragments (divalent $F(ab')_2$ [13,14] and monovalent Fab fragments [15,16]), which have reduced the incidence and severity of adverse reactions associated with antivenom administration. Among

many aspects involved in antivenom production, i) the quality of the venoms used for immunization, ii) the design of appropriate venom mixtures to obtain effective antivenoms, and iii) the preclinical assessment of antivenom efficacy, are key points that demand careful attention.

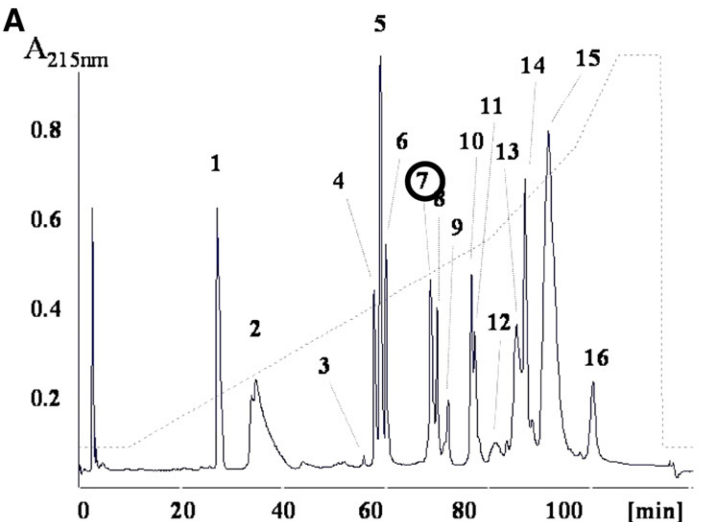
Besides the issues related with snake husbandry and maintenance, which are out of the scope of this review, two aspects of venom preparation are particularly relevant for producing effective antivenoms: the adequate selection of snake specimens for venom collection, and the correct handling, identification, characterization, and storage of venoms. The selection of a snake species as a candidate for antivenom production is based on various criteria, such as: (a) which are the snake species responsible for the largest burden of envenoming in a geographical region?; (b) what is the range of geographic distribution of these species?; (c) what is the extent of geographic and ontogenetic variation of the venoms of these species across their distribution range?; (d) what is the venom yield obtained from a particular species?; (e) what are the immunological relationships between these venoms and the venoms of other taxonomically related species?; and (f) which venoms have an immune suppressor effect?

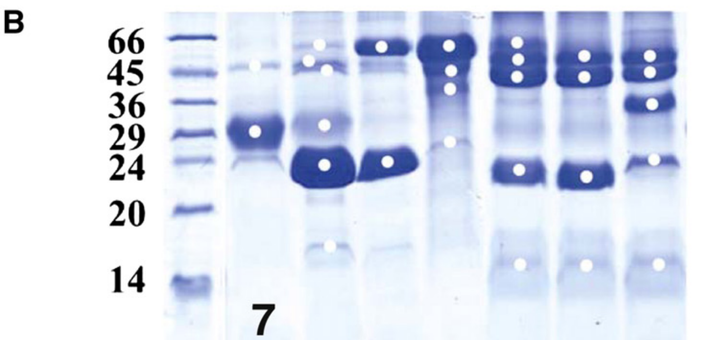
Antivenoms present a unique characteristic compared with other therapeutic immunoglobulin preparations, such as tetanus antitoxin, diphtheria antitoxin, gas gangrene antitoxin, and anti-rabies immunoglobulins. In these, the antigens used for immunization are the same in all regions of the world where these immunobiologicals are manufactured. By contrast, the immunization mixtures used for antivenom production are specific for every country or region, due to the intraspecific venom variability and to the fact that different snake species are responsible for the majority of envenomings in different countries. The inter- and intraspecies heterogeneity in venom composition may account for differences in the clinical symptoms observed in human victims of envenoming by the same snake species in different geographical regions [17,18]. Understanding the variation in antigenic constituents of venoms from snakes of distinct geographic origin represents thus a key challenge towards the design of novel, toxin-specific approaches for the immunotherapy of snake bite envenoming. On the other hand, the high levels of intra- and interspecific variation [17], which reflects local adaptations conferring fitness advantages to the snake population, and age-related (ontogenetic) changes in venom composition (possibly related to diet differences between juvenile and adults of the same species) [19], may also have an impact in the treatment of bite victims and highlights the need of using pooled venoms as a substrate for antivenom production. Intraspecific, geographic, and ontogenetic variability in venom composition can be conveniently analyzed using a combination of proteomic tools and toxicological and biochemical functional assays. Knowledge of inter- and intraspecies variability is necessary for the selection of the regions from which snake specimens have to be collected for the preparation of venom pools. The reference venom pool has to be obtained from a relatively large number of specimens collected from different geographic regions within the distribution range of the species.

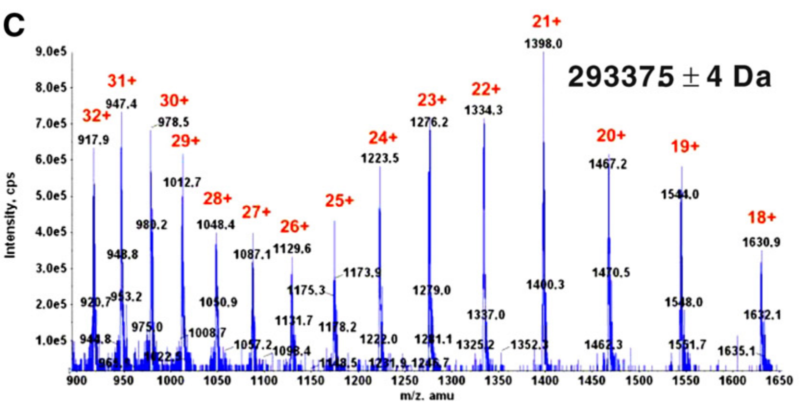
Venom immunization protocols have changed very little over a century and made no attempt to direct the immune response to the most toxic venom proteins (many venom proteins are not toxic and many low molecular mass venom proteins are highly toxic but weakly immunogenic). We believe that toxin-specific antivenoms would improve antivenom dose-efficacy and, by reducing the volume of heterologous IgG required to reverse venom-induced effects, would have a better safety profile. Clearly, the design and optimization of immunizing venom mixtures are critically dependent upon a detailed knowledge of the venom toxin profile. In order to explore the putative venom components, several laboratories have carried out transcriptomic analyses of the venom glands of viperid (*Bitis gabonica* [20], *Bothrops insularis* [21], *Bothrops jararacussu* [22], *Bothrops jararaca* [23], *Agkistrodon acutus* [24,25], *Agkistrodon piscivorus leucostoma* [26], *Echis ocellatus* [27] , *Lachesis muta* [28], and *Sistrurus catenatus edwardsii* [29]), elapid (*Oxyuranus scutellatus* [30] and *Austrelaps labialis* [31]), and colubrid (*Philodryas olfersii* [32]) snake species. Transcriptomic investigations provide catalogues of partial and full-length transcripts that are synthesized by the venom gland. However, transcriptomes include translated and non-translated mRNAs, transcripts encoding non-secreted, housekeeping, and cellular proteins, in addition to toxin precursor genes. Moreover, the transcriptome does not reflect within-species ontogenetic, individual and geographic heterogeneity of venoms, which may account for differences in the clinical symptoms observed in envenomings. With this in mind, we have developed proteomic-based protocols, venomics (reviewed in [33]) and antivenomics [34,35, but see also Calvete et al. in this issue], to define the protein composition and immunological profile of snake venoms, and have applied these approaches for analyzing two key aspects of the variation of venom composition (discussed below): geographic variation and ontogenetic variation, and for assessing the intra- and intergeneric cross-reactivity of heterologous monospecific and polyspecific antivenoms.

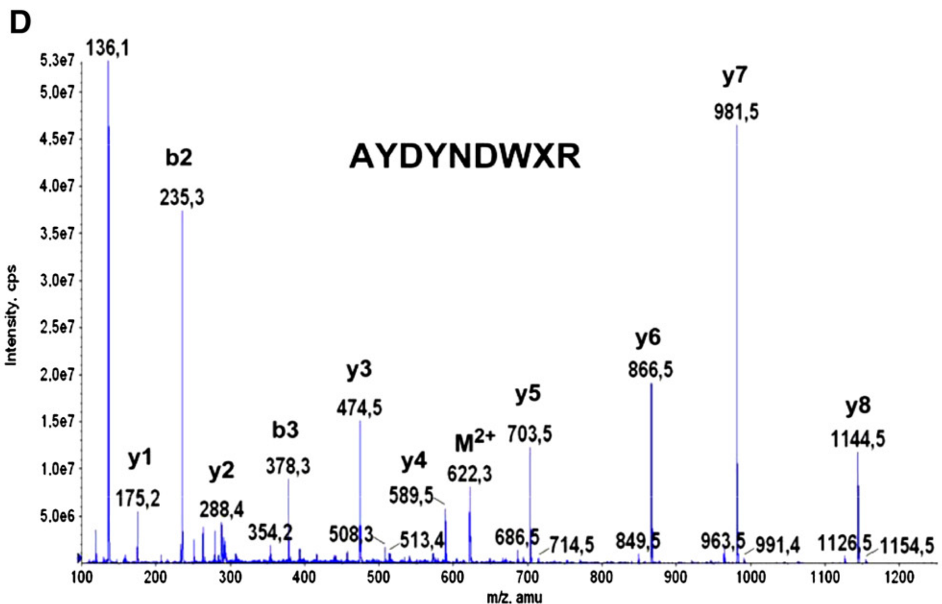
2.1. Snake venomics and antivenomics

Our snake venomics approach (Fig. 1) [33] starts with the fractionation of the crude venom by reverse-phase HPLC, followed by the initial characterization of each protein fraction by combination of N-terminal sequencing, SDS–PAGE (or 2DE), and mass spectrometric determination of the molecular masses and the cysteine (SH and S-S) content. Protein fractions showing single electrophoretic band, molecular mass, and N-terminal sequence can be straightforwardly assigned by BLAST analysis to a known protein family. Thus, although few toxins from any given species are annotated in the public-accessible databases, representative members of most snake venom toxin families are present amongst the ~1100 viperid toxin protein sequences belonging to 157 species deposited to date in the SwissProt/TrEMBL database (Knowledgebase Release 56.5 of November 2008; http://us.expasy.org/sprot/). On the other hand, protein fractions showing heterogeneous or blocked N-termini are analyzed by SDS–PAGE and the bands of interest subjected to automated reduction, carbamidomethylation, and in-gel tryptic digestion. The resulting tryptic peptides are then analyzed by MALDI–TOF mass fingerprinting followed by amino acid sequence determination of selected doubly- and triply-charged peptide ions by collision-induced dissociation tandem mass









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AltName: Full=Acutase; Flags: Precursor
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Sbjct  240  YDYNDWIR  247


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Length=257

 Score = 24.0 bits (49),  Expect =   204
 Identities = 5/6 (83%), Positives = 6/6 (100%), Gaps = 0/6 (0%)

Query  2    YDYNDW  7
            +DYNDW
Sbjct  237  FDYNDW  242
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spectrometry. Except for a few proteins, the peptide mass fingerprinting approach alone is unable to identify any protein in the databases. In addition, as expected from the rapid amino acid sequence divergence of venom proteins evolving under accelerated evolution [36,37], with a few exceptions, the product ion spectra do not match any known protein using the ProteinProspector (http://prospector.ucsf.edu) or the MASCOT (http://www.matrixscience.com) search programs against the 1097 UniProtKB/Swiss-Prot entries from taxon *Serpentes* (http://ca.expasy.org/cgi-bin/get-entries?view=full&KW=Toxin&OC=-Serpentes). Furthermore, it is not too unusual that a product ion spectrum matched with high MASCOT score to a particular peptide sequence corresponds actually to a tryptic peptide of a homologue snake toxin containing one or more nearly isobaric amino acid substitutions. Hence, it is necessary to revise manually all the CID–MS/MS spectra (to confirm the assigned peptide sequence or for performing *de novo* sequencing), and submit the deduced peptide ion sequences to BLAST similarity searches. Although the lack of any complete snake genome sequence is a serious drawback for the identification of venom proteins, high-quality MS/MS peptide ion fragmentation spectra usually yield sufficient amino acid sequence information derived from almost complete series of sequence-specific b-and/or y-ions to unambiguously identify a homologue protein in the current databases. The combined venomics strategy allows us to assign unambiguously all the isolated venom toxins representing over 0.05% of the total venom proteins to known protein families. This methodology has been applied to explore the venom proteomes (Table 1) of the medically relevant Tunisian vipers *Cerastes cerastes*, *Cerastes vipera*, *Macrovipera lebetina* [38]; African *Bitis gabonica* [39], *Bitis arietans* [40], and *Echis ocellatus* [41]; North American *Sistrurus miliarius barbouri* [42] and *Sistrurus catenatus* subspecies [43]; the South and Central American *Lachesis* sp [44]; the Armenian mountain vipers *Vipera raddei* and *Macrovipera lebetina obtusa* [45]; the arboreal Neotropical pitvipers *Bothriechis lateralis* and *Bothriechis schlegelii* [34]; to infer phylogenetic alliances within genus *Bitis* [46] and *Sistrurus* [42]; to rationalize the envenomation profiles of *Atropoides* [47] and *Bothrops* [35] species; to define venom-associated taxonomic markers [48]; and to establish the molecular basis of geographic, individual, and ontogenetic venom variations [49]. The state-of-the-art of snake venom proteomics has been recently revised by Serrano and Fox [50]. The long-term goal of our Snake Venomics project is a detailed analysis of all viperid venomes.

We have coined the term "antivenomics" for the identification of venom proteins bearing epitopes recognized by an antivenom using proteomic techniques [34,35,51]. Antivenomics is based on the immunodepletion of toxins upon incubation of whole venom with antivenom followed by the addition of a secondary antibody. Antigen–antibody complexes immunodepleted from the reaction mixture contain the toxins against which antibodies in the antivenom are directed. By contrast, venom components that remain in the supernatant are those which failed to raise antibodies in the antivenom, or which triggered the production of low-affinity antibodies. These components can be easily identified by comparison of reverse-phase HPLC separation of the non-precipitated fraction with the HPLC pattern of the whole venom previously characterized by venomics (Fig. 2). According to their immunoreactivity towards antivenoms, toxins may be conveniently classified as: C-toxins, completely immunodepletable toxins; P-toxins, partly immunodepleted toxins; and N-toxins, non-inmunodepleted proteins (Fig. 2B). Assuming a link between the *in vitro* toxin immunodepletion capability of an antivenom and its *in vivo* neutralizing activity towards the same toxin molecules, improved immunization protocols should make use of mixtures of immunogens to generate high-affinity antibodies against class P and class N toxins. On the other hand, our antivenomics approach [34,35,51] is simple and easy to implement in any protein chemistry laboratory, and may thus represent another useful protocol for investigating the immunoreactivity, and thus the potential therapeutic usefulness, of antivenoms towards homologous and heterologous venoms [51].

2.2. Geographic and ontogenetic variation in venom composition: implications for the preparation of venom pools

Intraspecies variation in snake venom composition has been extensively reported [17,18]. This phenomenon is particularly notorious among species that have a wide distribution range. Venom variations have been classically documented by using conventional electrophoresis systems, such as starch gel electrophoresis [52,53], agarose gel electrophoresis [54] and one-dimension polyacrylamide gel electrophoresis [55,56]. More recently, the application of proteomic-based approaches has brought novel possibilities to unveil the great complexity and variability of snake venom composition [33,49,50,57–59].

Bothrops asper, the medically most important viperid species from southern Mexico to northern regions of South America [60,61], is amongst the most thoroughly studied species concerning individual, geographic, and ontogenetic venom variability [49]. The uplift of the mountains of lower Central America, including the Guanacaste Mountain Range, Central Mountain Range, and Talamanca Mountain Range which presently separate the Caribbean and Pacific regions of Costa Rica, occurred in the late Miocene or early Pliocene some 8–5 million years ago (Mya) and culminated in the Pliocene with the closure of the Panamanian Portal. This uplift may have fragmented the original homogeneous lowland Costa Rican herpetofauna into allopatric Caribbean and Pacific populations. The occurrence of intraspecies variability in the biochemical composition and pathophysiological manifestations of envenoming by snakes from different geographical location and age has long been appreciated by herpetologists and toxinologists. Thus, variation among the venoms of specimens collected in the Caribbean and in the Pacific regions

Fig. 1 – Snake venomics. Schematic representation of the steps typically followed in a snake venomics project. (A) Reverse-phase chromatographic separation of the venom proteins; (B) SDS–PAGE of the RP–HPLC isolated proteins; (C) determination of the molecular masses of the proteins isolated in panel A; (D) amino acid sequence determination by nanospray-ionization CID–MS/MS of selected tryptic peptide ions; (E) or MS/MS-derived amino acid sequence through Basic Local Alignment Search Tool (BLAST). For more details consult [33].

Table 1 – Overview of the relative occurrence of proteins (in percentage of the total HPLC-separated proteins) of the toxin families in the venoms of *Sistrurus catenatus catenatus* (SCC), *Sistrurus catenatus tergeminus* (SCT), and *Sistrurus catenatus edwardsii* (SCE) from USA [43] *Sistrurus miliarius barbouri* (SMB) from USA [42] the Tunisian snakes *Cerastes cerastes cerastes* (CCC), *Cerastes vipera* (CV) and *Macrovipera lebetina transmediterranea* (MLT) [38] African *Bitis arietans* (BA) [40] *Bitis gabonica gabonica* (BGG) [39] *Bitis gabonica rhinoceros* (BGR), *Bitis nasicornis* (BN), and *Bitis caudalis* (BC) [46] *Echis ocellatus* (EO) [41] *Lachesis muta* (LM) [44] *Crotalus atrox* (CA), and *Agkistrodon contortrix contortrix* (ACC) from USA (Calvete et al., unpublished); Armenian vipers *Macrovipera lebetina obtusa* (Mlo), and *Vipera raddei* (Vr) [45] *Atropoides picadoi* (Api), and *Atropoides mexicanus* (Amex) [47] from Costa Rica; *Bothrops asper* (Bas) from the Caribbean (C) and the Pacific versants of Costa Rica [49] Lesser Antillean pitvipers *Bothrops caribbaeus* (Bcar) (Santa Lucía), and *Bothrops lanceolatus* (Blan) (Martinique) [35] Brazilian *Bothrops fonsecai* (Bfon), and *Bothrops cotiara* (Bco) [48] *Bothriechis lateralis* (Bolat), and *Bothriechis schlegelii* (Bosch) [34] from Costa Rica; and *Lachesis stenophrys* (Lste) [44] from Costa Rica

Protein family	Venom															
	SCC	SCT	SCE	SMB	CCC	CV	MLT	BA	BGG	BGR	BN	BC	EO	LM	CA	ACC
	% of total venom proteins															
Disintegrins																
-Long	–	–	–	–	–	–	–	17.8	–		–	–	–	–	–	–
-Medium	2.5	4.2	0.9	7.7	–	–	–	–	–		–	–	–	–	6.5	–
-Dimeric	–	–	–	–	8.1	<1	6.0	–	3.4	8.5	3.5	–	4.2	–	–	1.5
-Short	–	–	–	–	–	–	<1	–	–	–	–	–	2.6	–	–	–
Myotoxin	0.4	<0.1	–	–	–	–	–	–	–	–	–	–	–	–	–	–
C-type BPP/NP	–	–	<0.1	<0.1	–	–	<1	–	2.8	0.3	–	–	–	14.7	2.1	<0.1
Kunitz-type inhibitor	–	–	<0.1	<0.1	–	–	–	4.2	3.0	7.5	–	3.2	–	–	–	–
Cystatin	–	–	–	–	–	–	–	1.7	9.8	5.3	4.2	–	–	–	–	–
DC-fragment	<0.1	<0.1	<0.1	1.3	–	–	1.0	–	0.5	0.6	<0.1	–	1.7	–	–	<0.1
NGF/sv VEGF	<0.1	<0.1	<0.1	<0.1	–	–	2.1	–	1.0	–	–	–	–	–	–	–
Ohanin-like	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	<0.1
CRISP	0.8	1.3	10.7	2.9	–	–	–	–	2.0	1.2	1.3	1.2	1.5	1.8	4.2	–
PLA_2	29.9	31.6	13.7	**32.5**	20.0	21.1	4.0	4.3	11.4	4.8	20.1	**59.8**	12.6	8.7	16.3	18.5
Serine proteinase	18.2	20.4	24.4	17.1	9.1	20.0	9.2	19.5	**26.4**	23.9	21.9	15.1	2.0	**31.2**	10.1	13.8
C-type lectin-like	<0.1	<0.1	<0.1	<0.1	24.0	0.9	10.1	13.2	14.3	14.1	4.2	4.9	7.0	8.1	1.6	–
L-amino acid oxidase	4.2	1.6	2.5	2.1	12.0	9.0	–	–	1.3	2.2	3.2	1.7	1.4	2.7	8.0	2.2
Zn^{2+}-metalloproteinase	**43.8**	**40.6**	**48.6**	**36.1**	**37.0**	**48.1**	**67.1**	**38.5**	**22.9**	**30.8**	**40.9**	11.5	**67.0**	**31.9**	**51.1**	**63.6**

Protein family	Mlo	Vr	Api	Amex	Bas(C)	Bas(P)	Bcar	Blan	Bco	Bfon	Bolat	Bosch	Lste
	% of total venom proteins												
Disintegrins													
-Long	–	–	–	–	–	–	1.5	–	–	–	–	–	–
-Medium	–	–	<0.1	2.5	2.1	1.4	–	–	1.2	4.4	–	–	–
-Dimeric	8.5	9.7	–	–	–	–	–	–	–	–	–	–	–
-Short	2.8	–	–	–	–	–	–	–	–	–	–	–	–
Myotoxin	–	–	–	–	–	–	–	–	–	–	–	–	–
C-typeBPPP	5.3	6.0	1.8	8.6	–	–	–	–	–	–	11.1	13.4	14.7
Kunitz-type inhibitor	–	0.1	–	–	–	–	–	–	–	–	–	–	–
Kazal-type inhibitor	–	–	–	–	–	–	–	–	–	–	–	8.3	–
Cystatin	–	–	–	–	–	–	–	–	–	–	–	–	–
DC-fragment	1.7	0.1	<0.1	<0.1	<0.1	<0.1	<0.2	–	0.5	0.7	–	–	–
NGF/svVEGF	–	2.4	<0.1	<0.1	–	–	–	–	3.3	3.9	0.5	–	0.4
Ohanin-like	–	–	–	–	–	–	–	–	–	–	–	–	–
3-Finger toxin	–	–	–	<0.1	–	–	–	–	–	–	–	–	–
CRISP	2.6	7.4	4.8	1.9	0.1	0.1	2.6	–	3.6	2.4	6.5	2.1	–
PLA_2	14.6	23.8	9.5	**36.5**	**28.8**	**45.1**	12.8	8.6	–	**30.1**	8.7	**43.8**	12.3
Serine proteinase	14.9	8.4	13.5	22.0	18.2	4.4	4.7	14.4	14.4	4.1	11.3	5.8	25.6
C-type lectin-like	14.8	9.6	1.8	1.3	0.5	0.5	–	<0.1	<0.1	9.8	0.9	–	3.6
L-amino acid oxidase	1.7	0.2	2.2	9.1	9.2	4.6	8.4	2.8	3.8	1.9	6.1	8.9	5.3
Zn^{2+}-metalloproteinase	**32.1**	**31.6**	**66.4**	18.2	**41.0**	**44.0**	**68.6**	**74.3**	**73.1**	**42.5**	**55.1**	17.7	**38.2**

Major toxin families in each venom are highlighted in boldface.

of Costa Rica had been reported by Jiménez-Porras [53], and a marked variation between venoms of neonate and adult specimens was also described [54]. However, only recently, Alape-Girón and colleagues [49] used a venomics approach to define in detail the molecular bases of *B. asper* complex geographic and ontogenetic changes in venom toxin composition. Using a similarity coefficient, it was estimated that the similarity of venom proteins between the Caribbean and the Pacific *B. asper* populations may be around 52%. Notably, reverse-phase HPLC separation provided an unambiguous profile for tracing the geographic origin of Costa Rican *B. asper* snakes (Fig. 3). All major venom protein families appeared to be subjected to

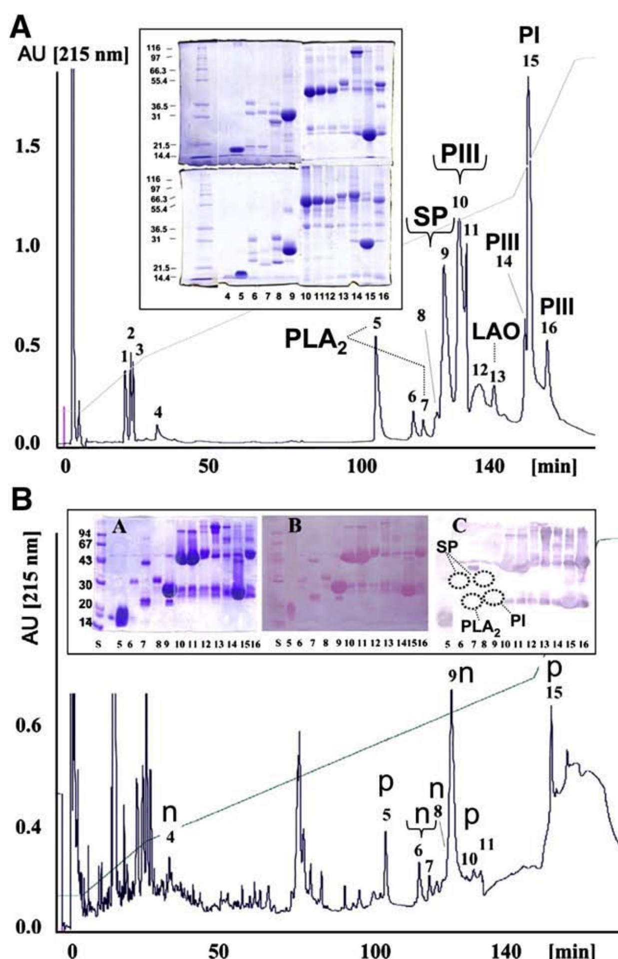


Fig. 2 – Venomics and antivenomics. Panel A displays the reverse-phase HPLC separation of the venom proteins from *Bothrops lanceolatus* (from Martinique). For the proteomic characterization of the venom, chromatographic fractions were collected manually and submitted to N-terminal sequencing, molecular mass determination by ESI mass spectrometry and SDS–PAGE under nonreduced and reduced conditions (insert) [35]. Protein bands were excised and characterized by mass fingerprinting and CID–MS/MS [33,35]. SP, serine proteinase; PI and PIII, snake venom Zn^{2+}-metalloproteinases of classes PI and PIII, respectively [117]; LAO, L-amino acid oxidase. Panel B, immunodepletion of *B. lanceolatus* venom proteins by polyvalent antivenom. Reverse-phase separation of soluble *B. lanceolatus* venom proteins recovered after incubation of the venom with the Costa Rican polyvalent (Crotalinae) ICP antivenom followed by rabbit anti-horse IgG antiserum and immunoprecipitation [35]. p, partly immunodepleted toxins; n, non-immunoprecipitated proteins. Insert: Western blot analysis of the reactivity of the antivenom. (A) SDS–PAGE separation of the various protein bands separated by HPLC. (B) Electrotransferred replica of A onto nitrocellulose membrane, followed by Ponceau Red staining. (C) Detection by Western blot of venom bands using the polyvalent ICP antivenom; protein bands not recognized by the antivenom are framed in broken-line circles. Adapted from [35].

individual variations within each *B. asper* geographic population. The occurrence of intraspecific individual allopatric variability highlights the concept that this species should be considered as a group of metapopulations. Analysis of pooled venoms of neonate specimens from Caribbean and Pacific regions with those of adult snakes from the same geographical region revealed prominent ontogenetic changes in both geographical populations. Major ontogenetic changes appeared to be a shift from a PIII-snake venom metalloproteinase (SVMP)-rich to a PI-SVMP-rich venom, and the secretion in adults of a distinct set of phospholipase A_2 (PLA_2) molecules (Lys49-PLA_2 homologues) present in very low amounts in the neonates (Fig. 3). In addition, the ontogenetic venom composition shift resulted in increasing venom complexity, indicating that the requirement for the venom to immobilize prey and initiate digestion may change with the size (age) of the snake. The higher content of P-III SVMPs in venoms of neonates than in those of adults is in line with their reported more potent hemorrhagic activity [54,62]. Further, the higher content of Lys49 phospholipase A_2 homologues in the venom of adults than in those of neonates is in agreement with the higher myotoxic activity of the former [54]. Besides its ecological and taxonomic implications, geographical venom variability may have an impact in the treatment of snakebite victims and in the selection of specimens for antivenom production. The occurrence of ontogenetic, geographic, and individual intraspecific venom variability highlights the necessity of using pooled venoms as a representative sample for antivenom manufacture.

Viperid venom variability appears to be the rule rather than the exception. Examples of intraspecies variability in the venoms of medically-relevant snakes are those of *Daboia russelli* [63], *Crotalus scutulatus* [64], *Echis carinatus* and *E. coloratus* [65], *Notechis ater* and *N. scutatus* [66], and *Bothrops jararaca* [56,67]. The venom of the subspecies of the neotropical rattlesnake *Crotalus durissus* represents a further and illustrative example of phenotypic variation [68–71] as an adaptive trait during *C. durissus* rattlesnake invasion of South America. The Central American population of adult *C. simus*, formerly classified as *C. d. durissus* [61], has a venom whose main activities are local tissue damage, hemorrhage, coagulopathy and cardiovascular shock. Adult *C. simus* venom is largely devoid of neurotoxic and systemic myotoxic activities [68–70]. On the contrary, the venom of newborn *C. simus* resembles that of the South American rattlesnake *C. d. terrificus* in that both are devoid of hemorrhagic activity and exert potent neurotoxic and myotoxic effects [68,70]. The characteristic pathophysiological picture of neurotoxicity, systemic myotoxicity and acute renal failure secondary to myoglobin accumulation in the kidneys, associated with *C. d. terrificus* envenomation [72], are all consequences of the neurotoxic and myotoxic effects of crotoxin, a neurotoxic PLA_2 heterodimeric complex, which causes progressive paralysis and myonecrosis [73,74]. The venoms of the subspecies *C. d. cumanensis* and *C. d. ruruima*, which inhabit northern regions of South America, exhibit a mixed pattern, as they induce neurotoxicity and hemorrhage [70,75,76]. Wüster and colleagues have traced the dispersal of *C. durissus* from Central to South America [77]. Their phylogeographical pattern is consistent with a stepwise colonization progressing from a northern centre of origin in Mexico, along the Central American Isthmus, followed by more rapid dispersal into northern South America and across

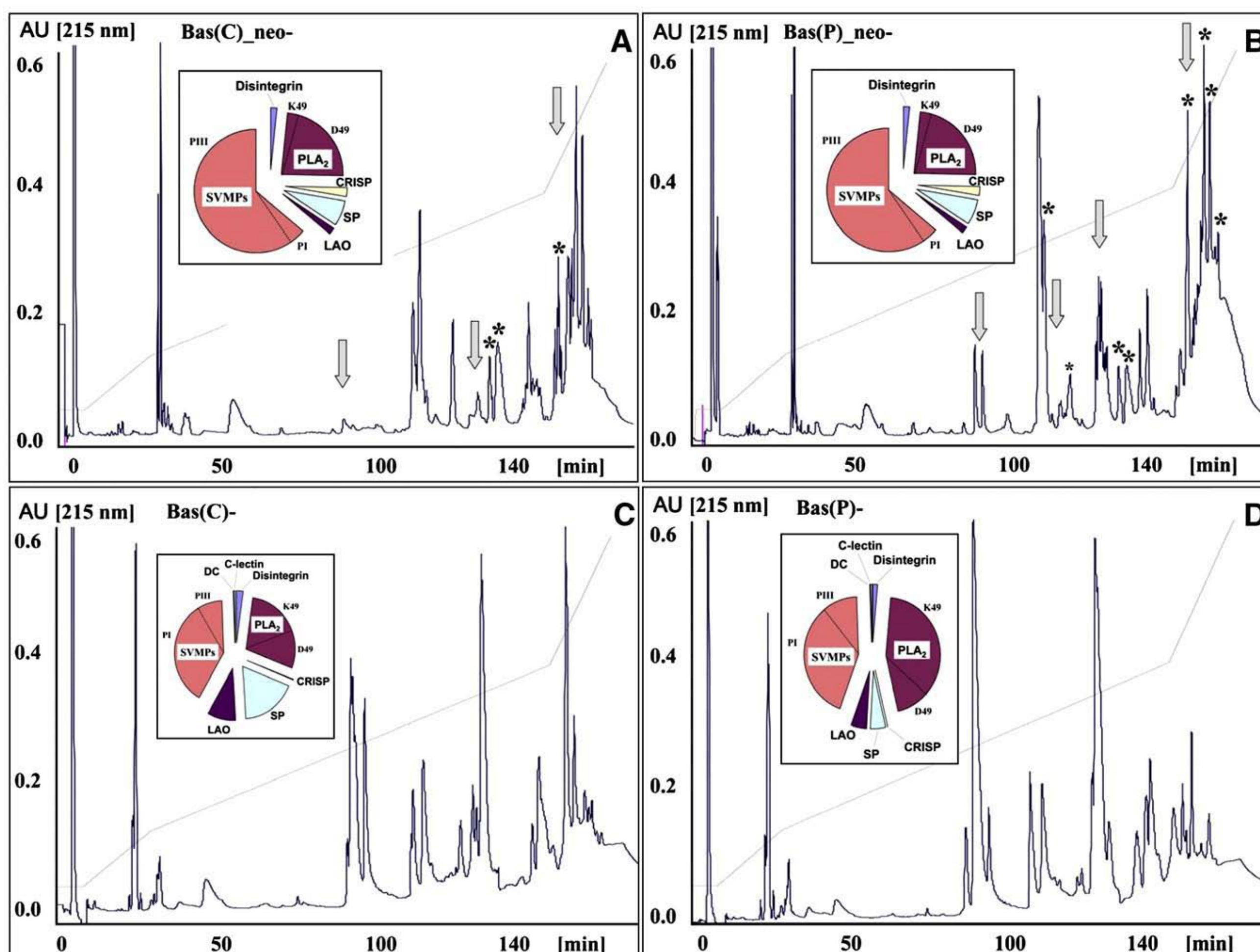


Fig. 3 – Geographical variation and ontogenetic changes in *B. asper* venom composition. Panels A and B, reverse-phase HPLC separations and overall protein compositions (insets) of the venoms from neonate *B. asper* specimens from the Caribbean and the Pacific versants of Costa Rica, respectively. Panels C and D, reverse-phase HPLC separations and overall protein compositions (insets) of the venoms from adult *B. asper* specimens from the Caribbean and the Pacific versants of Costa Rica, respectively. Prominent ontogenetic changes in both geographical populations, which are highlighted with arrows (proteins whose expression is greatly enhanced in adult venom) and asterisks (proteins uniquely expressed in the venom of neonates), involve a shift from a PIII-SVMP-rich to a PI-SVMP-rich venom, and the secretion in adults of a distinct set of PLA_2 molecules (K49 PLA_2s). In addition, the ontogenetic venom composition shift results in increasing venom complexity. Figure adapted from [49]. DC, Disintegrin-like/cysteine-rich fragment from PIII SVMPs; PLA2, phospholipases A_2; CRISP, cysteine-rich secretory proteins; SP, serine proteinases; LAO, L-amino acid oxidase; C-lectin, C-type lectin-like proteins.

the Amazon Basin after the uplift of the Isthmus of Panama. Our ongoing venomic analyses, which will be reported in detail elsewhere, indicate that the gain of neurotoxicity associated with increasing crotoxin expression represents the key axis along which overall venom toxicity has evolved during *C. durissus* invasion of South America (Fig. 4).

Assuming a link between venom toxicity and increased crotoxin concentration, the identification of evolutionary trends may have an impact in defining the mixture of venoms for immunization to produce effective antivenoms. At this respect, an antivenom manufactured in Costa Rica using venom of the Central American rattlesnake (*C. simus simus*) population, is ineffective for neutralizing both the venom of South American *C. durissus* subspecies and of newborn specimens of *C. simus simus* [70]. Similarly, antivenoms produced in South America against *C.d. terrificus* venom neutralize lethality of Central American venoms but are ineffective at neutralizing the hemorrhagic activity of venoms from genus Crotalus [70]. Such neutralizing profile is fully explained by the proteomic characterization of *Crotalus* (*simus* and *durissus*) venoms showing increasing amounts of crotoxin in the venoms of *C. durissus* subspecies along the north to south colonization pattern of this group of snakes (Fig. 4C). This trend points to crotoxin as an adaptive trait in the evolution of the South American rattlesnakes. Moreover, some populations of *C. d. terrificus* contain crotamine, a low molecular mass myotoxic polypeptide, whereas in populations east of the 49th meridian and south of the 22nd parallel the crotamine-negative *C. d. durissus* rattlesnakes predominate [71]. Qualitative individual differences in the venom

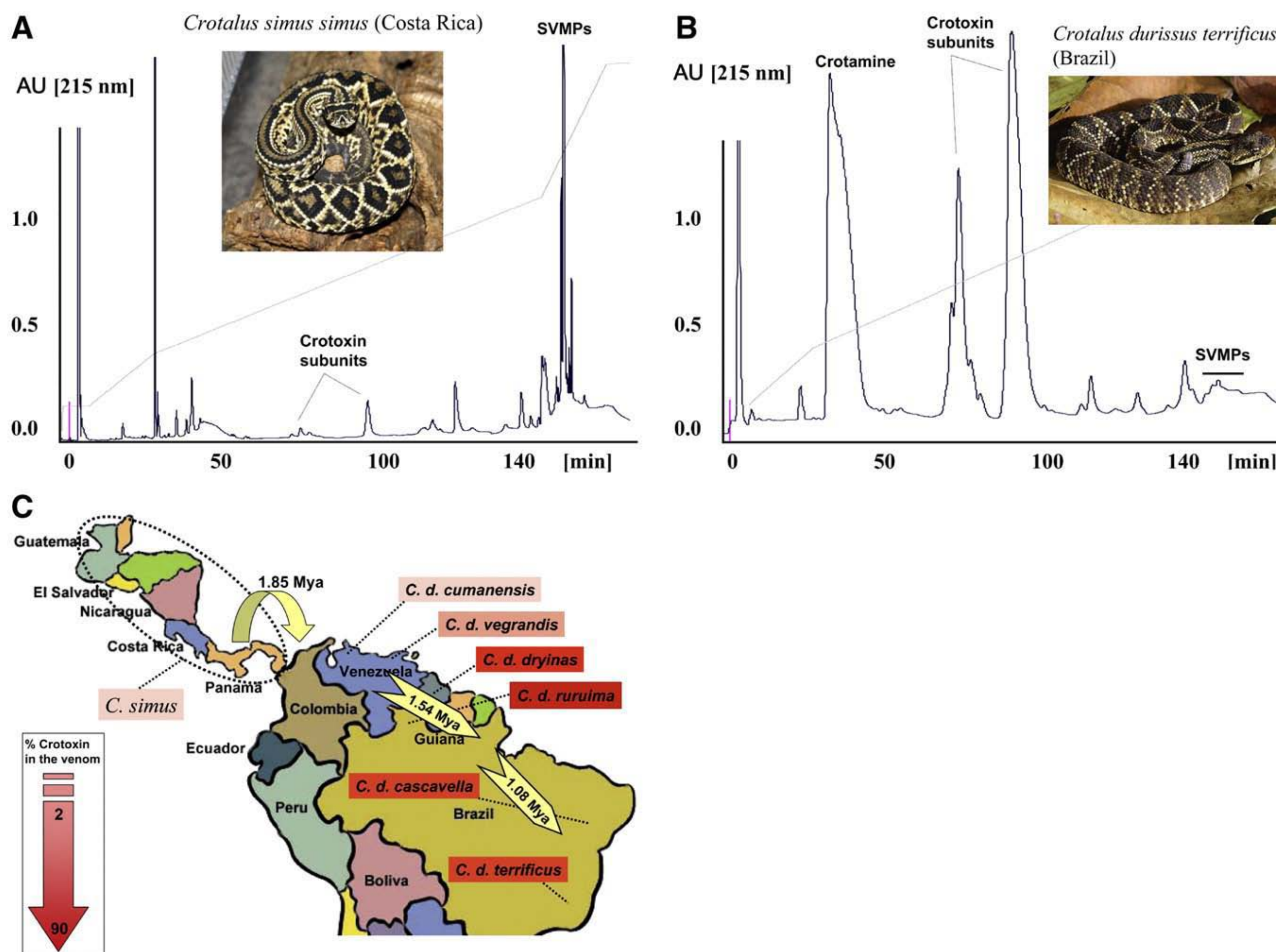
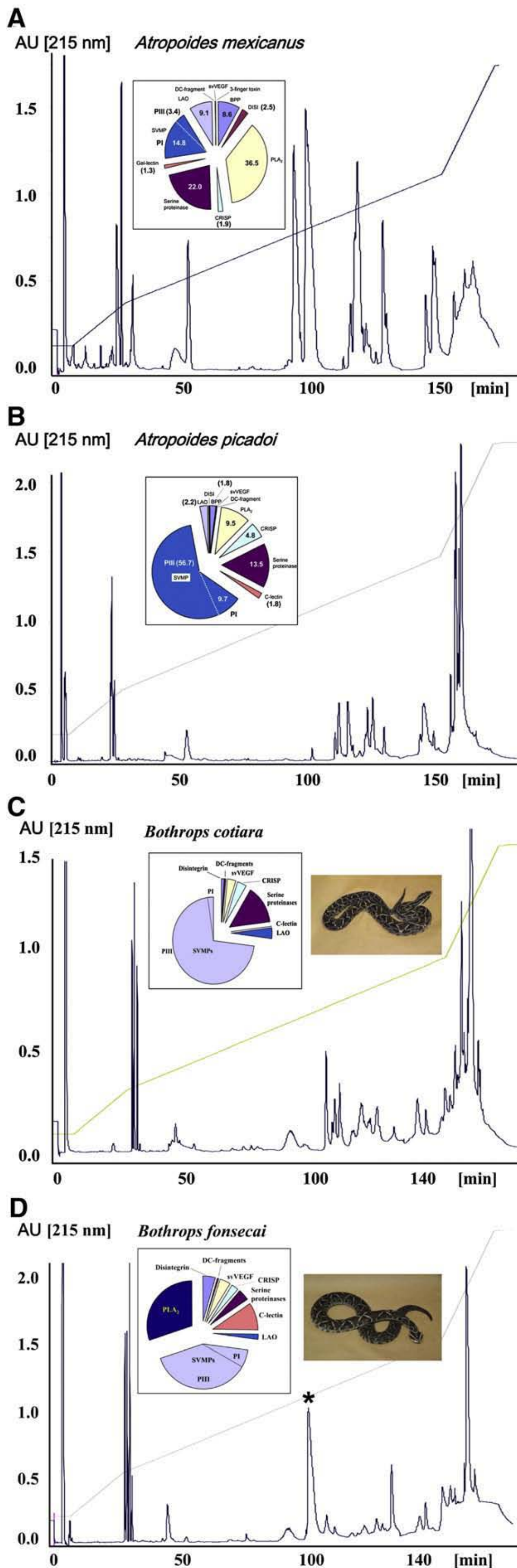


Fig. 4 – Crotoxin as an adaptive trait during *Crotalus durissus* speciation. Panels A and B display, respectively, reverse-phase HPLC separations of the venom proteins of *Crotalus simus simus* (Costa Rica) and *Crotalus durissus terrificus* (Brazil) highlighting peaks containing toxins responsible for the hemorrhagic (SVMPs), neurotoxic (crotoxin), and myotoxic (crotamine) activities of the venoms. In line with their main biological effects, the hemorrhagic and tissue-damaging venom of adult C.*s. simus* contains 72% of SVMPs and 4% crotoxin, while the neurotoxic and myotoxic venom of C.*d. terrificus* comprises 60% crotoxin, 20% crotamine, and only 5% SVMPs. Panel C, Geographical distribution of neotropical Crotalus species color-coding the crotoxin content of their venoms. Notice a correlation between increasing expression of crotoxin in the venoms of *C. durissus* subspecies along the north to south colonization pathway. Major dispersal events and their estimated dates in the evolution of the *Crotalus durissus* complex have been adapted from [77]. Mya, million years ago.

composition of the same snake species are also highly relevant in snakebite pathology and therapeutics since envenoming results from the venom of a single snake. The application of proteomic techniques provides useful tools to identify qualitative and quantitative individual differences in the composition of the venoms within populations [19]. The case of *Crotalus* illustrates how the knowledge of venom variations and their geographical distribution can lead to securing venoms with a more defined composition for preparing venom mixtures for the generation of antivenoms effective against the venoms of rattlesnakes from Central and South America.

2.3. Venomics aiding in taxonomy and in the correct identification of venoms in collections

The taxonomy of snakes is a highly dynamic field of research, and old classifications are being challenged and modified on the basis of novel molecular analyses. Such rapidly evolving taxonomic landscape has practical implications for antivenom production and use, since quite frequently the old nomenclature remains in use in medical circles, as well as within antivenom manufacturers. Since the sources of venoms used for the production and quality control of antivenoms are greatly diverse and heterogeneous, the correct taxonomic identification of venom samples provided by private companies or venom collections in public institutions is sometimes uncertain and difficult to determine (see for example [78]). Moreover, the geographic origin of the specimens from which venom pools are prepared may not be provided by venom suppliers. In some old venom collections, the identification of the containers where valuable venom samples are stored may have been damaged or partially erased, thus compromising the use of this precious biological material.



Snake venom composition retains information on its evolutionary history, and may thus have a potential taxonomic value [46,48]. Proteomic tools represent thus an excellent alternative to solve potential confusions in venom identification, since the HPLC venom separation profile or 2D SDS–PAGE constitute valuable 'fingerprints' of venoms. An example is the case of two closely related pit viper species from Central America, *Atropoides mexicanus* (*nummifer*) and *A. picadoi* [61,79]. These snakes have a relatively similar external appearance, which brings the risk of confusion in the identification of specimens in snake collections. Fortunately, the venoms of *A. mexicanus* (*nummifer*) and *A. picadoi* strongly diverge in their proteome composition [47] and in their biochemical features [80] (Fig. 5, panels A and B). We have estimated that the similarity of venom proteins between the two *Atropoides* taxa may be around 14–16% [47]. Hence, HPLC separation or 2D SDS–PAGE analysis of venom samples of uncertain origin can easily tell whether the venom belongs to one or the other species, or whether it is a mixture of both. Similarly, *Bothrops* represents a highly diversified genus [81] in which some species present very similar morphological features [61]. Species of genus *Bothrops* are responsible for more fatalities in the Americas than any

Fig. 5 – Snake venomics aiding in taxonomy. Panels A and B display, respectively, the reverse-phase HPLC separation of the venom proteins of *Atropoides mexicanus (nummifer)* and *Atropoides picadoi* [47] The overall protein compositions are displayed as inserts. Their distinct venom toxin compositions provide clues for rationalizing the low hemorrhagic, coagulant, and defibrinating activities, and the high myotoxic and proteolytic effects evoked by *A. mexicanus* snakebite in comparison to other crotaline snake venoms, and the high hemorrhagic activity of *A. picadoi*. Despite the efforts of numerous authors, phylogenetic relationships within the subfamily Crotalinae remain controversial, particularly at the intergeneric level. In particular, the genus Atropoides was inferred through Bayesian phylogenetic methods to be paraphyletic with respect to Cerrophidion and Porthidium, due to *Atropoides picadoi* being distantly related to other *Atropoides* species [132–134]. The proteomic characterization of venom composition [47] supports the large divergence among *A. mexicanus (nummifer)* and *A. picadoi*. Panels C and D show, respectively, the reverse-phase HPLC separation of the venom proteins of *Bothrops cotiara* and *Bothrops fonsecai* [48]. The pictures displayed in panels C and D illustrate the high morphological similarity between these Brazilian species. Comparative proteomic analysis has shown that compositional differences between their venoms can be employed as a taxonomy signature for unambiguous species identification independently of geographic origin and morphological characteristics [48]. In particular, PLA_2 molecules are not expressed in the venom of *B. cotiara* (Table 1) whereas venom of *B. fonsecai* contains an abundant PLA_2 protein (~30% of the total venom proteins) (Table 1) having the N-terminal sequence NLWQFGMMIQHTTRENPLFKYFSYGCYCG and an isotope-averaged molecular mass of 13889.9 ± 1.3 Da [48]. This protein (labelled with an asterisk in panel D) represents a taxonomic marker for differentiating *B. fonsecai* from *B. cotiara*.

other group of venomous snakes [1,61,82]. Nineteen *Bothrops* species are represented in the Brazilian herpetofauna. In this country, 28,597 snakebites were reported in 2005, of which 87.5% were related to *Bothrops* species (http://portal.saude.gov.br/portal/arquivos/pdf/situacao.pdf). *B. cotiara* is morphologically very similar to *B. fonsecai* (Fig. 5 panels C and D), although they are not sympatric. Comparative proteomic analysis has shown that compositional differences between their venoms can be employed as a taxonomy signature for unambiguous species identification independently of geographic origin and morphological characteristics ([48] but also compare panels C and D of Fig. 5).

2.4. *Quality control of venoms*

An adequate preparation of snake venoms is a delicate task that involves a correct identification and maintenance of specimens in captivity, as well as effective protocols for venom collection, preparation and storage. Since snake venoms contain proteinases, venom has to be rapidly frozen upon collection and should be properly stored following identification. Moreover, venoms should be thawed, homogenized and freeze-dried for the preparation of representative pools of many individuals. These processes, if not carried out properly, introduce the risk of degradation and denaturation of venom proteins. The process of freeze-drying is particularly critical, since an inadequate freeze-drying may significantly impair the quality of the venom. Furthermore, the storage of freeze-dried venoms for prolonged periods of time may also involve the risk of hydration, with the consequent impairment in venom quality. Thus, the quality control of venoms is a critical aspect in the preparation of venom samples for immunization and for the quality control of antivenoms.

The quality control of venoms should include both biochemical and pharmacological tests. Chromatographic patterns, along with 2DE, SDS–PAGE, and proteomic techniques, may be employed to detect degradation of individual venom proteins through the appearance of novel components, including low molecular mass hydrolysis products. These analyses can be performed in parallel with toxicological and functional tests such as the determination of the Median Lethal Dose (LD_{50}) or the quantification of enzymatic activities. It is recommended that venom preparations used in research and antivenom manufacture and control should be supported by a quality control analysis that ensures their appropriateness and correct identification to the users of the venoms.

3. Proteomics in the development and selection of adjuvants for immunization

Immunization of large mammals with venom from a single species (for the production of monospecific antivenoms) or from various species (for the production of polyspecific antivenoms) involves the use of adjuvants, aimed at fostering the immune response. Traditionally, most producers use Freund's complete and incomplete adjuvants in the first immunization stages, followed by aluminum salts or other adjuvants in subsequent immunizations [83,84]. More recently, a variety of novel adjuvants have been developed but have not been systematically assessed for antivenom production. One of the main functions of adjuvants is to promote a slow release of antigen molecules. Proteomic tools offer an excellent alternative to analyze the kinetics of release of various venom components in different adjuvants. Thus, the kinetics of toxin release from the venom–adjuvant mixture can be easily assessed *in vitro* by following the time-course release of venom proteins by HPLC profiling. In this way, the concentration and formulation of the adjuvant can be optimized to a toxin release pattern that better fits the desired immunization strategy.

4. 'Antivenomics' for immunoprofiling whole venoms and analyzing the extent of heterologous cross-reactivity of antivenoms

Adequate treatment of snakebite envenoming is critically dependent on the availability of antivenoms that effectively reverse the evolution of venom-induced local and systemic consequences of envenomation. Polyspecific antivenoms that cover the range of snakes in a given area are preferred to monospecific ones, because their use simplifies production, distribution, and therapeutic procedures. However, there are limits on the number of venoms that can be used to immunize horses or sheep, on the amount of total venom proteins that can be contained in a dose for immunization ("diluting" the concentration of each toxin in the immunizing mixture) and, once the antivenom has been produced, on the amount of total IgGs that comprise an antivenom dose. In addition, some venoms are difficult to obtain, and some countries do not have the adequate infrastructure or the market size to support the local production of antivenoms. The deficit ('crisis') of antivenom supply in some regions of the world can be addressed to a certain extent by optimizing the use of existing antivenoms and through the design of novel immunization mixtures for producing broad-range polyspecific antivenoms [1–3,83,85–88]. Therefore, the extent of immunological relationship between the venom proteins used for immunization and the proteins present in venoms of species not included in the immunization mixture has to be considered, in order to assess the coverage spectrum of existing or newly developed antivenoms. The rationale behind the concept of heterologous cross-reactivity of antivenoms is that in spite of the fact that viperid venoms comprise complex mixtures of a large number of distinct proteins [50,58], venom proteins belong to only a few major protein families, including enzymes (serine proteinases, Zn^{2+}-metalloproteinases, L-amino acid oxidase, group II PLA_2) and proteins without enzymatic activity (disintegrins, C-type lectins, natriuretic peptides, myotoxins, cysteine-rich secretory proteins (CRISP), nerve and vascular endothelium growth factors (svVEGFs), cystatin and Kunitz-type protease inhibitors) [33]. Members of a single family may depart from each other in their pharmacological effects but share remarkable structure similarity, including in many cases common epitopes.

The efficacy of monospecific and polyspecific antivenoms is well documented in the literature [88–97]. However, the extent of cross-protection of many monospecific or polyspecific antivenoms against heterologous venoms not included in

the immunizing mixtures remains largely unexplored. For instance, in Central America and the northern regions of South America, the species *Bothrops asper* and *B. atrox* inflict the vast majority of the bites [60,61,82,98]. However, other species from the genera *Bothrops*, *Bothriechis*, *Atropoides* and *Porthidium* also provoke a number of accidents [61,82]. Do antivenoms raised against *B. asper* or *B. atrox* protect against the venoms of these other species? This question is of utmost relevance, since the design of venom mixtures containing these additional species would complicate antivenom manufacture, not only because some of these species are difficult to obtain and maintain in captivity, but also because their venom yield is low [99]. A similar scenario occurs in Africa and Asia, where the immunological relationships between medically-relevant venoms has been studied only to a partial extent [88].

The potential value of antivenomics, together with preclinical neutralization tests, in assessing antivenom cross-reactivity is clearly illustrated by the following examples. A highly effective antivenom (Sanofi-Pasteur 'Bothrofav®') has been developed for the treatment of envenomings by *B. lanceolatus* [100,101], endemic to the Lesser Antillean island of Martinique. It exhibits an excellent preclinical profile of neutralization [95] and its timely administration prevents the development of the most serious effects of envenoming, including thrombosis [100,101]. However, the restricted availability of the antivenom in the neighboring island of Saint Lucia and in zoos and herpetariums where these species may be kept is a matter of concern. Gutiérrez and colleagues [35] have performed detailed proteomic studies of the venoms of *B. caribbaeus* and *B. lanceolatus* and have evaluated the immunoreactivity of a Crotalinae polyvalent antivenom produced in Costa Rica (by immunization of horses with a mixture of equal amounts of the venoms of *B. asper*, *C. simus*, and *Lachesis stenophrys*) towards the venoms of *B. caribbaeus* and *B. lanceolatus*. This study showed that the antivenom immunodepleted ~80% of the proteins from both *B. caribbaeus* and *B. lanceolatus* venoms, and was effective in neutralizing the lethal, hemorrhagic, PLA_2 and proteolytic activities of the two venoms. It also showed that a CRISP molecule and certain serine proteinases were not recognized by antivenom antibodies and were not immunodepleted from the venom. Major PLA_2 and PI-SVMP molecules displayed weak immunoreactivity towards the antivenom and were only partially immunoprecipitated [35]. Similarly, Lomonte et al. [34] have conducted an antivenomic study of the immunoreactivity of the Costa Rican polyvalent antivenom towards *Bothriechis* (*lateralis* and *schlegelii*) venoms, revealing that L-amino acid oxidase and SVMPs represent the major antigenic protein species in both venoms. The results provided a ground for rationalizing the reported protection of this polyvalent antivenom against the hemorrhagic, coagulant, defibrinating, caseinolytic and fibrin(ogen)olytic activities of *B. schlegelii* and *B. lateralis*) venoms. However, these analyses also evidenced the limited recognition capability of the polyvalent antivenom towards a number of Bothriechis venom components, predominantly BPPs, svVEGF, Kazal-type inhibitors, some PLA_2 proteins, some serine proteinases, and CRISP molecules. The toxicity and potential pathophysiological relevance of such non-recognized venom components (N-toxins) remain be assessed by *in vivo* toxicity tests.

Several authors [34,35,102] have employed Western blot analysis for assessing the immunoreactivity of antivenoms. Western blot and immunodepletion analyses yield complementary information. However, the immunochemical detection of blotted proteins provides a Yes/No response: a given protein is recognized or not by the antivenom, and it is essentially a non-quantitative technique. Further, proteins are denatured to an unknown degree when solubilized by boiling in sample buffer containing SDS. This treatment may introduce artifacts such as loss of conformational epitopes and/or artifactual recognition of non-native epitopes. On the other hand, the degree of recognition of native proteins by the antivenom IgGs can be easily quantitated by measuring the amount of non-immunodepleted proteins.

Proteomics-based immunochemical analysis (antivenomics) provides relevant information for outlining which venom mixtures cross-react with the most important components in medically-relevant venoms from a particular region. This type of approach may set the basis for the development of antivenoms on an immunologically sound basis. However, the actual spectrum of cross-neutralization has to be further investigated by using toxicity preclinical tests (see below). On the other hand, antivenomics may also be useful for analyzing differences in the immune response against venoms of those animal species usually employed for hyperimmunization, such as horses, donkeys, sheep or camelids [8,9]. It might be that some of these species develop a better immune response against particular venom components than others, and this can be easily investigated by assessing the profiles of immunodepletion of the different venom proteins by antivenoms raised in the various species. Likewise, the differences in toxin immunorecognition among individual animals of the same species may be also studied using an antivenomic protocol. The information gathered by this type of analysis might contribute to the selection of the best individuals in terms of immune response for antivenom production.

5. Preclinical tests to assess antivenom efficacy

Many antivenoms are produced in the world using different venoms in the immunization schemes [103]. Each of these antivenoms is effective against envenomations by snake venoms not included in the immunizing mixtures, demonstrating immunological cross-reactivity between related snake venoms. A practical consequence of this fortunate circumstance is the possibility of using these heterologous antivenoms to circumvent the restricted availability of species-specific antivenoms in some regions. However, before testing in clinical trials, antivenoms need to be evaluated experimentally, by a set of preclinical tests, to assess their neutralizing ability against the most relevant toxic and enzymatic activities of homologous and heterologous snake venoms [94,104,105]. The selection of the tests to be used depends on the pathophysiological profile of human envenomings characteristic of the species under investigation. For instance, for venoms of many elapid species of genera *Naja*, *Bungarus*, *Micrurus*, and *Dendroaspis*, whose main clinical manifestation is neurotoxicity (i.e. muscle paralysis leading to respiratory failure) [106,107], antivenoms should be tested against the lethal effect of the venom, since death is the ultimate manifestation of neurotoxicity. Other elapid venoms induce additional alterations, such as myotoxicity and coagulopathy (Australian

snakes), or local necrosis (some cobras) [106,107]. For these venoms, laboratory assays assessing myotoxicity, coagulopathy and dermonecrosis are required [104]. On the other hand, the scenario for most viperid venoms is different, since envenomings by these species are characterized by a complex series of local and systemic pathophysiological alterations that include hemorrhage, myonecrosis, dermonecrosis, defibrin(ogen)ation, renal alterations, cardiovascular shock and, eventually, death. In these cases, the study of the neutralization of lethality, albeit being the most important effect, should be complemented by the assessment of the neutralization of the additional toxic activities [94]. Only antivenoms showing a good neutralizing performance in preclinical testing should be authorized to go to the clinical phases. A similar situation occurs when an antivenom that has been approved for clinical use in a particular country for the treatment of envenomings by certain snake species is going to be introduced in other countries or for treating bites by other snakes, in which cases preclinical testing should be performed. The ample demonstration of inter- and intraspecies venom variability showed by proteomic studies strongly supports this point of view. The information provided by our antivenomics approach [34,35,51] may further contribute to interpret the results of neutralization assays, since it enables the identification of cross-reacting and non-reacting components in homologous and heterologous venoms.

A number of studies have been performed to assess the cross-reactivity of different antivenoms manufactured in different countries. In the case of Latin America, the investigations have revealed a high-degree of cross-protection between several antivenoms generated against *Bothrops* sp venoms [35,91,93,96,97,108,109]. On the other hand, similar studies have demonstrated that some *Bothrops* sp antivenoms are effective in the neutralization of lethality of *Lachesis* sp. venoms, but are ineffective in neutralizing their coagulant and defibrin(ogen)ating activities [95,110–112]. These results have direct implications for antivenom design: an antivenom aimed to treat envenoming by *Lachesis* sp. should include this venom in the immunizing mixture, or the coagulant enzyme purified from the venom or expressed as a recombinant protein. This example illustrates the relevance of performing additional assays to the mouse lethality test in the preclinical assessment of the neutralization profile of antivenoms.

6. Perspectives: possible contribution of proteomics in the selection of antigens for toxin-specific immunization

The study of venom proteomes may bring another possibility for improving immunization schemes for antivenom manufacture, since the experimental tools described above (venomics and antivenomics), coupled with the characterization of the predominant peaks and the toxicological analysis of their activities, offer the possibility of identifying the key toxic components in a venom. There are examples in which a single protein, or a small set of toxins, are responsible for the main pathophysiological activities of a venom. In such cases, an antivenom produced using these toxins as antigens would provide protection against the toxic effects of the whole venom. For instance, antivenoms against sphingomyelinase D, the main toxic component of the venoms of spiders of genus *Loxosceles*, effectively neutralize the toxicity induced by the crude venom [113,114]. A similar situation may apply to some snake venoms, whose toxicity is based on the action of few components. This is the case of the South American subspecies of rattlesnakes (*C. durissus* spp), whose venoms present high concentrations of the neurotoxic phospholipase A_2 complex 'crotoxin'. This toxin, which represents up to 83% of the total venom proteins in *C. d. ruruima* [Calvete et al., unpublished] (Fig. 3C), is responsible for three of the most important manifestations of these envenomings, i.e. neurotoxicity, rhabdomyolysis, and acute renal failure secondary to the accumulation of myoglobin in the renal tubules [73,74]. Anti-sera raised in rabbits against crotoxin and phospholipase A_2 from *C. d. cascavella* venom neutralize the neurotoxicity of the venom and crotoxin [115]. Some *C. durissus* venoms also induce defibrin(ogen)ation due to the action of a thrombin-like serine proteinase [116]. Identification of this toxin by proteomics and reverse-phase HPLC profiling may allow the preparation of immunization mixtures composed of crotoxin and this clotting enzyme, which theoretically would raise an antibody response effective in the neutralization of the most relevant toxicological effects induced by South American rattlesnakes.

The venoms of many species of elapid snakes of the genera *Naja*, *Bungarus*, *Micrurus*, and *Dendroaspis*, among others, exert a toxicological profile based on the action of pre- or post-synaptically-acting neurotoxins. In these cases, proteomics in conjunction with biological analyses are instrumental to identify the predominant neurotoxins present in each particular venom, which would be the optimal antigen candidates for immunization. Proteomic analyses have also revealed the high concentration of SVMPs in many medically relevant viperid snakes, such as *Echis ocellatus* [41], *Bothrops jararaca* [58], *Atropoides picadoi* [47], and *Bothriechis lateralis* [34] (Table 1). In agreement with these observations, SVMP-rich venoms exert a potent hemorrhagic activity, likely due to the action of potent multi-domain P-III hemorrhagins [117,118]. In these cases, immunization with isolated or recombinant SVMPs may be effective for generating antibodies able to neutralize hemorrhagic SVMPs in a variety of viperid venoms.

Large-scale isolation of specific toxins for toxin-specific immunization requires access to large amounts of venom, which is often not possible. Alternatively, the structural information gathered from venomic projects may allow cloning and subsequent recombinant expression of the relevant proteins. Another alternative is immunization with specific toxin-coding DNA [119–121] or with chimeric DNA molecules encoding a string of bioinformatics-designed multi-epitopes that are predicted to be highly immunogenic and stimulate antibodies that will neutralize toxin function [122]. Exploring technologies to develop toxin-specific antivenoms is critically dependent upon a detailed knowledge of the venom toxin profile.

7. The need to substitute animal tests for *in vitro* assays: can proteomics help?

There is a growing concern for the use of mice in antivenom testing, with the associated animal suffering and economic

burden that represents the massive use of laboratory animals. Sustained efforts have been performed to find alternative tests, such as *in vitro* assays or tests performed in chicken embryos before they develop pain sensitivity (reviewed in [123]). However, the cases in which *in vitro* tests have been shown to correlate with the conventional mouse test are limited [123–128]. One reason for this failure may have to do with the high complexity of snake venoms, whose toxicity is due to the action of several, often many, toxins exhibiting synergistic actions [129,130]. Proteomic tools may bring new light into this relevant field of *in vitro* antivenom testing by identifying the most relevant toxins in medically important venoms, while the antivenomic analysis of these venoms may be used to assess the presence in antivenoms of high-affinity antibodies against the relevant toxins. Once the most important toxins in a venom have been identified, *in vitro* functional tests may then be performed on nerve–muscle preparations [115,131], on plasma clotting assays [104], on enzymatic (proteolytic or PLA_2) activities [91,125], or by using enzyme immunoassays [127,128]. This review illustrates how the growing body of proteomic information on snake venoms might assist in the selection and design of *in vitro* tests for the assessment of the preclinical efficacy of antivenoms. The complexity of snake venoms implies that a single test can not be adapted for all venoms but, instead, that the proteomics-derived knowledge on particular venoms, coupled to the toxicological profiles of venoms and their individual toxins, may greatly help in the design of tests tailored for each venom or groups of venoms. In the long term, this would contribute to the introduction of different *in vitro* tests and in the reduction of animal use and suffering associated with the performance of *in vivo* assays.

8. Concluding remarks

There is an urgent need to strengthen a global initiative to confront the problem of snakebite envenoming and to reduce the human toll associated with this pathology. Multiple efforts are being promoted, and the involvement of multiple actors is required at different levels, from the basic scientific research realm to the public health intervention domain. Here we have reviewed the accumulating evidence showing the potential of proteomic tools for the improvement of antivenom design, manufacture and control, based on a detailed and profound knowledge of venom composition, variability and immunological cross-reactivity. Renewed efforts have to be promoted towards the study of the composition and actions of the medically most important snake venoms around the world. Since the proteomic technologies discussed here are still not available in many countries, the organization of international academic and public health partnerships should be fostered, involving laboratories with the necessary technological resources, but at the same time improving the endogenous scientific capacity in low-income countries of Asia, Africa and Latin America to develop proteomic research on venoms. Such an international cooperative scenario, along with other initiatives, would be a very positive step towards a better understanding of venoms and antivenoms, and consequently towards the effective reduction of the heavy burden associated with human envenoming by snakebite.

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