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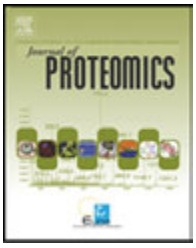

Review

Ending the drought: New strategies for improving the flow of affordable, effective antivenoms in Asia and Africa

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ABSTRACT

The development of snake antivenoms more than a century ago should have heralded effective treatment of the scourge of snakebite envenoming in impoverished, mostly rural populations around the world. That snakebite still exists today, as a widely untreated illness that maims, kills and terrifies men, women and children in vulnerable communities, is a cruel anachronism. Antivenom can be an effective, safe and affordable treatment for snakebites, but apathy, inaction and the politicisation of public health have marginalised both the problem (making snakebite arguably the most neglected of all neglected tropical diseases) and its solution. For lack of any coordinated approach, provision of antivenoms has been pushed off the public health agenda, leading to an incongruous decline in demand for these crucial antidotes, excused and fed by new priorities, an absence of epidemiological data, and a poor regulatory framework. These factors facilitated the infiltration of poor quality products that degrade user confidence and undermine legitimate producers. The result is that tens of thousands are denied an essential life-saving medicine, allowing a toll of human suffering that is a summation of many individual catastrophes. No strategy has been developed to address this problem and to overcome the intransigence and inaction responsible for the global tragedy of snakebite. Attempts to engage with the broader public health community through the World Health Organisation (WHO), GAVI, and other agencies

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have failed. Consequently, the toxinology community has taken on a leadership role in a new approach, the Global Snakebite Initiative, which seeks to mobilise the resources, skills and experience of scientists and clinicians for whom venoms, toxins, antivenoms, snakes and snakebites are already fields of interest. Proteomics is one such discipline, which has embraced the potential of using venoms in bio-discovery and systems biology. The fields of venomics and antivenomics have recently evolved from this discipline, offering fresh hope for the victims of snakebites by providing an exciting insight into the complexities, nature, fundamental properties and significance of venom constituents. Such a rational approach brings with it the potential to design new immunising mixtures from which to raise potent antivenoms with wider therapeutic ranges. This addresses a major practical limitation in antivenom use recognised since the beginning of the 20th century: the restriction of therapeutic effectiveness to the specific venom immunogen used in production. Antivenomic techniques enable the interactions between venoms and antivenoms to be examined in detail, and if combined with functional assays of specific activity and followed up by clinical trials of effectiveness and safety, can be powerful tools with which to evaluate the suitability of current and new antivenoms for meeting urgent regional needs. We propose two mechanisms through which the Global Snakebite Initiative might seek to end the antivenom drought in Africa and Asia: first by establishing a multidisciplinary, multicentre, international collaboration to evaluate currently available antivenoms against the venoms of medically important snakes from specific nations in Africa and Asia using a combination of proteomic, antivenomic and WHO-endorsed preclinical assessment protocols, to provide a validated evidence base for either recommending or rejecting individual products; and secondly by bringing the power of proteomics to bear on the design of new immunising mixtures to raise Pan-African and Pan-Asian polyvalent antivenoms of improved potency and quality. These products will be subject to rigorous clinical assessment. We propose radically to change the basis upon which antivenoms are produced and supplied for the developing world. Donor funding and strategic public health alliances will be sought to make it possible not only to sustain the financial viability of antivenom production partnerships, but also to ensure that patients are relieved of the costs of antivenom so that poverty is no longer a barrier to the treatment of this important, but grossly neglected public health emergency.

Contents

1. Introduction

Throughout the last decade, modern proteomic investigational tools have given toxinologists the key to revealing a dazzling array of biotoxins in the venoms of snakes, spiders, scorpions, wasps, caterpillars and a vast treasure trove of marine organisms. Never before have we been able to grasp the true complexity and diversity of venom proteins and peptides with such clarity and precision. With the aid of 2-dimensional polyacrylamide gel electrophoresis (2D-PAGE), high-performance liquid chromatography (HPLC), a variety of sophisticated mass spectrometry platforms, and the ability to unravel protein transcriptomes or probe complex solutions with well designed ligand libraries, we have entered a new world of discovery and understanding in our quest to penetrate fundamental biological processes at the microcellular level. In these few short years we have gone beyond the limits of basic protein separation and characterisation into the very foundations of the genes that are the design blueprints of protein structure and function and the scaffolds upon which we can build treatments for diseases of many causes.

Snake venoms are rich soups of protein and peptide diversity. Many authors have discussed means of deploying proteomic investigations to unravel the venomes of numerous species [1–32]. Proteomics has been used not only to explore individual species' proteomes [5,10,13,16,20,23,24,29,32–34] and sub-proteomes [19,22,25,35–38], but to compare and contrast differences at both intra- [17,20,30,33,39,40] and inter-species levels [3,9,11,14,15,17,18,36,40–43]. A number of excellent reviews are available [8,44–51]. Many of the recent papers have taken snake proteomics to its logical extension, antivenomics. Proteomic techniques are now being applied to the neglected field of immunotherapy for snake envenoming [8,39,40,43,47,49,51–55]. This has been a most welcome development, perhaps one of the most important new avenues in antivenom research to have been explored in the post-World War II era. Whilst the origins of immunotherapy for envenoming stretch back more than 120 years, a lack of financial incentives, dwindling markets and stagnant leadership from global public health organisations have made this a field of modest improvements and very little innovation. Although antivenoms are the most effective treatment for snakebites, they are frequently inaccessible to the millions of rural poor most at risk from snakebites in low and middle-income countries [56]. This tragic reality belies the fact that, with modest investments in innovation, antivenoms are potentially one of the most cost-effective and affordable treatments [57].

As the 20th century drew to a close, the future of antivenom treatment for snakebites looked anything but certain, particularly in the developing world where these essential medicines are most needed. In Africa, where some of the most exciting developments in antivenom research have been directed, many governments, overwhelmed by the HIV/AIDS epidemic and with numerous other issues of governance to contend with, allowed many less compelling health issues to be ignored, de-prioritised or merely neglected. Snake antivenoms became scarce or non-existent as poor economic viability forced some manufacturers to leave the market, and others to downscale production [58–60]. Although an important humanitarian and social issue, death from snakebite lacks the profile, status and assistance associated with other international health crises in Africa, and reputable manufacturers have been left to carry the burden with only limited support.

Widespread shortages of antivenom in Africa opened doors to opportunists. Poorly manufactured, ineffective products and in some cases complete counterfeits emerged [59,61,62]. Poorly equipped National Regulatory Agencies (NRA) lack the resources and skills to detect and exclude deleterious products. Faced with the choice between an expensive but effective product or an affordable but unproven alternative, many agencies have simply opted for the latter, with disastrous results [63].

Chronic shortages of effective antivenom were not confined to Africa. In Sri Lanka, where the incidence and mortality associated with snakebites are amongst the highest in the world, dependence on poor quality, low potency antivenoms made in India resulted in many patients' not receiving effective treatment, whilst suffering adverse reaction rates as high as 80% [64,65]. Shortages of antivenom have also become common elsewhere in Asia and Oceania, most notably in nations such as Cambodia, Laos, Malaysia and Papua New Guinea, which lack local producers. But deficiencies have also occurred in countries that do produce antivenom, such as Myanmar, where natural disasters have led to acute rises in demand [66,67] and Viet Nam where antivenom was originally introduced by Albert Calmette in the 1890s. These inadequacies have led both to the legal sale of inappropriate products and to thriving black markets [62]. Despite initial promise and some constructive intermediate outcomes, efforts to engage the wider public health community in programmes to address these challenges have been largely unsuccessful [68]. A positive step forward was the publication, following a wide consultation process, of the WHO Guidelines for the Production, Regulation and Control of Snake Antivenom Immunoglobulins, which should be used by manufacturers and regulators for the improvement of antivenom quality [68]. However, WHO's promise to advise national producers and help them achieve prequalification of antivenoms failed to attract funding due to the Global Financial Crisis. It became apparent that the toxinology community itself must assume the responsibility for drawing international attention to the plight of snakebite victims, and developing pragmatic, lasting solutions. To this end, the Global Snakebite Initiative (GSI) was founded in collaboration with the International Society on Toxinology (IST) in 2008 [69,70]. It is currently working towards the development of a strategic approach to improving access to antivenoms in sub-Saharan Africa and South and South-East Asia. In the 21st century, proteomics can play a vital role in the design, development and testing of antivenoms. This paper discusses some of the challenges, opportunities and practical considerations, and identifies broader issues that need to be addressed if the developing world's antivenom drought is to be ended.

2. A brief history of antivenoms

The first experimental snake antivenom was raised in pigeons against venom from a pit viper *Sistrurus catenatus*[71], but the pioneer of immunotherapy in the treatment of envenoming

was Albert Calmette. In October 1891, whilst Calmette was foundation director of the Vaccine Institute of Viet Nam, flooding along the Mekong river led to 40 snakebites including fatalities at Bac-Liêu near the river mouth [72].The District Governor sent Calmette a barrel-full of 19 monocellate cobras (*Naja kaouthia*), from which he harvested venom glands in the hope of raising an antiserum against their contents. Inspired by the earlier success of Emil Behring (diphtheria antiserum) and Shibasaburo Kitasato (tetanus antiserum), Calmette spent much of the remainder of his time in Saigon attempting to raise antiserum to cobra venom, with little success. Recalled to France, he continued his work, and in 1894 produced a protective *sérum antivenimeux* to cobra venom [73,74]. Although Césaire Phisalix and Gabriel Bertrand also demonstrated production of antisera against viper venom that same year [75], it was Calmette who persisted, scaling up from rabbits to produce his cobra antivenom first in donkeys, and then, after moving from Paris to Lille, in horses. In 1895, his antivenom had saved the life of one of his own Annamite snake collectors [76,77]. In 1897 he sent 100 doses to India, and snakebite immunotherapy was well and truly born [72].

Others soon followed Calmette's example. In São Paulo, Dr Vital Brazil started producing antivenoms against *Crotalus* and *Bothrops* species in 1897 [78], and in Australia, Frank Tidswell produced the first experimental equine antivenom against *Notechis scutatus* venom [79]. Significant contributions to early understanding of antivenom specificity and venom/antivenom pharmacodynamics were made between 1897 and 1904 by British scientist Sir Charles James Martin working in Australia [80–85]. Martin's rejection of Calmette's view that antivenom was universally effective against all snake venoms was especially valuable in establishing the principle of specificity, a guiding constraint to future antivenom production and clinical use [81]. Commercial antivenom production in Australia began in 1928 at the Commonwealth Serum Laboratories (CSL) in Melbourne. The first batches of CSL tiger snake antivenom were produced in 1930 [79].Asian nations also took up the production of antivenoms, with Thailand's Pasteur Institute (later Queen Saovabha Memorial Institute) and India's Central Research Institute both producing antisera against local snake species during the 1920s. In South Africa, Dr E. H. Cluver began production of a bivalent *Bitis arietans/Naja nivea* antivenom at the South African Institute of Medical Research, Johannesburg in 1929 [86]. This product had therapeutic potency against several other cobra venoms, as well as *Dendroaspis angusticeps*, *Hemachatus haemachatus* and *Causus rhombeatus*. The South African Institute of Medical Research (SAIMR) released its first commercial antivenom in 1932, and Poul Christensen and colleagues subsequently introduced C.G. Pope's method of pepsin digestion to produce $F(ab')_2$ antivenoms in a drive to improve product safety and quality [87]. Christensen also abandoned the use of formalin-derived venom toxoids as immunogens after experiments showed that native venom could be safely administered to horses in a bentonite adjuvant.

By 1955, there were more than 22 antivenom producers around the world, including 4 in Africa and 5 in Asia [86]. In the 1990s, one manufacturer turned to papain digestion of immunoglobulins raised in sheep, yielding small Fab fragments that were seen as more rapidly distributed, less reactogenic alternatives to $F(ab')_2$ [88,89], and had proved effective in treating digoxin poisoning. However, Fab fragment antivenoms have proved problematic due to their rapid clearance that allowed subsequent recurrence of envenoming due to continued absorption or redistribution of unneutralised venom [90,91]. Another strategy has been to return to the use of intact IgG whose longer clearance time reduces the risk of recurrent envenoming and has theoretical advantages in neutralising and eliminating venom toxins [92]. The use of caprylic acid to precipitate non-immunoglobulin proteins and to improve viral safety has been a valuable, cost-saving development since the late 1980s [93–95].A serious problem throughout the history of the use of antivenom has been the risk of early anaphylactic and late serum sickness-type reactions which in the vast majority of cases have nothing to do with IgE-mediated Type I hypersensitivity but are caused by complement activation by aggregates of IgG or its fragments. Pope's strategy of pepsin digestion, to remove the F_c fragment responsible for complement binding, was introduced in the 1930s to combat this problem but has been of uncertain benefit. However, in the case of autologous human immunoglobulin, 400–800 mg/kg are given intravenously every 4 weeks to patients with hypogammaglobulinaemia. Severe reactions were formerly common but scrupulous improvement in production methods, including chromatographic purification, led to a virtual elimination of these risks. This demonstrates what can be achieved for a product used in the West for which greater financial resources are available.

The fact that antivenom technology has remained relatively unchanged for most of the 20th century reflects the manufacturers' lack of interest and investment in a small scale, low yield production that delivers, at best, small commercial returns. This is vividly illustrated by the abrupt end to some 60 years of venom and antivenom research upon the privatisation of the former Australian Commonwealth Serum Laboratories in 1994. Upon becoming a public company, commercial realities forced CSL Limited to close its world famous antivenom research laboratories [96], although antivenom production continues under a 'National Interest' agreement with the Australian government which provides some subsidy. On the other hand, the German firm Behringwerke AG, abandoned production of all its antivenoms in the 1980s including several widely used African/Middle East polyvalent antivenoms, ostensibly for commercial reasons. In South Africa, the continued production of polyvalent antivenom by South African Vaccine Producers (SAVP) (formerly South African Institute of medical Research SAIMR) has also been fraught with difficulties as a result of financial and other pressures. Health Ministries of some African countries have turned to cheap, ineffective Indian substitutes, which are marketed at much lower prices than the South African product (Eugene Erulu, personal communication). Such competition and resultant market contractures threaten the viability of an effective antivenom product suitable for a wide region of sub-Saharan Africa.

3. The current global situation

3.1. *Sub-Saharan Africa*

The availability of antivenoms in Africa during the 1930s was associated with a decline in morbidity and mortality from

snakebite envenoming which encouraged their widespread use. Sadly, the last 30 years have been marked by critical shortages of this life-saving medication. By 1994, it was estimated that 1.6 million vials of antivenom were required annually for the whole of Africa, yet only 100,000 vials were produced and distributed. It has been suggested that the demise of the antivenom market in Africa was precipitated by the increased cost of improved production methods that, in turn, reduced demand, driving some producers out of business [97]. Within the last 10 years, it has been reported that as little as 2% of the annual requirement for antivenom has been fulfilled [62,97].

In the 1970s, three main manufacturers supplied antivenoms to sub-Saharan Africa: Institut Pasteur, Paris ("IPSER AFRIQUE" covering *Bitis–Echis–Naja–Dendroaspis*), Behringwerke, Marburg ("North Africa" covering *Cerastes–Bitis–Naja–Echis*, and "Central Africa" excluding *Echis* but including *Hemachatus* and *Dendroaspis*) and SAIMR, Johannesburg (Polyvalent and *Echis* monovalent). In Nigeria, Behringwerke antivenoms were the most widely available at a marked-up price to the recipient of as much as UK£60 per vial, but the North Africa antivenom proved disappointing in the treatment of envenoming by the most important species, *Echis ocellatus*. Even doses as high as 12 vials sometimes failed to correct coagulopathy [98]. Importation of SAIMR products was illegal because of anti-apartheid sanctions but it was smuggled in and proved highly effective against *E. ocellatus* envenoming [99]. Behringwerke (Hoechst) ceased production of all their antivenoms sometime in the 1980s, leading to shortages in Nigeria and elsewhere. In the aftermath, there were undocumented impressions of increasing case fatality and morbidity. The role of Pasteur (Pasteur-Mérieux, Aventis-Pasteur, Sanofi-Pasteur) as an alternative supplier was compromised by their decision radically to change the production method [100,101]. This resulted in a prolonged lapse in supply and yielded a product (Sanofi-Pasteur FAV-Afrique) that was unaffordable except to expatriate oil workers and wealthier patients. An average of 37 ± 4 mL of FAV-Afrique was required to cure cases of *E. ocellatus* envenoming in Cameroun [100,101]. In Nigeria, the untreated case fatality of *E. ocellatus* envenoming has been as high as 10–20%, resulting in hundreds of deaths each year [102]. In recent years, the increasing cost and scarcity of antivenom has put this treatment beyond the means of most patients [59,61,103,104], providing an opportunity for unscrupulous marketing of geographically-inappropriate products that have proved clinically disastrous [62,105,106]. Fortunately, several international manufacturers, in the UK, Costa Rica, Colombia and Mexico have taken up the challenge of raising safe and effective antivenoms against the venoms of medically important snakes of this region [107–110].

A 2007 survey of 46 commercial or government manufacturers of antivenom by one of us [N.I.B.], revealed that only 6 companies produced snake antivenom for sale in sub-Saharan Africa. These six companies combined reported manufacturing approximately 227,400 ampoules annually, which, according to product inserts, ought to be enough to treat an average of 55,000 envenomed patients. The cost of a complete course of antivenom therapy from these producers ranged from $40 to $640, with combined revenue from antivenom sales of ~$6 million. Despite the immense need for more antivenom across Africa, only three manufacturers were able to distribute their entire stock. More than 26,000 ampoules (equating to ~10,000 complete treatments) remained unsold. In 2010, further inquiries found that those same six companies had increased production to 410,500 ampoules, or approximately 96,000 effective treatments. All produced stock was sold, and the total sales of antivenom rose to ~$11 million. Unfortunately, not all of the distributed antivenom was regionally appropriate or as effective as claimed. Of the original 46 institutions surveyed globally in 2007, 9 have ceased antivenom production completely.

Although production figures indicate encouraging growth in the volume of snake antivenom being manufactured and utilised in Africa, it is widely known that a large percentage of the antivenom marketed in Africa is ineffective. In particular, the two largest manufacturers of African antivenom, which account for more than 350,000 ampoules and $9 million in sales, are known to use venom immunogens from snake species that are not relevant to Africa.

A further three companies based in Iran, Saudi Arabia and Egypt produce antivenoms intended for Middle-Eastern snakes species, but which may also be effective against venoms of some North African snake species. Three Latin American producers are currently developing or marketing new antivenoms for use against African snake species. Some of these new products have shown promising effectiveness and safety profiles compared to existing commercial products. Critically, these antivenoms are produced using less expensive manufacturing processes than some other commercial antivenoms.

The exact incidence of snakebite envenoming in Africa is unknown, but estimates range from 125,000 to 500,000. There is undoubtedly a great shortage of good quality antivenoms in Africa. Many of the currently available antivenoms have a poor record of safety and effectiveness. Deficient distribution networks, inappropriate clinical use, and a lack of community awareness of the potential benefits of antivenom treatment (compared to traditional remedies) further compound the problem [111]. Many of these traditional "cures" for snakebite have been proven ineffective [112], but their apparent success in cases of non-envenoming by "dry" snakebites earns them unwarranted credibility, and in the absence of an effective, affordable antivenom alternative, provides false-hope to many patients in sub-Saharan Africa.

3.2. *South and Southeast Asia*

In India, there are currently five manufacturers of polyvalent antivenoms for South Asia raised against venoms from the same four species (*Naja naja*, *Daboia russelii*, *Echis carinatus* and *Bungarus caeruleus*) for an average of INR250-280/vial (US $5.50–6.20/vial). Of these, only three produced antivenom for the Indian market in 2010 [VINS (1.2 million vials), Bharat Serums and Vaccines (600,000 vials) and Biological E Limited (60,000 vials)], whilst a fourth (Haffkine Pharmaceuticals Limited) exported undisclosed amounts to Nepal, Bangladesh and other neighbouring countries, but distributed none in India. The other producer, Serum Institute of India (SII) exported 4000 vials of polyvalent antivenom to Africa. There was no antivenom production by either the King Institute in Chennai or the Central Research Institute, Kasauli. For all of these products,

the manufacturers claim identical potency, but published preclinical assessment data are not available for any of them, and the potencies themselves are remarkably low (0.45 mg venom/mL antivenom for *B. caeruleus* and *E. carinatus*, and 0.6 mg/mL for *N. naja* and *D. russelii*). Historically it appears that Indian authorities once required antivenoms to be much more potent, with standards of 4.0 mg/mL for *D. russelii* venom and 2.0 mg/mL for *N. naja* venom being required prior to the mid-1950s [86]. It is not clear why these standards were relaxed, but the consequences have had a considerable impact in India and neighbouring countries. Patients may require massive doses of these antivenoms. In northern India, one study reported that an average of 51.2 (5–190) vials of Indian-made polyvalent antivenom was administered to patients bitten by *Naja* spp. and *Bungarus* spp., whilst patients bitten by *D. russelii* received an average of 32 (1–130) vials [113]. Another northern Indian report on neurotoxic snakebite quoted a median dose of 90 vials per patient, and in southern India, a study in Kerala State reported overall average usage of 22 vials per patient [114,115]. Administration of high doses of poorly refined Indian antivenoms have been associated with reports of high rates of pyrogenic and other early adverse reactions [64,65,116].

In Thailand, where antivenoms have been produced since 1923 by the Queen Saovabha Memorial Institute (QSMI), some products currently have similar potencies to those made in India, even though the species against which the products are raised are different. Until the mid-1990s there were a number of problems with the quality and effectiveness of QSMI antivenoms [117], but new production technologies and quality controls developed over the past 15 years have led to considerable improvements in quality, if not therapeutic potency [118–121]. Traditionally, QSMI produced only monospecific antivenoms which were difficult to use throughout wide areas of the country where three or more medically important species gave rise to almost identical clinical syndromes. Recently, QSMI has produced two polyspecific antivenoms (haemato-polyvalent and neuro-polyvalent) to cover viper and elapid envenomings respectively. Thailand's other antivenom producer, the Government Pharmaceutical Organization (GPO) has ceased production at present.

Elsewhere the problems are more diverse. A polyvalent antivenom manufactured by P.T. Bio Farma in Bandung, Indonesia uses venoms from *Calloselasma rhodostoma*, *Naja sputatrix* and *Bungarus fasciatus*, the last of which is a very rare cause of envenoming in Southeast Asia, whereas *Bungarus candidus* (not included) causes many more bites. Also missing from this antivenom is coverage against the Indonesian populations of *Daboia siamensis* and *Cryptelytrops insularis* and the Australasian elapids (*Acanthophis*, *Pseudechis*, *Pseudonaja*, *Oxyuranus*) occurring in the islands east of Weber's line. Potency is based on the number of murine LD_{50} doses of each venom neutralised by 1 millilitre antivenom, with 10–15 LD_{50}*C. rhodostoma* venom and 25–50 LD_{50}*N. sputatrix* and *B. fasciatus* venom per millilitre stated by the manufacturer. For example, based on the LD_{50} of *B. fasciatus* venom in mice (0.024–0.028 mg/mouse), the potency of Indonesian polyvalent antivenom against this species would be 6.0–14 mg/10 mL vial, however this does not accommodate regional venom variation [122].

In Burma (Myanmar), one of the SE Asian countries worst affected by snakebite, Myanmar Pharmaceutical Industry (MPI) has manufactured monospecific and bi-specific antivenoms covering *D. siamensis* and *N. kaouthia*. Unfortunately, production problems led them to dilute the antivenom in order to maintain output volume, but this was done without doubling the recommended dose and lead to patients receiving ineffective doses. Recently they have developed experimental chicken egg (IgY) and ovine antivenoms for *D. siamensis*. In response to cyclone Nargis (2008), a surge in cases of snakebite in Myanmar was predicted. To supplement the already stressed supply of MPI antivenoms, emergency importation of Indian polyvalent antivenom was initially suggested to WHO. However, rodent ED_{50} studies had shown these to be ineffective against Burmese *D. siamensis* venom (Tun-Pe personal communication) and, mercifully, this inappropriate advice was discarded in favour of Thai QSMI Russell's viper monospecific antivenom. In view of the greater clinical severity of *D. siamensis* envenoming in Myanmar compared to Thailand, double the normally-recommended initial dose of QSMI antivenom was recommended. In Viet Nam, two national producers have now emerged after a prolonged period during which antivenom was unobtainable.

3.3. *Papua New Guinea*

Papua New Guinea (PNG) uses Australian-made antivenoms, manufactured by CSL Limited (formerly Commonwealth Serum Laboratories). It was the perceived need in the 1950s for specific antivenom for bites by the Papuan blacksnake (*Pseudechis papuanus*) that led to the development of CSL's Black Snake Antivenom. Four of CSL's terrestrial snake antivenoms (Black Snake, Death Adder, Polyvalent and Taipan) and their Sea Snake Antivenom are currently in use in PNG. Clinical evidence supports a view that early administration of both CSL Polyvalent and Taipan Antivenoms prevents lethal presynaptic neurotoxicity after bites by Papuan taipans (*Oxyuranus scutellatus*) [123]. CSL Death Adder antivenom is effective at preventing or reversing postsynaptic neurotoxicity caused by smooth-scaled death adder (*Acanthophis laevis*) envenoming regardless of when it is given [124]. These are, however, very expensive products for a developing nation with a high burden of both infectious and non-infectious disease, and a myriad of institutional, logistic, policy and political challenges. Due to economic decisions by the PNG government, and product price increases the cost (per vial) of CSL Polyvalent to the PNG Department of Health increased from US$334 to US$1500 between 1987 and 2007, corresponding to a 40% decline in annual stock availability [125,126]. Over the same time the price of CSL Taipan Antivenom rose from US$320 to US$1430; that of CSL Death Adder Antivenom rose from US$210 to US$855; and CSL Black Snake Antivenom costs increased from US$247 to US$1094 per vial [125,126]. As a consequence, these antivenoms have become increasingly unaffordable to a health system already under enormous stress, leading to chronic antivenom shortages of antivenom and poor clinical outcomes. The high prices and relative scarcity have led to a flourishing black market, where stolen antivenoms are resold by private pharmacies and unlicenced wholesalers for up to US$3200 per vial [127]. Efforts involving local and international collaborators are now underway to develop new antivenoms for use in Papua New Guinea

that can reduce the cost burden without sacrificing safety or clinical effectiveness [128]. CSL are also exploring a variety of options for improving access to their antivenoms across PNG, and this very positive step, promises to ameliorate some of the supply shortages experienced in the past.

3.4. *Latin America*

Compared to antivenom manufacture and accessibility in Africa and some regions of Asia, the situation in Latin America and the Caribbean is more favourable. There are antivenom manufacturing laboratories in Mexico, Costa Rica, Colombia, Venezuela, Ecuador, Bolivia, Perú, Brazil, Argentina and Uruguay [129]. Most of these producers are public laboratories belonging to Ministries of Health or public universities, whereas there are private manufacturers in Mexico, Colombia and Argentina. Mexico, Costa Rica, Brazil and Argentina produce enough antivenom for national and, in some cases, regional needs, but in Ecuador, Perú, Colombia, Venezuela and Bolivia, production generally does not fulfil the national demand for antivenoms. Other countries, such as Paraguay, the Guyanas and some Central American countries have no local antivenom producers and rely on regional manufacturers, mostly in Brazil, Costa Rica and Mexico, to fulfil their needs [129].

Where antivenoms must be imported, careful quality control is essential to ensure that they are effective against the venoms of the most important snake species in the country. In general, a pattern of extensive cross-reactivity of antivenoms has been described in the region [130]. However, there have been cases where ineffective antivenoms have been imported and used, with poor clinical results. In Ecuador, in the 1990s, the national producer was unable to fulfil the country's antivenom requirements. As a result, a Mexican antivenom was imported that had been raised against Mexican venoms. The clinical results were disastrous in the Oriente region and subsequent pre-clinical testing against the principal species of medical importance demonstrated negligible efficacy [131]. In 2005, when the INS was producing only liquid antivenoms, the demand for freeze-dried antivenom for areas of the Amazon province of Loreto that had no cold chain led to the purchase of a Brazilian antivenom, which proved to be a veterinary product that had not been cleared for human use in its country of production. Subsequently, the INS has developed a freeze-dried product that is awaiting clinical evaluation (Armando Yarlequé personal communication). In the Lesser Antillean islands of Martinique and Saint Lucia, where severe envenomings by endemic *Bothrops* species occur, an antivenom manufactured in France (Bothrofav®; Sanofi-Pasteur) has proved effective [132]. An improved Bothrofav® has now been developed using the pooled venom from a larger group of *Bothrops lanceolatus* specimens and has produced encouraging preclinical results (Laurent Thomas personal communication).

In the recent years, a regional effort has been coordinated in Latin America to foster cooperation between public laboratories in the region, with the support of the programme CYTED and the Pan American Health Organization [129,133]. This has resulted in workshops and training programmes aimed at improving the local capacity for antivenom production and development. It is expected that such regional integration will contribute to improve the manufacture and quality control of antivenoms in the region, with the long term goal of fulfilling the total regional demand of high quality antivenoms in Latin America. Likewise, it is necessary to develop renewed efforts to improve the distribution of antivenoms, especially to vulnerable regions, such as those populated by indigenous groups, as well as to develop training programmes for health staff in charge of attending snakebite victims assisted by the publication of regional treatment guidelines similar to those available for envenoming in Africa and South-East Asia.

4. How can we best contribute to sustainable improvements?

The reality of the current antivenom situations in Africa, South and South-East Asia, and Papua New Guinea is such that many obstacles stand in the path of sustainable solutions. Many of the broader issues of governance, poor medicines regulation, corruption and social inequity apply generally to the societies in which these problems have become endemic, and will perhaps take many decades to resolve. By far the most surmountable challenges are those where we can deploy considerable experience; that is, in the design, production and assessment of new candidate antivenoms, and in the refinement of current products to better meet particular needs. If a multidisciplinary, international collaborative effort were to be mounted, new and improved antivenoms that meet high standards could be produced in sufficient volumes to serve not just the needs of one or two nations in an affected area, but those of whole regions.

There are already examples of constructive international efforts to provide improved antivenoms for sub-Saharan Africa, South Asia and elsewhere. MicroPharm UK (formerly an Anglo-American company, Therapeutic Antibodies) has developed improved antivenoms for Nigeria and Sri Lanka by working in collaboration with local Ministries of Health and with academic institutions, Liverpool School of Tropical Medicine and University of Oxford. Initially, their distinctive strategy was to raise antibodies in sheep rather than horses, and to refine them to Fab fragments in the hope of hastening tissue distribution and reducing the risk of anaphylactic reactions. This approach persists in the form of ViperaTAb used for treating *Vipera berus* envenoming in Sweden and CroFAb used for crotaline envenomings in the United States. However, the rapid renal elimination of Fab fragments gave rise to recurrent envenoming and has created the need for cumbersome and protracted dosage regimens for CroFAb. This problem was documented in early trials of EchiTAb for *E. ocellatus* envenoming in Nigeria [134] and PolongaTAb raised against Sri Lankan *D. russelii* venom for use in Sri Lanka [90]. Despite its specificity for the venom of indigenous *D. russelii* and encouraging results of clinical testing, PolongaTAb was not taken up by the Health Ministry in Sri Lanka, which continues to purchase antivenoms of Indian manufacture. In Nigeria, EchiTAb was redesigned as $F(ab')_2$ and finally whole IgG ("EchiTAb-G"), eliminating the problem of

recurrent envenoming and ensuring that, in most cases, a single dose treatment was effective in permanently correcting coagulopathy [135].

5. So what does proteomics and biotechnology have to do with all of this?

A great deal of the interest in subjecting snake venoms to rigorous proteomic investigation has its foundations in the quest to discover, characterise and harness their bioactive constituent toxins in the fields of drug discovery and design [8,49]. But researchers have not ignored the fact that these venoms are principally diverse offensive weapons systems, and that humans are all too often the subject of their onslaughts. Many of these victims are amongst the most impoverished inhabitants of some of the least developed nations on earth [56]. Whilst a lack of comprehensive reporting systems, and a multitude of logistical challenges currently hinder attempts accurately to estimate the true global burden of envenoming, the current consensus is that somewhere between 421,000 and 2.5 million cases of envenoming occur annually, leading to 20,000–125,000 deaths [136,137]. These lower totals are questionable, since recent reports, based on representative community studies, of 6000 snakebite deaths from 590,000 bites per year in Bangladesh [138] and 46,000 snakebite deaths per year in India [139], eclipse them. Many of these lives could be saved, and considerable disability prevented though the early use of appropriate, well-designed, rigorously tested antivenoms. Proteomics offers the exciting possibility of improving both the design of immunogen mixtures, and antivenoms, and of providing a mechanism for assessing the suitability of antivenoms for existing and potentially new markets.

5.1. *Proteomic tools for studying venoms*

5.1.1. *Snake venomics*

Venom profiling by reverse-phase HPLC separation, followed by determination of the homogeneity of the chromatographic fractions by combination of SDS-PAGE, accurate molecular mass determination (by MALDI-TOF-MS or ESI-MS), and N-terminal Edman sequencing, allow an initial, global insight into the number and classes of toxins present in the venom [8]. Since toxins likely belong to a restricted set of protein families [140], accurate mass figures significantly reduce the number of candidate proteins (Table 1). In addition, most venom proteins are characterised by a high and protein-family-specific cysteine content [8]. S—S bonds are particularly common in proteins, such as venom toxins, which are too small to have a well defined hydrophobic core, and are thus important for fold stability. The stabilising effect of a disulfide bond (2.5–3.5 kcal/mol) is proposed to come from the decrease in conformational entropy of the unfolded state. The large structural impact at low energy cost of engineering disulfide bonds represents an opportunity for the structural (and functional) diversification of proteins during evolution. Hence, determining the number of cysteines represents a useful tool for the preliminary classification of toxins into protein families (Table 1), and mass spectrometry is perhaps the best-suited technique for counting cysteine residues in proteins [8]. Some venom proteins form complexes containing subunits from the same or different toxin classes held in position by noncovalent interactions like hydrogen bonds, ionic bonds, van der Waals forces, and hydrophobic interactions. Each subunit can have one or more polypeptide chains linked by covalent disulphide bonds. Multimeric proteins exhibit much higher levels of pharmacological activity than individual components and play an important role in pathophysiological effects during envenomation [141]. Covalent arrangements of polypeptide chains can be easily detected by analysing the RP-HPLC-separated venom fractions via non-reduced and reduced SDS-PAGE [8] (Fig. 1). The isolation of non-covalent complexes, which dissociate during RP-HPLC and SDS-PAGE, is more demanding: it requires the application of "old-fashioned" classical separation techniques, such as non-denaturing chromatographic or electrophoretic protocols, that preserve their native quaternary conformations.

Separation of venom components by RP-HPLC allows the quantitative recovery of all venom components present in the molecular mass range of 7–150 kDa. Although the same components can also be separated by conventional 2D

Table 1 – Classification of snake venom toxins to protein families according to their cysteine content. [a], Intersubunit disulfide bonds; [b], intrasubunit disulfide linkages; C-NP, C-type natriuretic peptide; 3Ftx, three-finger toxin; CRISP, cysteine-rich secretory protein; SVMP, snake venom metalloproteinase; DC, disintegrin-like/cysteine-rich domains of PIII-SVMP; svVEGF, snake venom vascular endothelial growth factor; LAO, L-amino acid oxidase.

Molecular mass range	Total cysteine residues		Protein family
(kDa)	—SH	S—S	
1.6–3	–	1	C-NP
–	–	2	Sarafotoxin
4–5	–	3	Myotoxin
–	–	4	Short disintegrin
6–9	–	2	Waprin
–	–	3	Kunitz-type inhibitor
–	–	3	Kazal-type inhibitor
–	–	4	3Ftx
–	–	6	Medium-sized disintegrin
–	–	7	Large disintegrin
10–12	1	–	Ohanin
13–16	–	2	Cystatin
–	–	3	sv nerve growth factor
–	–	(2^a+4^b)	Dimeric disintegrin
–	–	7	PLA_2
23–33	–	8	CRISP
–	1	4	PI-SVMP
–	–	6	Serine proteinase
–	–	(1^a+3^b)	αβ snaclec (C-type lectin)
–	–	(1^a+4^b)	svVEGF
–	–	13	DC-fragment
46–58	–	3	LAO
–	1	18	PIII-SVMP
100–110	–	(2^a+3^b)	$(αβ)_4$ snaclec (C-type lectin)

electrophoresis, the initial part of the acetonitrile gradient of the reverse-phase chromatography resolves peptides and small proteins (0.4–7 kDa), which would not be recovered from a 2DE gels. Moreover, for the accurate mass spectrometric determination of toxin-specific features, such as the native molecular mass, the quaternary structural arrangement, and the number of sulfydryl groups and disulfide bonds, toxins need to be available in solution. In addition, given that the wavelength of absorbance of a peptide bond is 190–230 nm, protein detection at 215 nm allows the estimation of the relative abundances (expressed as percentage of the total venom proteins) of the different protein families from the relation of the sum of the areas of the reverse-phase chromatographic peaks containing proteins from the same family to the total area of venom protein peaks in the reverse-phase chromatogram. The relative contributions of different proteins eluting in the same chromatographic fraction can be estimated by densitometry after SDS-PAGE separation. According to the Lambert–Beer Law ($c\,[M] = A/\varepsilon l$), the calculated figures correspond to the weight % (g/100 g) of peptide bonds. To estimate the relative contribution of each toxin family expressed as protein molecules/100 molecules of total venom proteins, the weight percentages of peptide bonds of each family should be normalised for the number of peptide bonds (amino acids) in the full-length sequence of a representative member of the protein family (Fig. 1).

Venom protein fractions showing single electrophoretic band and N-terminal sequence can be straightforwardly assigned by BLAST analysis to known viperid protein families. On the other hand, protein fractions showing heterogeneous or blocked N-termini are analysed by SDS-PAGE and the bands of interest subjected to automated reduction, carbamidomethylation, and in-gel tryptic digestion. The resulting tryptic peptides are then analysed 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 spectrometry [8,49]. CID spectra are manually interpreted or using a licenced version of the MASCOT programme (http://www.matrixscience.com) against a private database containing the viperid protein sequences deposited in the SwissProt/TrEMBL database plus the previously assigned peptide ion sequences from snake venomic projects carried out in our laboratories. The outlined snake venomic protocol allows the unambiguous assignment to known protein families of all isolated venom toxins representing ≥0.05% of the total venom proteins [8,49]. Abundant venom proteins may perform generic killing and digestive functions that are not prey specific whereas low abundance proteins may

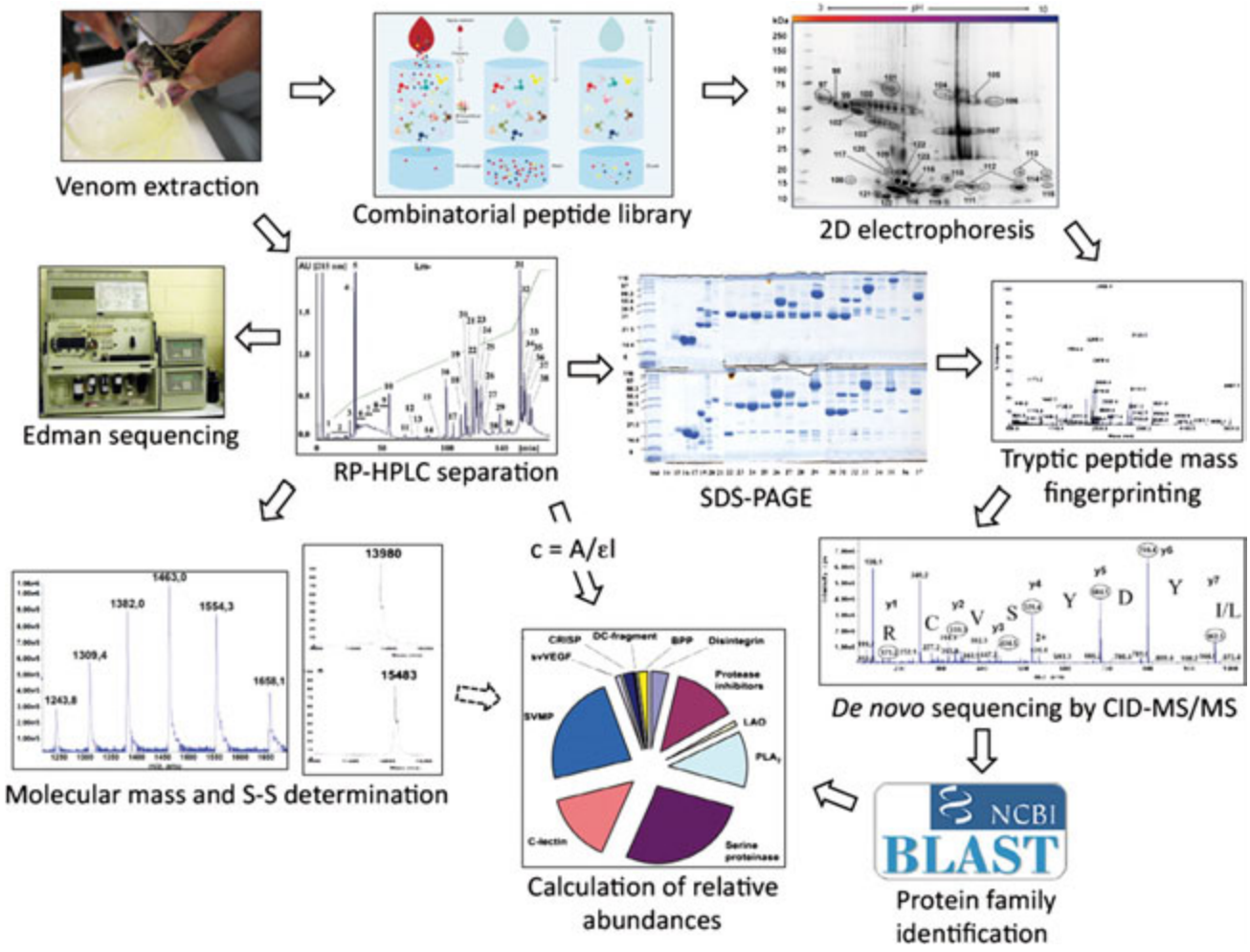

Fig. 1 – Scheme of the steps typically followed in a snake venomic project.

be more plastic either in evolutionary or ecological timescales. Low abundance proteins may serve to "customise" an individual venom to feeding on particular prey, may represent orphan molecules evolving functionally unconstrained "*in search of a function*", or molecules involved in venom gland functioning. Due to the wide dynamic range of proteins in complex proteomes, visualising every component of a proteome is not possible using a single proteomic technique, and compromises are always required. Recent contributions to the literature of snake venomics have underscored the need for multifaceted approaches for maximising proteome coverage [45]. Removal of high-abundance proteins has extensively been used for decomplexing proteomes. However, this approach might remove several minor species together with the target proteins. Recently, the Combinatorial Peptide Library approach (commercialised as Proteominer™) [50] has emerged as a powerful tool for mining below the tip of the iceberg, and complements the data gained using the snake venomic protocol towards a complete visualisation of the venom proteome [24,29] (Fig. 1).

5.1.2. *Antivenomics*

The deficit of antivenom supply in some regions of the world can be addressed to a certain extent by optimising the use of existing antivenoms and through the design of novel immunisation mixtures for producing broad-range polyspecific therapeutic antivenoms. Toxins from the same protein family present in venoms from snakes belonging to different, even phylogenetically distant, genera often share antigenic determinants [47,49]. Assessing the cross-reactivity of a polyvalent antivenom against venoms not included in the immunisation mixture may thus aid in expanding their range of clinical application.

Western blot and ELISA analysis are the most popular techniques for assessing the immunoreactivity of antibodies. However, the immunochemical detection of blotted proteins provides a Yes/No response: a given protein is recognised 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 SDS-sample buffer. This treatment may introduce artefacts such as loss of conformational epitopes and/or artefactual recognition of non-native epitopes. On the other hand, although antibody binding levels are quantified in an ELISA-based protocol, this technique does not discriminate between binding and non-binding molecules present in a mixture. To circumvent the limitations of Western blotting and ELISA, we have developed "antivenomics" for the qualitative and quantitative analyses of the immunoreactivity of antivenoms [43]. The original antivenomic protocol is based on the immunodepletion of toxins upon incubation of whole venom with purified antivenom IgGs, followed by the addition of a secondary antibody or immobilised IgG-binding moiety, such as protein-A or protein-G [43]. 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 characterised by a venomic approach. According to their immunoreactivity towards antivenoms, toxins may be conveniently classified as: C-toxins, completely immunodepletable toxins; P-toxins, *p*artly immunodepleted toxins; and N-toxins, *n*on-immunodepleted proteins [47,49]. Assuming a link between the in vitro toxin immunodepletion capability of an antivenom and its in vivo neutralising activity towards the same toxin molecules, improved immunisation protocols should make use of mixtures of immunogens to generate high-affinity antibodies against class P and class N toxins. A further aim of antivenomics is to define the intra- and interspecific complexity of venoms in terms of common and unique antigenic determinants. This information is relevant for defining the minimal set of venoms containing all epitopes necessary to generate therapeutic broad-range polyvalent antisera. Antivenomics 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.

Another recently developed antivenomic technique in RAH's laboratory utilises affinity column chromatography, rather than immunoprecipitation, to isolate/identify venom components that do not bind to antivenom. This method was developed following the successful use of a simple, cheap and easily replicable column chromatography technique to measure the specific antibody content of an antivenom, by binding and eluting immunoglobulins from venom-coupled columns [89,142,143]. For antivenomics, this technique was reversed by coupling antivenom to HiTrap NHS-activated HP affinity columns, before varying concentrations of reconstituted venom were sequentially introduced to the column. Subsequently, columns were washed with a sodium phosphate buffer (pH 7.5) and then eluted with 0.1 M glycine (pH 2.5). Fractions containing the washed (i.e. unbound) and eluted (bound) material were collected and visually analysed using reduced and native PAGE, before immunoblotting with antivenom to confirm unbound toxins are present due to the absence of immunoreactivity, rather than saturation of antibody binding sites. Unbound proteins visualised by gel electrophoresis were then excised from the gel, de-stained and in-gel trypsin digested [144], before protein identification by LC–MS and tandem mass spectrometry as previously described [30]. Using these techniques, multiple snake venom metalloproteinase (SVMP) and cysteine rich secretory protein (CRISP) venom toxins from four *Echis* venoms (*E. ocellatus*, *Echis coloratus*, *Echis pyramidum leakeyi* and *Echis carinatus sochureki*) that were not bound by the monospecific anti-*E. ocellatus* antivenom EchiTAbG were identified (Table 2). The antivenom utilised was recently determined to be highly efficacious in both pre-clinical and clinical trials [135,142,145]. In total, ten protein bands were present in the unbound fractions; these bands were not observed in eluted fractions at any venom concentration. Notably, in reduced form each protein band was reactive with IgG when immunoblotted with EchiTAbG antivenom. However, in native form the unbound fractions showed complete absence of immunoreactivity, yet whole venom samples exhibited high reactivity to IgG. These results suggest that whilst these venom proteins are not recognised by antivenom immunoglobulins in their native form, protein denaturation exposes epitopes recognised by antibodies for binding. These results perhaps account for observations that some immunoassays, whilst providing comprehensive and

Table 2 – Antivenomic identification of venom proteins from four *Echis* species which failed to bind to the *E. ocellatus* antivenom EchiTAbG. Cluster identifications arise by BLAST sequence similarity to translated expressed sequence tags derived from the four *Echis* venom gland transcriptomes [148,149]. In all cases 100% sequence similarity was observed. Cluster representation is expressed as the percentage of toxin encoding ESTs each cluster represents in the respective species venom gland transcriptome [149].

Species	Band	Protein family	Cluster identified	Cluster representation	GenBank accession number	Peptide ion m/z (Da)	z	MS/MS derived sequence	Mascot		Sequest	
									Ion score	Exp value	Probability	XCorr
E. ocellatus	Eoc1	CRISP	EOC00029	0.29%	DW361159	595.055	+2	SVNPTASNMLR	37	0.00176		
						777.605	+2	MEWYPEAAANAER	34	0.00199		
						603.100	+2	SVNPTASNMLR	32	0.00419		
						777.605	+2	MEWYPEAAANAER			37.62	2.92
						603.100	+2	SVNPTASNMLR			37.62	2.26
	Eoc2	PII-SVMP	ECO00011	5.74%	GU012238	487.510	+2	NNGDLTAIR	52	0.00005		
			EPL00005	17.65%	GU012274	487.050	+2	NNGDLTAIR	46	0.00017		
						487.050	+2	NNGDLTAIR			43.39	2.68
						487.510	+2	NNGDLTAIR			31.58	2.41
E. p. leakeyi	Epl1	PII-SVMP	EPL00005	17.65%	GU012274	606.235	+2	QSVGIIENHSK	38	0.00110	–	–
						620.650	+2	HDNTQLLTGLK	35	0.00247	–	–
						515.990	+2	EYQSYLTK	–	–	19.20	2.01
						1032.325	+1	EYQSYLTK	–	–	6.49	1.47
						1031.295	+1	EYQSYLTK	–	–	21.84	1.42
	Epl2	No sig. hit	–	–	–	–	–	–	–	–	–	–
E. coloratus	Eco1	No sig. hit	–	–	–	–	–	–	–	–	–	–
	Eco2	PII-SVMP	ECO00020	5.74%	GU012246	494.190	+2	NKGDLTAIR	36	0.00244	–	–
						494.625	+2	NKGDLTAIR	35	0.00275	–	–
						494.190	+2	NKGDLTAIR	–	–	5.72	2.59
	Eco3	PI-SVMP	ECO00047	1.51%	GU012229	527.010	+2	YNSDLTAIR	47	0.00017	–	–
						527.010	+2	YNSDLTAIR	–	–	32.94	2.28
E. c. sochureki	Ecs1	CRISP	ECS00168	1.83%	GR950013	603.065	+2	SVNPTASNMLR	29	0.00915	–	–
						603.065	+2	SVNPTASNMLR	–	–	34.70	2.39
						595.570	+2	SVNPTASNMLR	–	–	21.21	2.22
	Ecs2	PII-SVMP	PII-SVMP	1.47%	GU012265	754.025	+2	DLINVVSSSSDTLR	33	0.00350	–	–
			ECS00253			754.025	+2	DLINVVSSSSDTLR			26.75	2.90
	Ecs3	PIIISVMP	EOC00001	2.60%	AM039691	1535.880	+3	XNHDNTQLLTGMNFDGPTAGLGYVGTMCHPQFSAAVVQDHNK	21	0.00910	–	–

informative immunological profiles of an antivenom, may not be good predictors of pre-clinical and clinical efficacy [142,146,147]. Eight of the ten unbound proteins observed here were successfully identified against translated transcriptomic databases [148,149] as members of SVMP and CRISP toxin families (Table 2).

Surprisingly the results found using these affinity chromatography techniques differ from those obtained by immunoprecipitation antivenomic analysis of *Echis* venoms with the polyspecific antivenom EchiTAbG-Plus-ICP, where disintegrins and phospholipase A_2 (PLA_2) were the main unbound venom components [54] (Table 3). Clearly, the difference in immunogens and animals used for immunisation (equine EchiTAb-Plus-ICP: raised against *E. ocellatus*, *B. arietans* and *Naja nigricollis*, ovine EchiTAbG: *E. ocellatus* only) may be responsible for these differences, although the absence of unbound SVMPs and CRISPs by EchiTAb-Plus-ICP and disintegrins and PLA_2 by EchiTAbG suggests the diversity of immunising venoms or perhaps an inherent difference in equine and ovine immunoglobulins may be responsible. Also, without a direct comparison, we cannot rule out that the technical differences between these antivenomic approaches accounted for the distinct results. Considering the potential of antivenomic techniques to identify (i) venom components of poor immunogenicity and (ii) venom components for "supplemental-immunisation" to improve antivenom efficacy, the existence of at least two distinct antivenomic methods — more research is required to comprehensively evaluate these techniques to meet the described objectives (i and ii above). In addition, antivenomic studies should be complemented with neutralisation assays, in order to correlate immunoreactivity with the neutralisation of the most relevant toxic activities in a venom. The combination of these two approaches provides robust information to assess antivenom efficacy and cross-reactivity.

5.2. *Getting the names and places right: systematics, phylogenetics and snake distributions*

A key component in the development of a strategy for the improvement of the snakebite situation has to be an improved understanding of the systematics, identification, phylogeny, distribution and behaviour of venomous snakes. In addition, the identification of evolutionary and immunological trends amongst venoms may aid in (a) defining the appropriate mixture of venoms for immunisation to produce effective polyvalent antivenoms, and (b) expanding the clinical range of currently existing antidotes.

At its most basic, formulating an antivenom strategy for Asia and Africa requires identification of the venomous species of greatest public health importance, documenting their distribution and their public health impact. This in turn necessitates as a fundamental precondition the accurate identification of the species involved, which must depend on robust taxonomic knowledge. Although the venomous snake fauna of both Africa and Asia have received extensive taxonomic attention in recent years, the steady stream of discoveries of new taxa, including large and conspicuous venomous species (e.g., *Bungarus slowinskii*[150]; *Naja ashei* [151]), has shown no sign of abating, and revisions of genera invariably lead to substantial increases in species numbers [150,151]. The rate of discovery and redefinition of species has been stimulated by the advent of molecular approaches, particularly mitochondrial DNA sequencing, and increasingly the use of nuclear DNA markers. Genetic and morphological data are mutually enlightening: for instance, congruence between morphology and mtDNA can confirm that morphologically differentiated populations represent different evolutionary lineages (=species) rather than populations within a single gene pool [152–154], or that different mtDNA haplotypes denote separate lineages rather than constituting relics of matrilineal descent within a single panmictic population [155]. Similarly, molecular approaches have provided increasingly robust evidence on the phylogenetic relationships at higher taxonomic levels, such as the evolution of major clades of venomous snakes [156–160] and indeed the phylogeny of snakes as a whole [161,162].

Elevation to species status of taxa previously considered as subspecies deserves special attention, in particular for establishing venom activity correlations based on published reports. The case the taxonomic confusion surrounding "*N. nigricollis* α-neurotoxin" serves to illustrate this point. This neurotoxin, termed α-toxin, was purified from the venom of "*N. nigricollis* collected in Ethiopia in 1961" [163] and maintained since then at the serpentarium of the Pasteur Institute (France). The α-toxin, a potent competitive antagonist of the nAChRs at the skeletal muscle neuromuscular junction, has been the subject of detailed structure–function studies [164], in particular by André Ménez's group [165]. However, a recent proteomic investigation of the toxin profile of African spitting cobra venoms failed to find α-toxin in *N. nigricollis* venom, but identified it in the venoms of *Naja katiensis* (4.4% of the total venom proteome), *Naja pallida* (2.8%), and *Naja nubiae* (12.6%) [166]. Furthermore, the polyspecific EchiTAb-Plus-ICP® antivenom, produced by hyper-immunisation of horses with a mixture of venoms from *E. ocellatus*, *B. arietans* and *N. nigricollis*, effectively neutralised the dermonecrotic and PLA_2 activities of all African *Naja* venoms, whereas lethality was eliminated in the venoms of *N. nigricollis*, *Naja mossambica* and *N. pallida*, but not in the venoms of *N. nubiae* and *N. katiensis*. The African spitting cobras have a long history of taxonomic instability. Relevant for understanding the "α-toxin paradox", Branch [167] and Hughes [168] elevated *pallida* to full species status [167,168]. This was later confirmed by Wüster and Broadley in 2003, who also described populations previously assigned to *N. pallida* from northern and north-eastern Africa as a new species, *N. nubiae* [169,170]. Hence, the lack of neutralisation of lethality of *N. nubiae* and *N. katiensis* by the EchiTAb-Plus-ICP® antivenom may be due to the occurrence of high concentrations of α-toxin in these venoms and the lack of this antigen in *N. nigricollis*. This suggests that the immunisation mixture used to produce an improved antivenom should probably include an α-toxin-containing venom, i.e. *N. nubiae*.

Epidemiological and systematic studies can be mutually enlightening: collections of snakes brought in by snakebite patients have contributed much new knowledge to our understanding of snakebite epidemiology, and the preserved specimens have contributed significantly to improving snake taxonomy in some countries: for instance, the identification and redefinition of medically important species such as *Naja siamensis* in Southeast Asia [171,172]. Today, the existence of

Table 3 – Assignment of reverse-phase isolated proteins from the non-immunodepleted HPLC fractions of the venoms of *Echis ocellatus*, *E. leucogaster*, *E. pyramidum leakeyi*, *B. arietans* (Ghana), *B. arietans* (Nigeria), *Bitis nasicornis*, *B. rhinoceros*, and *B. gabonica*, to protein families by N-terminal Edman sequencing, mass spectrometry and collision-induced fragmentation by nESI-MS/MS of selected peptide ions from in-gel digested protein bands. X, Ile or Leu; Z, pyrrolidone carboxylic acid. Unless otherwise stated, for MS/MS analyses, cysteine residues were carbamidomethylated; molecular masses were determined by electrospray-ionisation mass spectrometry or SDS-PAGE of reduced (▼) samples; n.p., non peptidic material found.

HPLC fraction	N-terminal sequence	Molecular mass	Peptide ion	MS/MS-derived sequence	Protein family	% of immunodepletion	
			m/z	z			
Echis ocellatus							
Eo-1	Blocked	444.1	444.1	1	ZKW	Inhibitor of SVMP	42
Eo-2	DCESGPCCDNCKFLK	5494, 5592	–	–	–	Disintegrin ocellatusin [Q3BER]	62
Eo-3	SVVELGKMIIQETGKS	13,825	–	–	–	PLA_2 [CAQ72890]	67
Eo-4	SVIEFGTMIIEETGRSPF	13,866	–	–	–	PLA_2 [CAQ72891]	45
Echis leucogaster							
El-1	DCESGPCCRDCKFLK	5458	–	–	–	Disintegrin leucogastin B [P0C7A8]	60
El-2	Blocked	444.1	444.1	1	ZKW	Inhibitor of SVMP	38
El-3	DCASGPCCRDCKFLE	5426	–	–	–	Disintegrin leucogastin A [P0C7A7]	65
El-4	NLYQFGKMIKNKTGK	14,066	–	–	–	PLA_2	40
El-5	SVIELGKMIIQLTNK	13,696	–	–	–	PLA_2	25
Echis pyramidum leakeyi							
Ep-1	DCASGPCCRDCKFLKEGT	5555	–	–	–	Disintegrin pyramidin A [P0C6R7]	66
Ep-2	Blocked	444.1	444.1	1	ZKW	Inhibitor of SVMP	48
Ep-3	DCASGPCCRDCKFLEE	5434	–	–	–	Disintegrin pyramidin B [P0C6R8]	60
Ep-4	NLYQFGKMIKNKTGK	14,103	–	–	–	PLA_2 [P59172]	40
Ep-5	SVIELGKMIIQLTNK	13,696	–	–	–	PLA_2	30
Bitis arietans (Ghana)							
BaG-1	SPPVCGNKILEQGED	8991	–	–	–	Disintegrin bitistatin D1 [P17497]	61
BaG-2	Blocked	6942	396.2	2	TPEECR	Kunitz-type inhibitor	72
BaG-3	SLVEFGQMIQEETER	13,905	–	–	–	PLA_2	66
Bitis arietans (Nigeria)							
BaN-1	SPPVCGNKILEQGED	8991	–	–	–	Disintegrin bitistatin D1 [P17497]	65
BaN-2	SPPVCGNEELEEGEE	8950	–	–	–	Disintegrin bitistatin D3 [Q4JCS0]	79
BaN-3	HLNQFMEMIQ	14,038	–	–	–	PLA_2	63
BaN-4	SLVEFGQMIQEETER	13,905	–	–	–	PLA_2	72
Bitis nasicornis							
Bn-1–5	n.p.	–	–	–	–	–	–
Bn-6	SLLEFAKMIKEETGF	13,828	–	–	–	PLA_2	68
Bitis rhinoceros							
Br-1	KKRPNFCYLPADPG	7 kDa▼	–	–	–	Kunitz-type inhibitor	66
Br-2	NSAHPCCDPVTCK	15,184	–	–	–	~Disintegrin Bitisgabonin-1 [Q6T6T3]	62
Br-3	NSAHPCCDPVTCK	15,111	–	–	–	~Disintegrin Bitisgabonin-1 [Q6T6T2]	80
Br-4	SLEEFAKMIKEETG	13,891	–	–	–	PLA_2	45
Bitis gabonica							
Bg-1	KKRPDFCYLPADTGP	7 kDa▼	–	–	–	Kunitz-type inhibitor [Q6T6T5]	85
Bg-2	NSAHPCCDPVTCKPK	15,184	–	–	–	Disintegrin Bitisgabonin-1 [Q6T6T3]	95
Bg-3	HLEQFGNMIDHVSGR	13,967	–	–	–	PLA_2 [Q6T7B8]	–

substantial and rapidly growing publically available DNA sequence data (especially mitochondrial DNA gene sequences) for most medically relevant genera can facilitate the identification of species from tissues as well as experimental venoms [173], Moreover, tissue samples collected from biting snakes during epidemiological studies can also contribute to taxonomic research. Collaboration between clinicians, epidemiologists and snake systematists will therefore be beneficial for all concerned. Indeed, with the increasing ease and reliability of DNA sequencing technology, the identification of series of snakes responsible for bites is often easier through molecular means than morphological, at least where baseline sequence data already exist. The establishment of a taxonomically comprehensive reference databank of the most commonly used mtDNA

genes for venomous snakes (cytochrome *b*, NADH dehydrogenase subunit 4 [ND4], 16s rRNA) should be a priority research goal for herpetological systematists.

Variation in venom composition is one of the key complicating factors in toxinological research and the design and use of antivenoms [174,175]. The relationship between systematic affinities, phylogeny, venom composition and antivenom cross-neutralisation is highly variable: phylogenetic relationships have been able to predict the symptoms of envenoming in some cases, such as the unique thrombotic syndrome of envenoming shared by the closely related Caribbean island pitvipers *B. lanceolatus* and *Bothrops caribbaeus* [176]. In other cases, the relationship is much less clear. Clinical syndromes bear no relationship to taxonomy or phylogeny in *D. russelii* and *D. siamensis*[177] (Fig. 2). Amongst medically important genera in Asia (Fig. 3), such as *Bungarus* and *Naja*, the ability of antivenom to provide para-specific protection has been poorly tested (Fig. 2). Toxin-specific antibodies against the haemorrhagic metalloproteinase, jararhagin, from *Bothrops jararaca*, failed to neutralise the haemorrhagic activity of venoms of a few populations of the phylogenetically distant *Bothrops atrox* complex, whereas the activity of most venoms of that complex was effectively neutralised [178]. Similarly, amongst African spitting cobras, the ability of EchiTAb-Plus-ICP® antivenom (raised against *N. nigricollis* as well as the viperids *B. arietans* and *E. ocellatus*) to neutralise the lethal activity of other species of spitting cobra is variable and not clearly related to the relative phylogenetic position of the species involved [179] (Fig. 4). Amongst African *Echis*, Casewell et al. [142] demonstrated good neutralising ability of the monospecific EchiTAbG antivenom, raised against *E. ocellatus*, against the venoms of the more closely related *E. coloratus* and *E. pyramidum*, but not against the venom of the phylogenetic outlier *E. carinatus* (Fig. 4), a result corroborated by repeated clinical experience in Africa [105,142]. However, other antivenoms raised against African *Echis* species failed to provide clinical benefit to a patient bitten by a Tunisian *Echis leucogaster*, a taxon closely related to *E. pyramidum*[180,181], illustrating the capricious nature of the relationship between phylogeny and venom composition.

However, despite the fact that species and phylogenetic affinities do not necessarily predict antivenom cross-neutralisation, they do provide some guidance for the investigation of venom composition and differences. At the very least, phylogenetic affinities provide a default hypothesis for venom variation, in that the discovery of new species and previously unsuspected genetic divergence should prompt the investigation of patterns of venom variation and antivenom compatibility. Similarly, understanding the phylogeny of a group of venomous snakes can provide a background of predictive value to problems of antivenom incompatibility: the reciprocal lack of cross-reactivity between antivenoms raised for *E. ocellatus* and *E. carinatus* [142] and their respective venoms is easily explained by their distant phylogenetic relationship [181], and this knowledge should help avoid further loss of life due to inappropriately marketed antivenom [105,127]. However, there are also examples of convergence in venom toxin composition between distantly-related taxa. For example, the venom proteome of *Bothriechis nigroviridis* is uniquely characterised amongst*Bothriechis* venoms by its high content of crotoxin-like PLA_2 subunits [34]. Neutralisation of the lethal activity of *B. nigroviridis* by an anti-*Crotalus durissus terrificus* antivenom manufactured by Instituto Butantan points to a major role of crotoxin-like PLA_2 in *B. nigroviridis* venom-induced lethality, and highlights the relevance of in vivo neutralisation assays and antivenomic profiling for expanding the clinical use of heterologous antivenoms on an immunologically sound basis.

Finally, understanding the phylogenetic relationships between species and populations of snakes forms an essential background towards understanding the causes and correlates of the evolution of venom composition, be they phylogenetic [182], natural selection for diet [183,184] or the evolution of neoteny [185,186]. For instance, mapping both diet variation and venom activity onto a phylogenetic tree for the genus *Echis* allows the identification of repeated coincident evolutionary shifts in diet and corresponding diet specific venom activity in this genus [184].

Similarly, a phylogenetic foundation allows the detection of evolutionary and immunological trends amongst venoms that may aid in (a) defining the appropriate mixture of venoms for immunisation to produce effective polyvalent antivenoms, and (b) expanding the clinical range of currently existing antidotes. For instance, snake venom population studies superimposed on a published phylogeny [187] have suggested paedomorphism (the retention in the adult of juvenile characters) as a selective trend during the southward expansion of *C. durissus* in South America [185]. Antivenomic results showed that antivenoms raised in Instituto Clodomiro Picado (San José de Costa Rica) and Centro de Biotecnología (Universidad Central de Venezuela) against different venom mixtures, which include venoms of adult *Crotalus simus* and *C. durissus cumanensis*, respectively, had impaired reactivity towards the major toxins (crotoxins and crotamines) of juvenile *C. simus* and *C. d. cumanensis* and adult *C. d. terrificus* venoms [185]. Conversely, antivenom raised at Instituto Butantan (São Paulo, Brazil) failed to neutralise the SVMP-induced haemorrhagic activity of adult *C. simus* and *C. d. cumanensis* venoms [188]. The evolutionary trend defined by venomic analyses in conjunction with phylogenetic analyses, along with the outcome of the antivenomic studies, aid establishing the spectrum of possible clinical use of current available anti-crotalic antivenoms.

5.3. *The use of proteomics in the design of venom mixtures for immunisation and in the preclinical assessment of antivenoms*

One of the key aspects of improving antivenom development and manufacture is how to harness the enormous wealth of scientific information gathered on venom biochemistry, immunology and toxicology. The designs of the vast majority of mixtures of venoms used in immunisation in antivenom-manufacturing laboratories were established several decades ago. A very large body of information on venom composition, variability and toxicity has emerged during the last decades, and this should be harnessed for the improvement of antivenom manufacture in several ways. Following the collection of sound epidemiological evidence highlighting the most relevant species responsible for snakebite envenoming in the various regions and sub-regions of the world, efforts should be aimed at establishing the most appropriate mixtures of venoms for immunisation. This process should be performed through analysis of inter- and intra-species venom variability and of

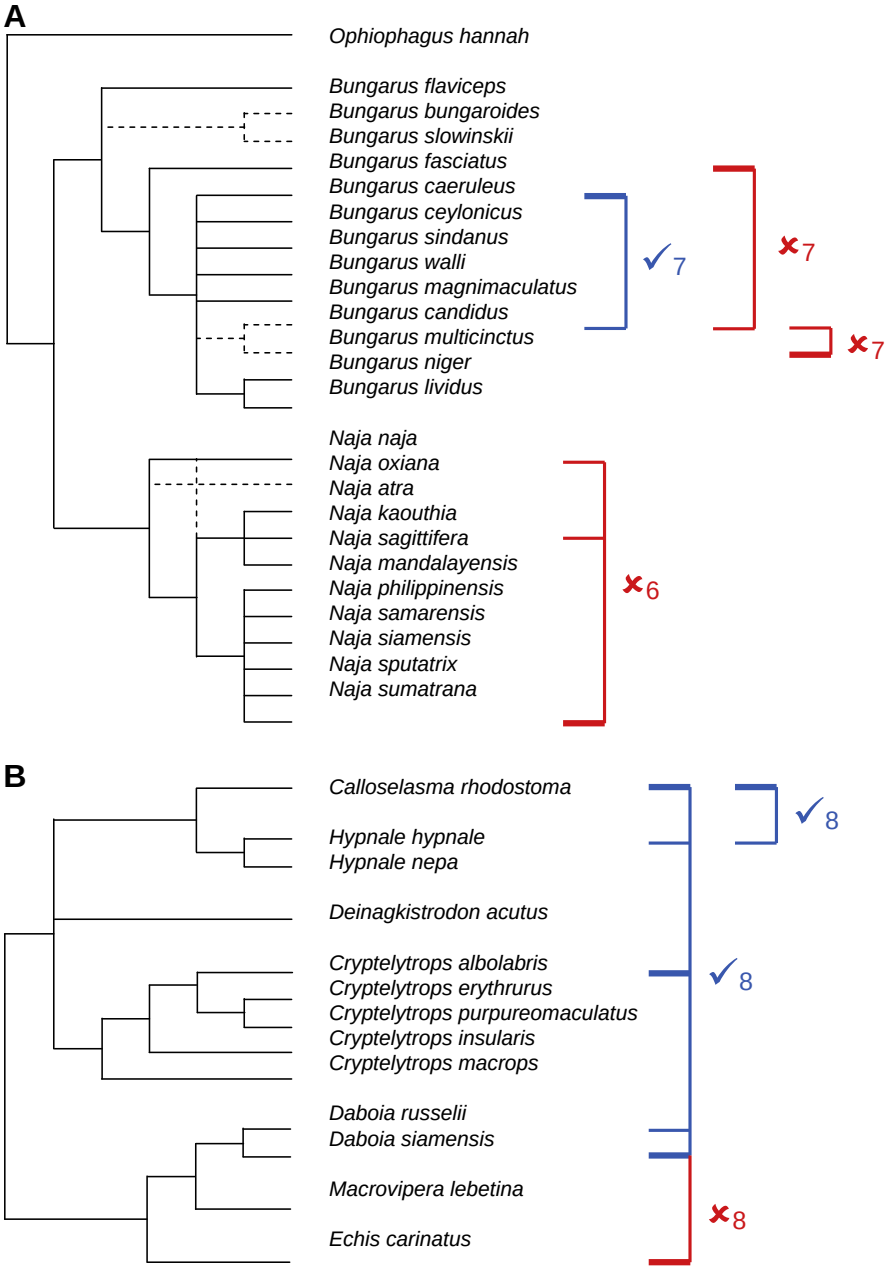

Fig. 2 – Phylogeny of (A) medically important Asian elapids and (B) viperids, and reported antivenom cross-neutralisation outcomes. Modified from [150,156,158,169,181,248–251] and Wüster (unpubl. data). There is no comprehensive, robustly supported, published phylogeny for *Bungarus*, and all arrangements within this genus should be regarded as tentative. Brackets on right of tree indicate cross-neutralisation. Bold lines indicate venoms used to raise antivenom, blue with tick mark indicates effective cross-neutralisation, and red with cross mark indicates ineffective cross-neutralisation as reported by (6) [252], (7) [253], and (8) [254].

the immunological cross-reactivity in the venoms. Modern laboratory platforms, including antivenomics and venom phenotyping [51], should be used to fulfil this goal.

In the characterisation of venoms, the issue of intraspecies variability, both at the regional and ontogenetic levels, should be investigated. This is particularly relevant in species of wide geographical distribution, such as *D. russelii* and *D. siamensis* in Asia [189], *B. arietans* in Africa [30], *B. atrox*[39] and *Bothrops alternatus* [190] in South America, and some *Crotalus* species in the Americas [191], to cite a few examples. Furthermore, the ontogenetic variability has to be also considered, as a number of studies have revealed a drastic ontogenetic-based shift in venom composition [33,186,192]. To perform these analyses, traditional biochemical tools can be used, such as polyacrylamide gel electrophoresis, both one - and two-dimensional, high-performance liquid chromatography (HPLC), and the

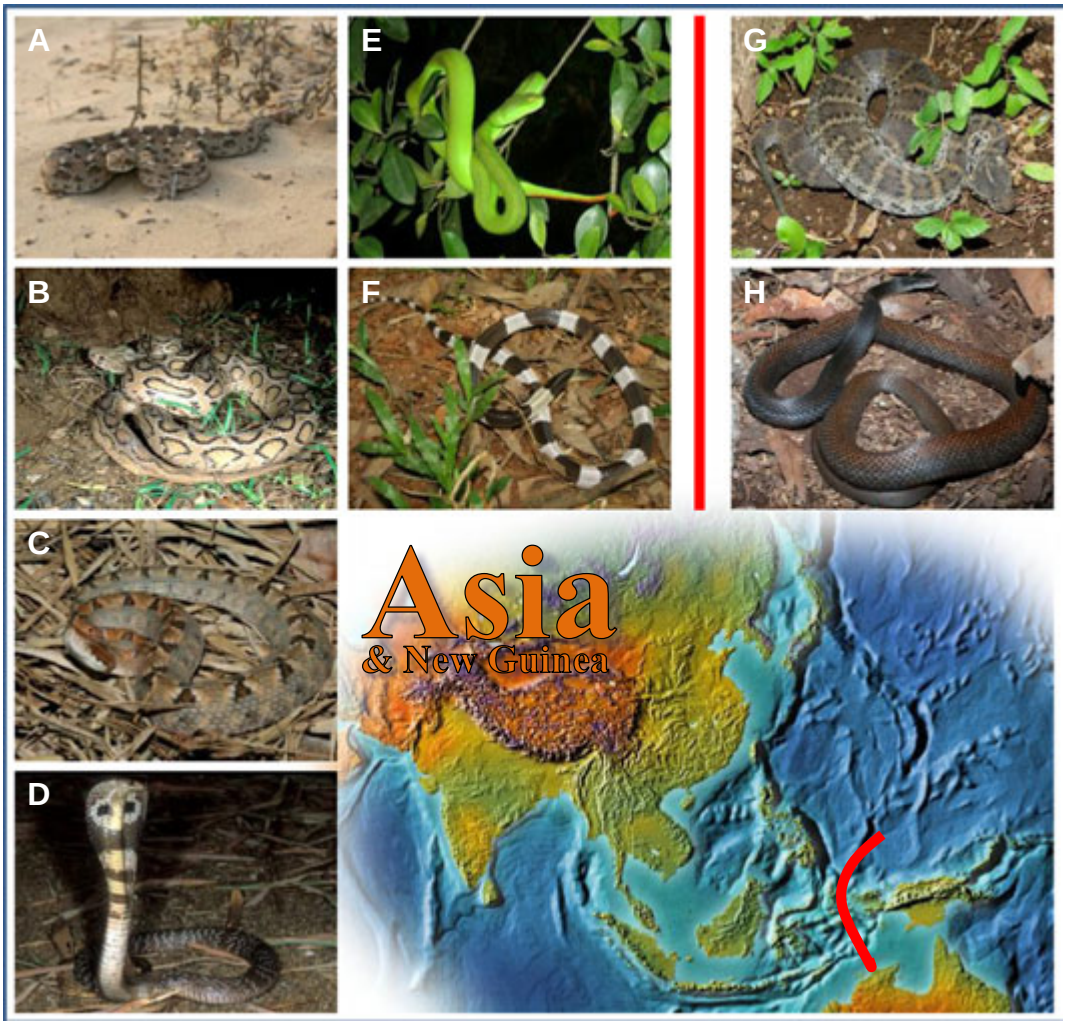

Fig. 3 – Major genera or species of South and Southeast Asian, and New Guinean snakes important to antivenom production: (A) saw-scaled viper (*Echis carinatus*), (B) Russell's vipers (*Daboia* spp.), (C) Malayan pit viper (*Calloselasma rhodostoma*), Asian cobras (*Naja* spp.), arboreal pit vipers (*Cryptelytrops* spp.), and the kraits (*Bungarus* spp.). In New Guinea the two most important species for antivenom production are (G) smooth-scaled death adder (*Acanthophis laevis*) and the Papuan taipan (*Oxyuranus scutellatus*). NB: Physical biogeographical separation of these two groups by Wallace's line marked in red. [Photos: A: Wolfgang Wüster; B, D: Mark O'Shea; C, E–H: David Williams].

methodologies associated with proteomics, i.e. mass spectrometry and N-terminal sequencing, amongst others (see reviews by [8,45,49]). A considerable body of knowledge has been published in recent years concerning the proteomic analysis of snake venoms, a field named 'venomics' [8,49]. Such knowledge has greatly expanded our view of venom composition and variability. It is necessary to continue these efforts in the study of the venoms of additional species, particularly those exerting a heavy toll of envenomings in different regions of the world.

The study of venom proteomics should go in parallel with the toxicological characterisation of snake venoms and with the identification of the most relevant toxins in the species causing the highest incidence in envenomings. In other words, the basic toxicological profile of venoms has to complement venomics. With these various types of information in hand, it is now possible to undertake the design of the most appropriate venom mixtures for antivenom development and production in the various regions of the world from a sound knowledge-based perspective. Moreover, the design of the traditional venom mixtures currently used to produce antivenoms need to be re-examined in the light of this knowledge. When a venom mixture is designed, it is then necessary to validate it through careful experimental studies. Pilot batches of antivenom should be prepared, and they should be assessed preclinically in terms of their ability to neutralise the most relevant toxic activities of the most important venoms in a particular region or country. The spectrum of toxicological effects whose neutralisation needs to be tested would depend on the pathophysiology of envenomings by the species. For example, in the case of *Bothrops* sp. venoms in Latin America, the neutralisation of lethal, hemorrhagic, myotoxic, coagulant and defibrinogenating activities should be investigated [130]. In the case of elapid

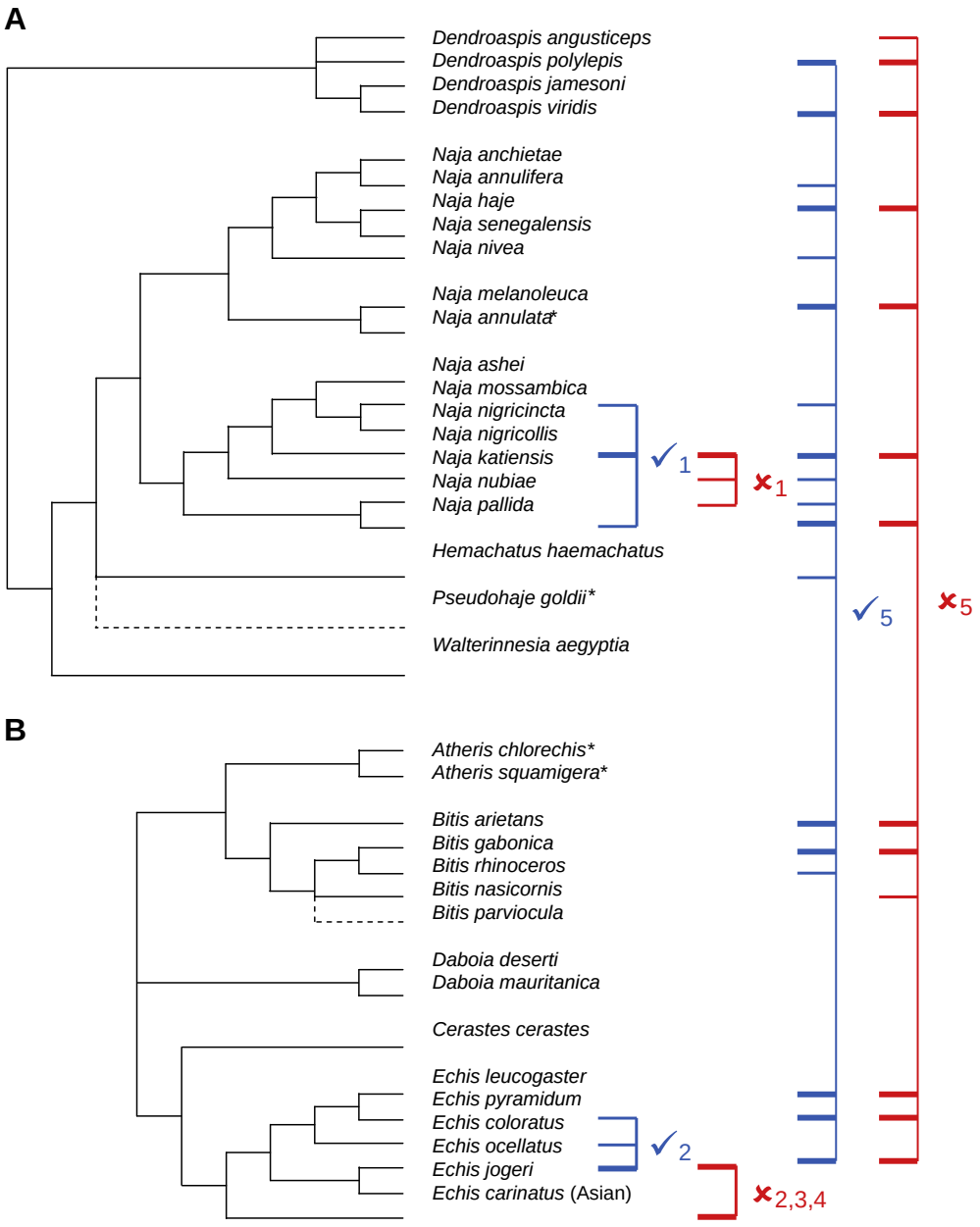

Fig. 4 – Phylogeny of (A) medically important African elapids and (B) viperids and reported antivenom cross-neutralisation outcomes. Phylogenetic trees modified from [158,169,181,250] and Wüster (unpubl. data). *Bitis parviocula* and *Pseudohaje goldii* have not been included in any comprehensive phylogenetic analyses, and their placement should be regarded as tentative. Brackets on right of tree indicate cross-neutralisation. Bold lines indicate venoms used to raise antivenom, thin lines paraspecific activity, blue with tick mark indicates effective cross-neutralisation, and red with cross mark indicates lack of cross-neutralisation as reported by (1) [166], (2) [142], (3) [255], (4) [105], and (5) [256].

venoms inducing predominantly a neurotoxic envenoming, the neutralisation of lethality is a good parameter to determine the preclinical efficacy of an antivenom [47].

The evaluation of the ability of antivenoms to react with homologous and heterologous venoms can greatly benefit from antivenomic protocols. By this approach, the immunological reactivity of antivenoms against particular venom components can be precisely determined, thus expanding the possibilities to investigate the patterns of cross-reactivity of these immunotherapeutics [47,51]. An example of such analysis has to do with the ability of *Crotalus* antivenoms in Central and South America to react and neutralise the venoms of the species *C. simus*, from Central America, and various subspecies of *C. durissus* from South America. The proteomic analysis of venoms revealed that venom from adult specimens of *C. simus* is rich in metalloproteinase but poor in the neurotoxic complex crotoxin [185]. In contrast, venoms of various subspecies of *C. durissus* have a high content of crotoxin and very low amounts of metalloproteinases. Moreover, some populations of *C. durissus* contain the toxin

crotamine, whereas others do not [40]. As a consequence, an antivenom produced using the venom of *C. simus* for immunisation is able to neutralise the lethal and hemorrhagic activities of *C. simus*, but not the lethal effect of *C. durissus*[188]. In contrast, antivenoms produced against *C. durissus* neutralise toxicity of the homologous venom, but are ineffective in the neutralisation of hemorrhagic activity of *C. simus* venom [188]. Antivenomic analyses clearly correlate with this neutralisation profile, as the anti-*C. simus* antivenom does not immunodeplete crotoxin, and the anti-*C. durissus* antivenom is unable to immunodeplete the metalloproteinases of *C. simus* venom [185]. Consequently, the proteomic analysis of these venoms paves the way for a knowledge-based design of the optimal immunising mixture for the manufacture of a crotalid antivenom effective in South and Central America, by using a mixture of the venom of *C. durissus*, of a crotamine-positive population, plus the venom of *C. simus*[51]. The issue of ontogenetic variation is also present in this example, as venoms from neonate and juvenile specimens of *C. simus* contain high amounts of crotoxin [185] and their lethality is not neutralised by an antivenom raised against adult specimens of this species, which lack crotoxin [188], thus requiring the introduction of crotoxin-rich venoms in the immunising mixtures.

It is therefore necessary to gather basic information on venom proteomics and on the cross-reactivity of antivenoms, using both neutralisation assays of the most relevant toxic effects and antivenomic analysis of the spectrum of reactivity [47]. This information would enable antivenom developers and manufacturers to select the most appropriate venom mixtures for immunisation. Moreover, the issue of venom quality control analysis can also benefit from these methodologies, since both laboratories working on venom collection and preparation of venom pools, and manufacturing laboratories, including quality control centres, could guarantee the quality and identification, ideally through mitochondrial DNA sequences of the animals [173] providing the venoms used in immunisation and quality control tests. The need to incorporate proteomic and neutralisation assessment of venoms and antivenoms demands international cooperative efforts, since some of the analytical platforms described are not easily accessed by laboratories in many developing countries. Thus, the integration of international partnerships, involving laboratories working in proteomics, with those dealing with the toxicological characterisation of venoms, and others focusing on the development, manufacture and quality control of antivenoms, should be promoted in order to fill the existing gaps in the generation and assessment of antivenoms. An example of such cooperation is the recent development of antivenoms for sub-Saharan Africa, involving the cooperation of groups of Nigeria, Costa Rica, the United Kingdom and Spain [54,108,110,135].

5.4. *The quest for 'universal' antidotes to elapid postsynaptic neurotoxins (PSNTs)*

Postsynaptic neurotoxins (curaremimetic or α-neurotoxins) are members of the 3-finger toxins in the superfamily of non-enzymatic proteins produced by elapid (cobra, krait, mamba) and sea snakes. As the name implies, they bind specifically and quasi-irreversibly to muscle (α1) nicotinic acetylcholine receptor (nAChR) at the muscle end plate and inhibit the ion-channel [193]. This inhibition results in neuro-muscular blockage, muscle paralysis and death by respiratory failure. Although elapid venoms may contain other toxins, e.g., presynaptic neurotoxins, the PSNTs are often the most lethal toxins of the venoms and are important targets for neutralisation to save the lives of the victims

PSNTs can be divided into 2 groups based on their structures: the short neurotoxin with 60–64 amino acids and 4 disulfide bridges, and the long neurotoxin with 70–74 amino acids with 5 disulfide bridges. Both groups of PSNT bind to the same target i.e., the α subunit of nAChR with comparable affinity of about 10 pM and 1 nM. The PSNTs all show similar secondary structure of the 3 finger toxins with β-sheets and a hydrophobic core [194]. Although chemically and pharmacologically similar, these PSNTs are distinct immunochemically. Thus an antivenom produced against an elapid venom generally fails to neutralise another elapid venom [195]. Although occasionally some paraspecific cross-reactions have been observed between heterologous venoms [196], it can be concluded that the majority of the dominant epitopes of these PSNTs in different elapid venoms are dissimilar.

5.4.1. *The active site of PSNTs in the binding to nAChR*

Various studies carried out by Andre Menez's group using site-directed mutagenesis have identified the amino acids in the PSNTs involved in the interaction with the *Torpedo* nAChR [197–199]. For the short-chain (erabutoxin a) and the long chain (α-cobrotoxin) toxins, these amino acids are Lys23/Lys27, Asp27/Asp31, Arg33/Arg33, Lys49/Lys4, Try25/Try29 and Phe29/Phe32, and reside in all the three loops of the toxins.

The amino acids on the nAChR involved in the binding of PSNTs have been studied by X-ray crystallography of the toxin–receptor complex. The binding complex of α-cobrotoxin and a water soluble acetylcholine binding protein (AChBP) from *Lymnaea stagnalis* was studied by Bourne [200]. More recently, data from the crystal structure of the complex between α bungarotoxin (αBTX), the major PSNT of *Bungarus multicinctus*, and mouse muscle nAChR subunit were obtained [201] and shown to be similar to those from AChBP. The amino acids Tyr93, Tyr190, Tyr193, Thr148 and Arg149 on the receptor are involved in the interaction with Asp30, Phe32 and Arg36 of the toxins [202].

5.4.2. *Strategies for finding a 'universal' inhibitor/antibody against elapid PSNTs*

Conceptually, two different approaches can be used, and in fact have been studied. The first approach is to search for peptides that can bind specifically and with high affinity to the active site of the PSNTs. The second is to produce antibodies or their fragments that bind to conserved and common amino acid sequences (epitopes) of the PSNTs.

5.4.2.1. *Peptide inhibitors that bind specifically and with high affinity of all PSNTs.* Since all PSNTs bind specifically and mainly to the α subunit of nAChR, and since the structure and topology of human muscle nAChR are conserved, peptides which mimic the surface topology of the toxin binding site on the receptor should bind with high affinity to PSNTs. Using a combinatorial phage display library, a 13 amino acid lead

peptide which bound moderately (10^{-6} M kDa) to αBTX was obtained [203]. The peptide showed similar amino acid sequence to the toxin binding surface on the α-subunit of nAChR. By systematic replacement of amino acids on the above lead mimotope, several peptides which bind at nanomolar affinity to αBTX have been identified. Some of the peptides bind to the toxin with even higher affinity than that observed with peptides with the same sequence as the homologous region on the receptor. A modified cyclic analogue with a disulfide bridge, synthesised as directed by NMR analysis, has been found to improve the binding by two orders of magnitude [204]. These peptide inhibitors have been shown to neutralise the lethality of αBTX and the crude venom when the inhibitor is given concomitantly with the toxin/venom. The cyclic peptide at 2.5 mg/mouse could neutralise the lethality of 2.5 μg of the crude venom [204]. A general approach for the design and synthesis of peptides that bind to toxins has been proposed [205].

The same approach has been used to find inhibitors of αBTX [206]. Peptide mimotopes obtained from combinatorial peptide library were shown to bind αBTX with higher affinity than peptides reproducing the native *Torpedo* receptor sequence. However, the in vivo neutralising activity of the mimotopes was quite low [204]. A branched peptide mimotope containing 4 peptides on a polylysine core in the form of multi antigen peptide (MAP) was synthesised. This branched peptide mimotope was found to greatly increase the in vivo neutralising activity, largely as a result of the change in the pharmacokinetics of the inhibitor [207].

This peptide inhibitor approach is very attractive, and if a more potent inhibitor with higher neutralising activity could be found, it should serve as a 'universal' inhibitor of all elapid PSNTs. It is also important that such inhibitors be of rather small molecular sizes and in themselves not immunogenic. Otherwise, antibodies produced against such inhibitors are likely to cross-react with nAChR and result in autoimmune disease like myasthenia gravis.

5.4.2.2. *Production of antibodies or antibody fragments that bind specifically to conserved and common epitopes of PSNTs.* As mentioned above, although elapid PSNTs are chemically and pharmacologically similar, they are distinct immunochemically. Thus antivenom against one elapid venom usually fails to neutralise the lethality of other elapid venoms [195]. Since various amino acids in the PSNTs are conserved for structural and/or functional purpose, there should be areas (epitopes) on the PSNTs that are common to most or all PSNTs. These conserved epitopes are important for the preparation of antivenom that cross-neutralise the toxins of other venoms [208]. For example, those amino acids involved in the interaction with the nAChR discussed above. It is not necessary, however, that the epitope be at the active site of the toxin, since binding of antibody, especially IgG or the F(ab′)$_2$ against other areas of the toxin should, at least by steric effect, alter the pharmacokinetics and/or hinder the interaction of the toxin to the receptor. If these common epitopes can be identified, the peptide mimotopes can be prepared and used as immunogen to raise 'universal' antivenom that can recognise, interact and neutralise the native PSNTs of various elapid venoms.

The epitopes of various proteins e.g., lysozyme, sperm whale myoglobin, recognised by polyclonal antibodies have been studied using a synthetic chemical approach [209]. For αBTX, they synthesised cyclic peptides representing the amino acid sequences of the 3 loops of the toxin: L1 (residues 3–16), L2 (26–41) and the C-terminal tail (66–74). It should be mentioned that these peptides do not represent the precise boundaries of the epitopes. These peptides were shown to bind antibodies obtained by αBTX immunisation and contain immunodominant epitopes of the toxin [210]. Antibody generated against L2 peptide bound most strongly to native αBTX and found to protect mice. When a mixture of the 3 peptides was used as an immunogen, the antibody offered even more protection. Interestingly, when the 3 peptides were conjugated to a common carrier protein ovalbumin, and used as an immunogen, the antibody generated showed 2 fold higher neutralising activity than that of the antibody raised against the native αBTX, even though the dose of each peptide in the conjugate was lower than that when free peptide was used [211].

It has been recognised that in a homologous set of proteins, epitopes are often present in the same structurally equivalent locations [209]. Thus the protecting epitopes on the αBTX may be extrapolated to equivalent regions on other PSNTs; and the corresponding peptides may be used, in the form of conjugates, for the production of 'universal' antivenom against PSNTs.

5.4.2.3. *Phage display libraries.* Another approach that has been used more recently to identify epitopes on various proteins and toxins is to use a phage display library to find peptides which bind to toxins (mimotopes of antibody paratopes) or bind to antibody specific against toxins (mimotopes of toxin epitopes). One advantage of this approach is that conformation as well as linear epitopes could be identified from their corresponding mimotopes.

The epitopes of Neuwiedase, a metalloproteinase from *Bothrops neuwiedi* venom, have been studied using rabbit polyclonal antibody against Neuwiedase as the target in a recombinant peptide library [212]. By bio-panning, peptide mimotopes were identified and sequence alignment showed them to correspond to two conformational epitopes which are structural motifs common to several snake venom toxins. Immunisation of mice with these mimotopes resulted in antibody that recognised the toxin. In other studies, mimotopes have been shown to induce production of protective antibodies [213–215]. In another example, bio-panning of a library of human single chain variable antibody fragment (*huscfv*) using a long chain PSNT of *N. kaouthia* as target resulted in the isolation of the toxin binding *huscfv*[216]. This *huscfv* was shown to neutralise the lethal activity of the PSNT. To identify the epitope on the toxin, the *huscfv* was used in a phage library to obtain peptides which bind the *huscfv* paratope. The amino acid sequences of the *huscfv* binding peptides (the mimotopes) were found to be similar to the *N. kaouthia* PSNT residues 51–54 (TVKT) which are conserved in many other elapid PSNTs. The authors suggested that the *huscfv* may be effective as a pan-neutralising antibody fragment against PSNTs of various elapid venoms. Although this approach is interesting and promising, a single chain Fv may not be very effective in neutralising PSNTs in cases of

envenoming for various reasons. First, pharmacokinetically the small *scfv* may be cleared from the body very rapidly and need to be administered repeatedly to neutralise the toxin which is slowly and continuously released from the bite site. Since the affinity of the PSNT for the nAChR is extremely high (1 nM–10 pM) and is likely to be higher than that of most *scfv* and monoclonal antibody for an epitope on the toxin, a mixture of the antibodies/fragments may be needed to increase, by way of cross-linking, the neutralising activity towards the toxins. All these factors need to be considered for the preparation of 'universal' antivenom against elapid PSNTs.

5.5. *Another big challenge: the neutralisation of toxins responsible for local tissue damage*

Envenomings by the majority of viperid species and by a number of elapid snakes are often associated with the rapid development of tissue damage at the site of venom injection. Viperid venoms induce tissue necrosis, blistering, haemorrhage, and oedema, whereas bites by some elapids, especially of the genus *Naja*, result in local necrosis [217,218]. The rapid development of these effects, together with the frequent delay in antivenom administration, causes extensive tissue damage in a percentage of these cases which end up in permanent physical and psychological sequelae. In the case of viperid snake venoms, such local pathology is mostly due to the combined action of hemorrhagic metalloproteinases, myotoxic phospholipases A_2 and, probably, hyaluronidases [219], whereas local necrosis after cobra bites is secondary to the action of phospholipases A_2 and cytotoxins ('cardiotoxins') [220]. The pathogenesis of local tissue damage induced by crude venoms and purified toxins has been elucidated in a number of venoms [221], and the proteomic analysis of wound exudates collected from animals injected with locally-acting toxins has been recently proposed as a tool to further investigate the mechanism of tissue damage [222].

Clinical observations indicate that antivenoms are only partially effective in the neutralisation of these local pathological alterations [223]. The reasons for this therapeutic failure are based, in the case of some antivenoms, in the low antibody titres against locally-acting toxins. However, in many cases antivenoms are effective in the neutralisation of these toxins when venom and antivenom are mixed prior to injection, thus showing the presence of neutralising antibodies. However, when antivenoms are administered after venom injection in experimental animals, neutralisation of these effects is only partial (see for example Gutierrez et al.[224]). The extremely rapid onset of tissue damage induced by these toxins is therefore the main culprit for the poor therapeutic success of antivenoms. The use of antibody fragments, such as $F(ab')_2$ and Fab, which would diffuse more readily to the affected tissues than whole IgG molecules, does not improve the neutralisation of local pathology [225,226].

Such a complicated scenario demands actions at, at least, three levels: (i) improving the titres of antibodies against hemorrhagic and cytotoxic components, especially against low molecular mass toxins such as elapid cytotoxins. The selection of the most appropriate venoms for immunisation, in terms of cross-neutralisation of hemorrhagic and necrotising components, is a relevant task which demands research on venomics and antivenomics. (ii) Promoting public health interventions aimed at ensuring a rapid access of patients to antivenom administration in health posts, especially in rural areas where envenomings mostly occur; a rapid infusion of potent antivenoms should be able to reduce the extent of local tissue damage, thus reducing the magnitude of tissue loss. (iii) Searching for natural and synthetic inhibitors of metalloproteinases, phospholipases A_2 and hyaluronidases, which could be administered in the field rapidly after the snakebite, thus abrogating the action of these toxins in situ. Such an approach has been supported by experimental findings with metalloproteinase, myotoxin and hyaluronidase synthetic inhibitors [227–231]. Moreover, potent PLA_2 and metalloproteinase inhibitors, belonging to various protein families, have been purified and characterised from the sera of snakes and mammals [232,233]. Structure–function studies will identify the molecular determinants responsible for this inhibitory effect, and such knowledge may pave the way for the design of synthetic or recombinant peptides with great potential in the therapy of snakebite envenoming, particularly regarding the inhibition of toxins responsible for local tissue damage. Additional discussion of other research approaches to improve the immunotherapy of snakebite is presented by Harrison et al. elsewhere in this issue.

6. A new approach

Despite strident efforts to engage the broader public health community in seeking long-term sustainable solutions to the perennial problems facing the victims of snakebites in the developing world, success remains elusive and antivenom scarcity persists in many regions as a result of many of the factors outlined in this review. The toxinology community is now taking a much more active role in advocacy about these issues [57,59,62,69,70,234]. Efforts are also being made to develop new antivenoms to fill some of the gaps in coverage [107,108,135,145]. But more must be done to get effective antivenoms into those parts of the world that are most in need. Some of the fundamentals of effective antivenom design need to be revisited, traditional concepts of antivenom production challenged [235], and novel financing, marketing, distribution and long-term surveillance strategies reconsidered.

One approach being explored by the Global Snakebite Initiative (GSI) is the development of a multinational collaborative project to develop new regional polyvalent antivenoms in Asia and Africa using phylogenetic, proteomic and antivenomic tools to optimise immunogen selection to ensure wide polyspecific coverage. This is but one thread in a multifaceted approach GSI is advocating to tackle snakebite problems in the developing world. Other components of the approach involve improved epidemiological surveillance, specific snakebite treatment training for medical personnel, the development and promotion of new snakebite treatment protocols for specific regions, promotion of snakebite prevention strategies, better regulatory control of antivenoms, support to laboratories already producing antivenoms in developing countries, first aid rationalisation, public

education, and engagement with the disability community to improve rehabilitation access for snakebite victims [69,70].

Combining a modern understanding of the biogeographical and phylogenetic relationships of venomous snake species with the vast information that can be assembled from proteomic and antivenomic investigations of venoms and candidate antisera to design a new generation of antivenoms that can be produced to high standards, creates a rational pathway for resolving some of the current problems. In Africa, the World Health Organisation has identified 26 species as being of the highest priority (Category 1) for antivenom production, whilst in South Asia 12 species as listed, and a further 16 species are given the same ranking in Southeast Asia and New Guinea [68]. Of the 26 species listed for Africa, 24 occur in 5 (*Bitis*, *Cerastes*, *Dendroaspis*, *Echis* and *Naja*) widely distributed genera (Fig. 5). The South and Southeast Asian species are represented by 9 genera, but with 25 of these in 6 (*Bungarus*, *Calloselasma*, *Cryptelytrops*, *Daboia*, *Echis* and *Naja*) widely distributed genera (Fig. 3). The reality however is that venoms from just four species are used in South Asia by Indian and Pakistani antivenom producers, and venoms from seven other Category 1 species are used in Southeast Asia by producers in Indonesia, Myanmar, the Philippines, Thailand and Viet Nam. There have been few studies of paraspecific activity in the region, yet clearly, with so few species being used in antivenom production, there is a pressing need for robust antivenomic investigations to determine the true value of the current antivenoms in clinical practice. Given that 8 species from the genus *Bungarus*, and 10 species from the genus *Naja* are listed as Category 1 priority species in South and Southeast Asia by WHO [68], the value of subjecting these venoms to proteomic and antivenomic analyses cannot be understated. Likewise published data on the preclinical efficacy of antivenoms in South Asia is lacking, and formal clinical trials of antivenoms suggest that the products lack efficacy and have poor safety profiles.

Current antivenoms produced in South Asia from *B. caeruleus*, *D. russelii*, *E. carinatus* and *N. naja* venoms, have specific potencies of 0.45–0.6 mg venom/mL antivenom depending on the species concerned. Effective treatment requires early administration of multiple vials of antivenom, and there are many cases of exorbitant volumes being administered to patients [65,113,236]. Considering the volumes of venom that may be expressed by some species (Table 4), it might be reasonable to expect that any new antivenom designed for use in Asia should have a minimum (per vial) potency adequate to neutralise the average volumes of venom injected by the target species during defensive bites. Such an approach is not new. Australia's antivenom producer, CSL Limited dispenses antivenoms based on delivering at least the minimum volume required to neutralise the average volume of venom obtained during manual extraction, and a new antivenom for *O. scutellatus* bites in Papua New Guinea also follows this approach [128]. The key advantage of administering a concentrated antivenom in a volume sufficient to neutralise the average amount of injected venom is the delivery of an adequate therapeutic dose. This strategy reduces the likelihood of suboptimal treatment since the dose is contained in a single vial. Where species coverage is dichotomous, with some taxa producing small volumes of venom, whilst others have large yields, dispensing potent antivenoms in 20 mL and 50 mL doses might be a reasonable compromise to minimise the number of patients receiving large antivenom boluses unnecessarily, such as following *E. carinatus* bites or juvenile *D. russelii* bites in South Asia. This is also highly relevant in western Africa, where *Echis* spp., (with venom yields typically under 25 mg) occur in habitats with *B. arietans* (100–350 mg) and *N. nigricollis* (200–400 mg) and hence the volumes of antivenom needed depending on the species responsible are very different. Relatives often have to go out to an external pharmacy to buy antivenom, returning with a single (expensive) vial that simply does not contain enough antibodies to adequately neutralise the injected venom. Clinical studies suggest that the current formulations of EchiTAbG (Micro-Pharm) and EchiTAbG-Plus-ICP (ICP) are effective in a single initial dose of 1 and 3 vials respectively in more than 75% of cases of *E. ocellatus* envenoming. The appropriate dose for envenoming by other species must be established through clinical trials or experience. Whilst increasing the concentration of immunoglobulin to raise neutralising potency carries with it an increased risk of inducing adverse reactions, modern production techniques are available to maximise removal of aggregates and other extraneous material from antivenom, and could be cost-effectively explored in the production of large volumes of polyvalent antivenoms for multinational markets.

The aim in producing new Pan-Asian or Pan-African antivenoms would be to introduce the greatest possible degree of production efficiency to the process by designing antivenoms that give the widest polyspecific coverage using as few specific venoms as possible. Incorporating a robust proteomics approach into the antivenom design process might facilitate using the least combination of carefully selected venoms as immunogens to produce antibodies that can neutralise the widest possible range of medically important toxins from a large number of species in a region. For example, is it really necessary to use venom from three different species of *Dendroaspis* in order to prevent death in patients bitten by any of the four species in this genus? Could the same result be obtained using just one or two of these venoms, and if so, from which intraspecific populations should the venoms be obtained to maximise paraspecific coverage? Likewise we can deploy a range of antivenomic analysis tools to refining the production of antivenoms once initial batches are ready for preclinical assessment.

One approach taken already in the development of new polyvalent antivenoms has been to adopt a syndromic strategy. Workers at the Queen Saovabha Memorial Institute in Bangkok have produced both polyvalent anti-neurotoxin antivenom [119], and polyvalent anti-haemorrhagic antivenoms, and this strategy may be advantageous, since theoretically more specific antibody against the primary lethal components of particular venoms are delivered in these preparations, rather than more heterogeneous mixtures containing large amounts of nonspecific antibodies.

The Global Snakebite Initiative would like to create a multidisciplinary, multi-centre collaboration that could bring some of these concepts for future antivenoms to life. The GSI approach would be to facilitate the design, production, evaluation and clinical validation of new polyvalent antivenoms for Asia and for Africa, initially on a small scale; using locally produced venoms from a number of target countries in both regions, with

Fig. 5 – Major genera of African snakes important to antivenom production: (A) desert horned vipers (*Cerastes* spp.), (B) carpet vipers (*Echis* spp.), (C) mambas (*Dendroaspis* spp.), (D) giant African vipers (*Bitis* spp.), and (E) the cobras (*Naja* spp.). [Photos: A–B, E: David Williams; C–D: Wolfgang Wüster].

various collaborative laboratories conducting quality control and venom selection using validated proteomic methods to create the best possible venoms pools. These venoms would then be used for immunogen optimisation and preparation. Host-animal immunisation and antivenom production would be undertaken by several different antivenom manufacturers following a standard protocol and adhering to GMP standards. Preclinical assessment and quality assurance would be conducted centrally, followed by decentralised clinical trials in host nations conducted by local medical personnel (Fig. 6). In order to attract the sort of donor funding necessary to such a large-scale project, the GSI model calls for parallel establishment of an Expert Committee drawn from a number of international regulatory agencies to inspect and validate all of the production processes, as a means of prequalifying the product for donor funding. Such a committee would also provide the independent product validation necessary to support the process of product registration by National Regulatory Agencies (NRAs) in target nations. If this approach were successful, new regional polyvalent antivenoms would be routinely manufactured on a large scale, following the same production pathway in which proteomic profiling of venoms from various supplies in each region would become a fundamental step in the selection of, and standardisation of the immunogen mixtures. Post-production quality assurance using antivenomic analyses of batch lots, and WHO-recommended preclinical assessment assays would also become part of a routine production cycle designed to ensure that the standards established during prequalification are maintained. Antivenom producers would be licenced to produce the new products under a quota system, at a price that balances profitability, sustainability and affordability, with funding for the purchase of their batch lots obtained through a network of institutional, governmental, private and public donors.

This approach not only holds promise for addressing the need to develop new regional polyvalent antivenoms to service coverage gaps or replace currently available, but ineffective products. There is an immediate need to improve

Table 4 – Venom yields of some medically important African and Asian snake species.

	N	No. of extractions	Mean yield (mg) [a]	Max. yield (mg) [b]
Vipers				
Bitis arietans (Nigeria and Ghana)	4–8	16	52.7 (±26.1) [c]	120.0 [c]
Bitis arietans	2	33	166.0 (±76.0) [257]	290.0 [246]
	17	–	71.0 [258]	165.0 [247]
Bitis gabonica	3	6	1050.0 (±170.0) [259]	2400.0 [259]
Bitis rhinoceros (Ghana)	5–7	6	54.7 (±20.8) [c]	121.0 [c]
Bitis rhinoceros	2	11	507.0 (±210.0) [246]	848.0 [246]
Bitis nasicornis	7	96	138.0 (±76.0) [246]	353.0 [246]
Cerastes cerastes (Egypt)	4–5	8	85.3 (±34.1) [c]	132.0 [c]
Daboia russelii (Sri Lanka)	2	5	86.4 (±18.5) [c]	115.0 [c]
Daboia siamensis (Myanmar)	25	–	127.0 (±13.0) [260]	268.0 [249]
Echis carinatus sochureki (UAE)	4–14	11	11.1 (±5.1) [c]	20.3 [c]
Echis carinatus sochureki (Iran)	18,227	–	15.0 (±2.0) [261]	40.0 [261]
Echis coloratus (Egypt)	4–6	18	5.8 (±1.8) [c]	13.7 [c]
Echis pyramidum leakeyi (Kenya)	6–8	25	7.4 (±2.6) [c]	13.3 [c]
Echis ocellatus (Nigeria)	200+	8	10.2 (±4.9) [c]	16.4 [c]
	–	–	–	24.8 [145]
Elapids				
Acanthophis antarcticus (Australia)	29	153	45.0 (±21.0) [246]	113.0 [246]
Bungarus caeruleus (Sri Lanka)	2	22	7.68 (±6.8) [c]	24.3 [c]
Dendroaspis angusticeps (E Africa)	3–5	18	16.6 (±6.5) [c]	75.0 [c]
Dendroaspis polylepis (E Africa)	2–3	14	13.8 (±7.2) [c]	26.1 [c]
Naja kaouthia (Thailand)	14	140	266.0 (±123.0) [246]	742.0 [246]
Naja nigricollis (Nigeria)	3–7	9	159.7 (±86.2) [c]	362.0 [c]
Naja mossambica	10	80	355.0 (±149.0) [246]	656.0 [246]
Naja oxiana (Iran)	6091	–	87.0 (±25.0) [261]	208.0 [261]
Naja siamensis (Thailand)	11	8	341.0 (±157.0) [246]	738.0 [246]
Oxyuranus scutellatus (Australia)	78	2543	146.0 (±102.0) [246]	882.0 [246]

[a] Pooled venom extractions.
[b] Maximum yield from a single venom extraction.
[c] Data from Alistair Reid Venom Research Unit, Liverpool School of Tropical Medicine, UK.

the flow of safe, effective antivenom preparations into Africa and Asia, and at the same time there exists a compelling challenge to actually determine the true coverage of existing products against venoms from species in many different countries, and determine once and for all, which products are being legitimately marketed, and which are not. Such a project has wide scope for the involvement of the proteomics and toxinological community, working collaboratively under the auspices of the Global Snakebite Initiative to examine all of the currently available antivenoms in Africa and Asia and their interactions with venoms from snake species in the countries where manufacturers and their agents market them for sale. Products that provide effective protection could then be subjected to careful preclinical assessment, and those that meet adequate standards, could in turn then be subjected to clinical studies. By again independently validating the processes through the scrutiny of an external Expert Committee, it might be possible to establish an ongoing pathway through which these antivenoms can be scrutinised, approved for specific markets and specific species populations, and through donor funding, purchased centrally, distributed and subjected to ongoing compliance checks and post-marketing surveillance (Fig. 7). Along the same lines, the information gathered with these multicentric exercises will provide useful information to existing manufacturing laboratories to improve their immunising mixtures in order to generate antivenoms of a higher cross-reactivity and potency. By purchasing the antivenoms under agreed supply contracts at prices which protect the sustainability of production by the manufacturers, the GSI proposal contributes not only to keeping important producers in the market, but is also likely to provide the financial stimulus needed to drive further innovation.

Under both of these approaches, antivenoms would be distributed by the GSI through strategic partnerships with other organisations operating in Africa and Asia, under agreements which guarantee free treatment of snake bite victims with these products. In rural Tanzania, it has been shown that making antivenom available freely significantly improved treatment-seeking behaviour, with some patients being willing to travel long distances to gain access to antivenom [237]. Elsewhere in Africa, provision of free or subsidised drugs and medical care has led to improved health seeking behaviour, and reduced reliance on traditional medicine in favour of conventional medical care [238,239]. Snakebite is an illness that drives many victims to seek traditional care over modern medicine [240–242], but the extent to which this is based on cultural norms as opposed to absence of access (to antivenoms or good quality health services) has not been investigated. What is well known is that the worst outcomes from snakebite occur in the world's most poverty-stricken communities [56]. The Tanzanian experience certainly suggests that the availability of free antivenom treatment results in greater utilisation and improved outcomes [237]. A GSI initiative

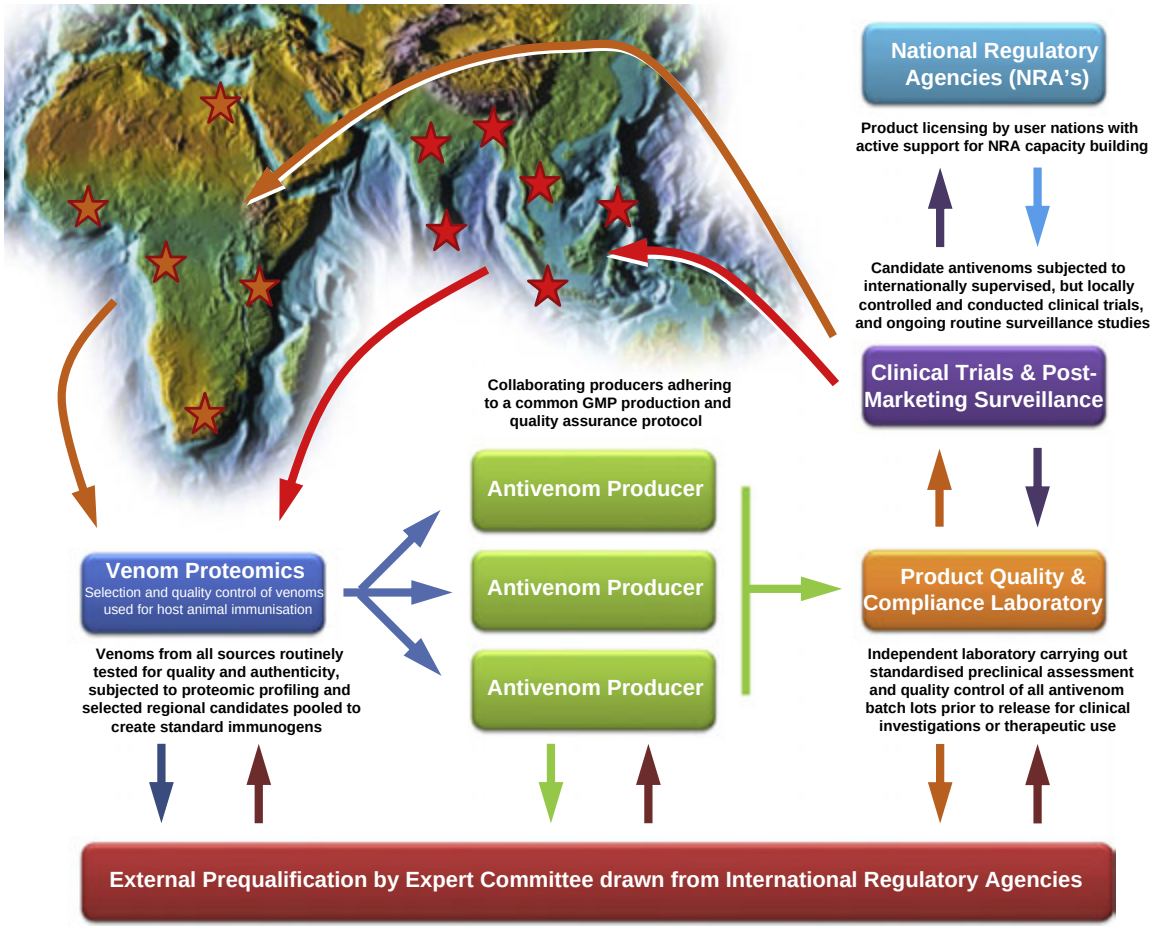

Fig. 6 – Pathway to creating new regional polyvalent antivenoms. Venoms obtained (★) in a variety of locations within each region (Africa and Asia) would be subjected to robust proteomic analysis and cataloguing. Representative venoms offering the best possible potential for broad polyspecific antivenom coverage would be selected, and standardised in batches for use as immunogens. These would be prepared and supplied to the group of antivenom manufacturers. Immunisation and subsequent production of the antivenoms would be carried out according to a standard protocol based on GMP production and QA requirements, and the batch lots forwarded to a centralised, independent laboratory where preclinical assessment, antivenomic studies and QC functions would be undertaken. All of this would be under the supervision of an external Prequalification Committee made up of regulators from major global NRAs. The function of this committee would be to document, validate and certify all of the production processes so that the products can be 'prequalified' to the donor organisations as meeting all of the required standards for injectable therapeutic immunoglobulins for human use. Clinical trials would be conducted under international supervision by local researchers in the countries where the antivenoms are to be licenced, and all of the data acquired for each product would be published to ensure transparency. Support would be offered to assist local NRAs in establishing appropriate capacity to regulate antivenoms in their jurisdictions. A two-way flow of information is envisaged between all parties. Once products are licenced, routine production would follow the same pathway, with the clinical trial function being replaced by one of routine post-marketing surveillance.

to deliver free antivenoms to at-risk communities, that works with donors to ensure that (a) manufacturers are fairly compensated for producing high quality antivenoms, and (b) that no patient who needs antivenom is denied access through poverty, has the potential to stabilise a fragile industry, and save countless lives.

As humanitarian exercises with sound scientific foundations and practical, achievable, objectives, these concepts could potentially attract strategic funding from a variety of sources, including partnerships derived from events such as the Clinton Global Forum, philanthropic contributions and the overseas aid budgets of wealthy governments. The potential to engage with non-profit pharmaceutical companies to learn more about novel financing strategies for bringing essential medicines to developing world markets should not be ignored [243,244]. Likewise there is also the potential to encourage large, financially secure antivenom manufacturers to contribute their efforts towards bringing antivenoms to end-users at less than cost prices as part of their individual commitments towards being socially responsible corporate citizens. In

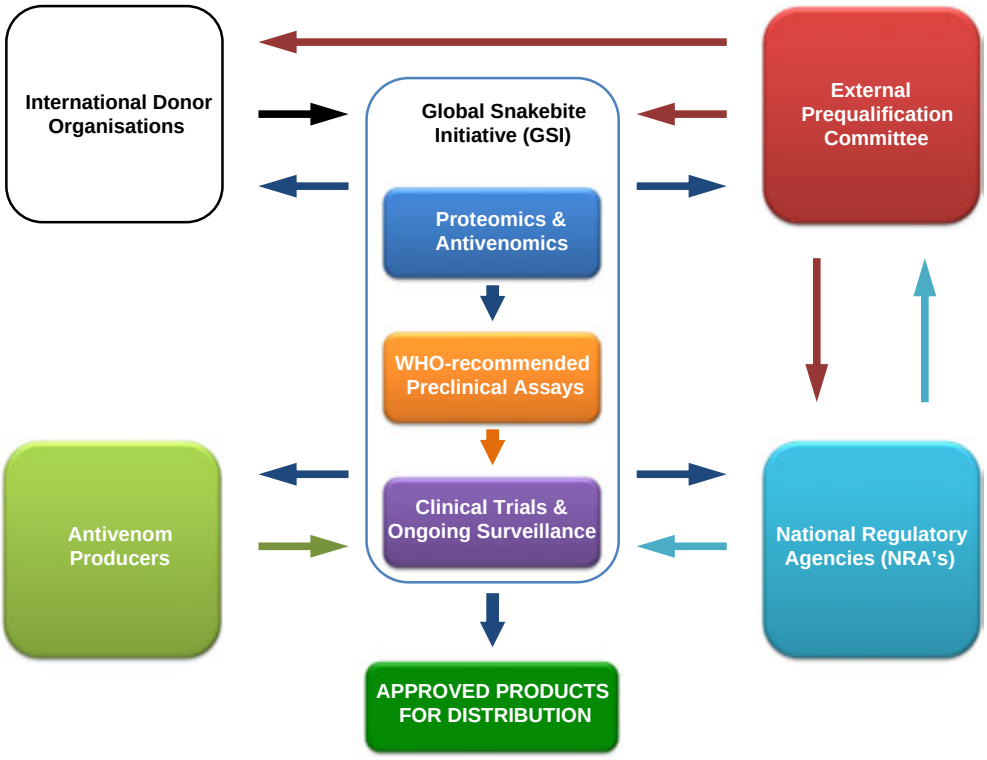

Fig. 7 – Flow diagram for evaluation of existing antivenoms. Donor funding would be used to establish a process wherein (a) antivenoms were obtained from producers, (b) subjected to proteomic and antivenomic assessment, followed by preclinical assessment of those antivenoms which show potential for therapeutic use in an intended market, and (c) if necessary supervised in clinical studies in the intended market destination. The evaluation process would be monitored by an external Prequalification Committee, reporting back to the donor organisations, the GSI, and to NRAs in the destination countries. All results would be published transparently and openly to provide feedback to antivenom producers, NRA's donors and the broader community. Products which have been validated for a particular market, and against specific venoms in those countries, could then be deemed "approved products" and their purchase and distribution funded by the donors.

parallel, there also needs to be a concerted effort to gain wider recognition of snakebite as a Neglected Tropical Disease (NTD). Although WHO included it on the list of NTDs in 2009, it is yet to feature in any NTD programme managed by WHO. Only an improvement in the political profile of snakebite envenoming will alter this, and may also give rise to finally securing the interest of philanthropic public health champions, such as the Bill & Melinda Gates Foundation, which supports a number of other NTD projects [245]. Even within Africa and Asia, there is the potential for neighbouring governments to form consortia to purchase a prequalified, clinically trialled antivenom in bulk in order to reduce unit costs and achieve significant savings. There is increasing evidence of the importance of working within health systems, rather than in parallel, or in competition, and this is particularly relevant to 'single issue' health initiatives [246], but at the same time, there also exists the potential to collaborate and work with other organisations to value-add to already functional infrastructure and projects [247]. The effective distribution of antivenoms to key health facilities where they can be deployed is a major logistical challenge. In many cases, surmounting this obstacle does not require the invention of a parallel system. There are a number of organisations who have established effective cold-chain distribution pathways particularly, in partnership with African governments, and some of these may be able to help with the distribution of antivenoms.

7. Summary

This paper looks pragmatically at the problems associated with obtaining adequate supplies of safe, affordable, effective antivenoms for communities in Africa and Asia. Many of these problems are complex political, good-governance and policy-related issues that are beyond easy resolution. This review acknowledges those obstacles and considers what can and cannot be achieved. It explores in detail how some of the technological problems associated with producing effective antivenoms can and are being resolved using modern proteomic and antivenomic approaches, and other methods. We propose, under the leadership of the Global Snakebite Initiative, a multidisciplinary, international collaboration to create pathways for (a) the development of novel immunogens based on proteomic comparisons of key regional venoms, that will be the basis for production of new regional polyvalent antivenoms, subject to preclinical and clinical assessment,

and (b) the evaluation of the suitability of existing antivenoms for new markets under a parallel system of applied proteomics coupled with functional preclinical and clinical studies. Under both of these frameworks, antivenoms would be deployed only after passing through an independent 'prequalification' system that provides confidence not just to donors, but also to National Regulatory Agencies and Health Ministries in target nations. We invite fellow scientists and clinicians, public health organisations and antivenom producers to join our collaboration and work with us to implement this new strategy. Whilst access to antivenoms of adequate safety and effectiveness is only one of the core challenges posed by the neglected tropical disease of snakebite, it is a fundamental problem that currently denies equality to millions of the world's poorest peoples who are at risk of death or lifelong disability.

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