Review Article

Indian J Med Res 138, July 2013, pp 38-59

New approaches & technologies of venomics to meet the challenge of human envenoming by snakebites in India

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Received February 14, 2013

The direct estimate of 46,000 snakebite deaths in India in 2005 (1 for every 2 HIV/AIDS deaths), based on verbal autopsies, renders unrealistic the total of only 47,000 snakebite deaths in the whole world in 2010, obtained indirectly as part of the "Global Burden of Disease 2010" study. Persistent underestimation of its true morbidity and mortality has made snakebite the most neglected of all the WHO's "neglected tropical diseases", downgrading its public health importance. Strategies to address this neglect should include the improvement of antivenom, the only specific antidote to envenoming. To accommodate increased understanding of geographical intraspecific variation in venom composition and the range of snake species that are medically important in India, the design of antivenoms (choice of venom sources and species coverage) should be reconsidered. Methods of preclinical and clinical testing should be improved. The relatively new science of venomics involves techniques and strategies for assessing the toxin composition of snake venoms directly through proteomics-centred approaches or indirectly via high-throughput venom gland transcriptomics and bioinformatic analysis. Antivenomics is translational venomics: a proteomics-based protocol to quantify the extent of cross-reactivity of antivenoms against homologous and heterologous venoms. These approaches could revolutionize the preclinical assessment of antivenom efficacy, leading to a new generation of antivenoms that are clinically more effective.

Key words Antivenoms - cobra - envenoming - krait - preclinical efficacy - Russell's viper - snakebite - venom - venomics

Introduction

When most people involved in public health think of neglected tropical diseases (NTDs) that affect the lives of men, women and children throughout the developing economies of the world, their minds usually turn to a conflagration of tropical infectious diseases including guinea worm, leishmaniasis, dengue, onchocerciasis, Chagas disease, leprosy and lymphatic filariasis. The World Health Organization (WHO) agrees. Supported

by drug makers and special interest groups, WHO initiated a coordinated effort to address a number of these diseases in 2005. That effort has steadily mushroomed, resulting in publication of a landmark report on these problems in 2010, that gave rise to a strategic plan for eradication and control efforts[1]. Today WHO lists 17 NTDs on its website as targets for eradication. At the WHO's World Health Assembly on 27th May 2013, resolution EB132.R7 was adopted, urging member States to expand their programmes to prevent, control, eliminate and eradicate all 17 of these NTDs[2]. But what few people will know is that in March 2009, WHO added snakebite to its official list of NTDs, and then quietly demoted it, along with podoconiosis and strongyloidiasis to a separate listing of what it calls "other neglected conditions"; a list that is included in none of WHO's plans to eradicate NTDs, or even mentioned in either its 2010 report, or the recently available 2013 report[1,3]. Such a move seems to be the way in which the public health community considers snakebite, begrudgingly admitting to it, but then quietly relegating it to the category of being too difficult to achieve, despite the reality that this forgotten tropical disease continues to impose a tremendous cost of suffering and chronic disability on many of the world's poorest and most disenfranchised communities[4].

Persuading the global community to take serious notice of the extent to which families and even whole communities are fractured by snakebites is a challenge that is being taken up by scientists, doctors and others who have been confronted by the tragedy of lives lost or bodies crippled by what is essentially a preventable and treatable illness. In 2008, members of the international toxinology community established the Global Snakebite Initiative (GSI), since when its members have worked towards developing an integrated new approach to improving therapeutic management of snakebite, and addressing issues ranging from prevention, to health-worker training and victim rehabilitation[5-7]. At the core of the GSI strategy is the desire to couple modern proteomic, immunological, pharmacological and molecular biological techniques to the quest for improved therapeutics and the understanding of the underlying pathophysiology and biomechanics of snakebite injuries[7-10]. Among the links being forged by the GSI, are partnerships with Indian researchers and institutions interested in the challenge of improving both the treatment of snakebite in India, and the outcomes for the million or more people bitten each year.

India has had a long and tumultuous relationship with serpents, which are both revered and feared. Some of the earliest reports of snakebite from India during British colonial rule spoke of fatalities in the tens of thousands. Sir Joseph Fayrer who championed the destruction of India's snakes as the best means to curb human deaths, wrote in a report to Nature published on December 28, 1882, that snakebite had caused the deaths of 11,416 people across seven administrative regions representing about half of what was then British India (including modern Burma and Pakistan) in 1869, but he believed this to be an under-estimate of the true burden[11]. Fayrer believed between 150,000 and 200,000 deaths due to snakebite had occurred in India from 1870-1882, citing 19,060 human deaths in 1880 and 18,610 in 1881. During this two year period the government paid out 23,623 rupees in bounties for 467,744 snakes killed under eradication programmes that Fayrer had fought to have instituted. Numerically, the highest numbers of fatalities in 1880/81 were recorded in the heavily populated Bengal (19,272), and the North-West Provinces/Aoudh (9,733), which also had the highest mortality rates relative to population size (15.93/100,000/yr and 11.04/100,000/yr, respectively) along with the Central Provinces (11.50/100,000/yr) and the small population of Ajmere-Marwara (10.30/100,000/yr)[12]. Using population data from the time provided in a letter to the British Medical Journal by Dr Edward Nicholson in 1883[12], the mortality rate for snakebite right across British India for 1880/81 averaged 10 deaths per 100,000/yr, a figure which if true today would equate to more than 163,504 deaths/yr across the former British India (excluding Sri Lanka) of which more than 122,000 would occur in present day India. Fayrer's claim of up to 200,000 deaths from 1870-1882 is supported by what were said to be incomplete annual figures from the British Administration in India[13]. It reported that 21,391 deaths occurred in 1875, 19,279 in 1876 and 22,125 in 1882. The 1876 figure is cited as the lowest number between 1875-1882. Thirty years of reports published in 1913 continued to put the annual death toll from snakebite at more than 20,000 per year[14]. In 1909, there were 21,634 deaths across the British colonies, and 22,478 were recording in 1910, with 7,767 of these occurring in Bengal[14]. Bombay (now Mumbai) saw far fewer snakebites than Bengal. In 1902 and 1903 there were 1,288 and 1,074 deaths from snakebites, respectively representing 16.7 per cent of all the deaths from injury in that 2 year period[15]. In 1910, snakebites caused 1,247 deaths in Bombay[11]. In 1924, there were 19,867

deaths from snakebites reported across British India, accounting for 2.35 per cent of all deaths recorded in the Public Health Commissioners annual report to government[16].

This review presents a modern summary of snakebite in India, and discusses how real improvements in the quality of snake antivenoms as front-line therapeutics can be achieved by embracing the opportunities presented by modern scientific technologies when applied to this age-old problem.

The global burden of snakebites

The recently published Global Burden of Disease 2010 estimated, from a large number of different sources, that the global total of deaths from animal contact (venomous) decreased from 54,900 (95% uncertainty intervals 30,100-89,300) in 1990 to 47,000 (25,600-84,700) in 2010 and that deaths from "other neglected tropical diseases", which include snakebites (*http://www.who.int/neglected_diseases/diseases/en/*)[17], increased from 22,900 (14,300-29,500) in 1990 to 23,700 (16,600 - 30,900) in 2010[18]. The absurd inadequacy of these estimates is immediately apparent if one considers the direct estimate of 46,000 (99% confidence interval 41,000 - 51,000) snakebite deaths in India in 2005[19]. Over the last half century, there have been a few attempts to discover the incidence of snakebite and its associated morbidity and mortality worldwide. In 1954, Swaroop and Grabb, using national hospital returns, suggested half a million bites with 30,000-40,000 deaths each year but they had no data from China, USSR, or Central Europe[20]. In 1998, Chippaux used results of household surveys, together with hospital and health authority data to increase these figures almost five-fold to 5 million bites, 125,000 deaths and 100,000 severe sequelae each year[21]. Evaluation of overall disease burden using the concept of disability adjusted life years (DALYs) produced the high estimate of 2 million DALYs per year for sub-Saharan Africa[17]. In the most recent WHO-sponsored effort, Kasturiratne *et al*[22] dredged the so-called "grey literature", combining these suspect sources with hospital returns and by extrapolation between adjacent (and some not-so-adjacent) countries produced a figure of 420,549–1,841,158 envenomings (bites in which venom was injected) and 19,886 - 93,945 deaths each year. Assuming that envenoming occurred in about one in every four snakebites, they estimated that between 1.2 and 5.5 million snakebites might occur annually. In all likelihood the only way in which the true burden will ever be known is if snakebites are made a reportable event by governments willing to invest in properly defining the impact of snakebite on public health and community well-being.

Snakebite incidence and mortality in India

With the growth of human populations in South Asia since Sir Joseph Fayrer and others produced their first calculations of snakebite impact more than 130 years ago, one might expect that snakebite figures in India would have climbed with the burgeoning of humanity. Community-based surveys in Burdwan district, West Bengal[23], and a neighbouring area just across the border in Nepal's eastern Terai[24], found surprisingly high annual incidences of snakebite deaths, 16.4 and 162 per 100,000 population per annum, respectively. However, between 2004 and 2009, hospitals in 31 of the 35 States and Union Territories in India reported an average of only 1,350 snakebite deaths each year to the Government of India (*http://cbhidghs.nic.in/index2.asp?slid=985&sublinkid=694*). These national totals were lower than older figures from single States. For example, in 1971, 1,476 snakebite deaths were recorded in Bombay Province, Maharashtra State and Ratnagiri district[25]. Controversy about the true burden of snakebite mortality in India should at last have been resolved by results of the Registrar General of India's "Million Death Study" (MDS)[19]. All causes of all deaths were ascertained in 6,671 randomly chosen sample areas, each including about 1000 people. Verbal autopsy was used to identify snakebite deaths. This involves a structured interview of the relatives or close associates of the deceased conducted by non-medical staff with central medical coding by at least two doctors. Verbal autopsy is reliable for snakebite because the fatal event is distinctive, dramatic and therefore memorable. However, some deaths may have been missed since some victims of nocturnal krait bites do not realize that they have been bitten and present with mysterious 'early morning paralysis' or seizures that would not be associated with snakebite[26]. In 2005, an estimated 46,000 people (99% CI 41,000 - 51,000) died of snakebite in India, approximately one for every two HIV/AIDS deaths[19]. Assuming that there are 100 non-fatal bites for each fatal bite (from results of a community-based study in Bangladesh)[27] there could be as many as 4.6 million snakebites in India each year. The MDS results indicate which States of India are worst affected: the highest numbers of deaths were in Uttar Pradesh (8,700), Andhra Pradesh (5,200), and Bihar (4,500), while Andhra Pradesh had the highest

incidence of mortality due to snakebite (6.2/100 000 population/year)[19]. Snakebite accounts for 3 per cent of all deaths in children of the age of 5-14 yr. Ninety seven per cent of the victims of snakebite die in rural areas, 77 per cent of them outside health facilities, because they preferred traditional therapy from *tantriks*, *vaidyas* and *ojhas*. These figures should encourage the Ministry of Health to reassess the public health priority of snakebite and deploy its resources where these are most needed.

Approaches to preventing snakebites

Snakebite in India is an occupational and environmental disease mainly affecting rural agricultural workers and their families. Prevention is all important. Like any form of health promotion, community education by all available means and media is crucial. The aim is to alert communities to the types of environments most frequented by dangerous species, and to advise them how to avoid being bitten. For example, cobras are most commonly found near water or in irrigated paddy fields, while Russell's vipers frequent these areas especially at times of harvest when rodents are most abundant. The most dangerous time of year is usually during the monsoon and hot weather. Many bites are inflicted as people walk home or collect firewood in the dark after dusk or before dawn. Safer walking and safer working can be achieved by wearing protective shoes, boots or gloves and by carrying a light after dark. The risk of nocturnal krait bites can be mitigated by sleeping in a hammock, on a raised bed or under a well-tucked-in mosquito net[28]. Communities should be persuaded to abandon their preference for traditional treatments and, instead, to transfer bite victims to dispensaries, clinics or hospitals as quickly as possible. Steps must be taken to improve the medical treatment of snakebite (including the effectiveness and safety of antivenoms) to generate confidence in conventional medicine. Where there are no roads and limited means of transport, village-based motorcycle volunteers have proved effective for transporting obstetrical emergencies in Sierre Leone and patients with neurotoxic snakebites in the eastern Terai of Nepal "SK Sharma, personal communication"[29]. Once the patient has arrived in hospital, prevention of morbidity and mortality will depend on the training of medical staff and provision of necessary equipment, antivenom and other drugs. Guidelines for the SEARO region, including India, have been published by WHO[30].

Indian snakes of medical relevance and the design of Indian polyvalent antivenoms

India and its surrounding seas are inhabited by more than 60 species of venomous snakes some of which are sufficiently common to cause frequent bites and whose venoms are capable of severe envenoming[31]. Four species, common krait (*Bungarus caeruleus*) (Fig. 1a), spectacled cobra (*Naja naja*) (Fig. 2a), Russell's viper (*Daboia russelii*) (Fig. 3a), and saw-scaled viper (*Echis carinatus*) (Fig. 4a) are distributed throughout virtually the whole country and have long been recognized as the most important causes of bites, deaths and

Fig. 1. Envenoming by common krait (*Bungarus caeruleus*): **(a)** the snake (specimen from Pune, Maharashtra); **(b)** bilateral ptosis, external ophthalmoplegia and facial paralysis; **(c)** fang and tooth puncture marks without local swelling in the same patient as 1b.

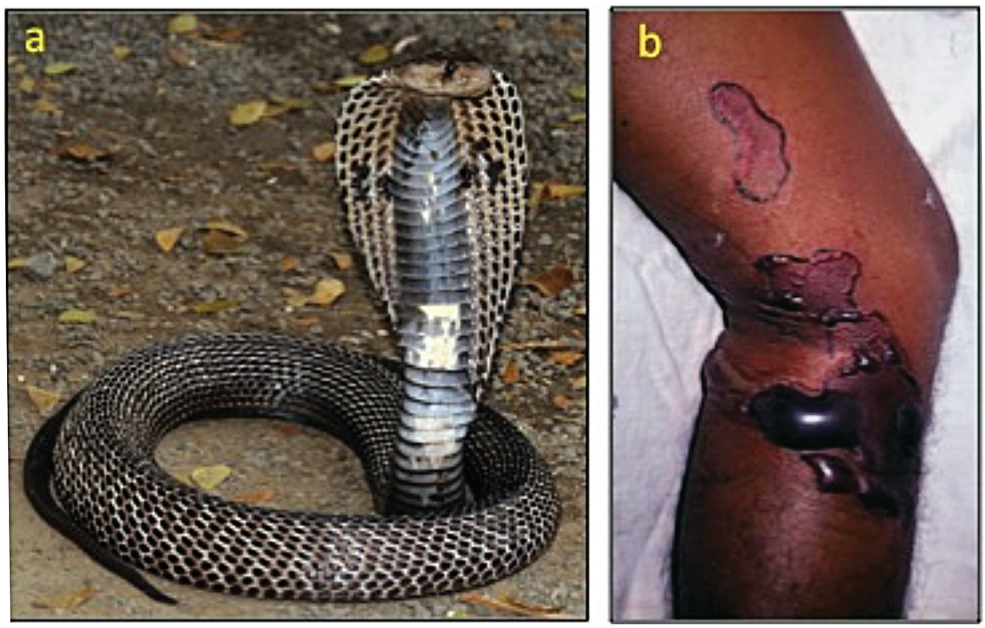

Fig. 2. Envenoming by common cobra (*Naja naja*): **(a)** the snake (specimen from Pune, Maharashtra); **(b)** local swelling, blistering and demarcated areas of dermonecrosis in a patient.

Fig. 3. Envenoming by Russell's viper (*Daboia russelii*): **(a)** the snake (specimen from Pune, Maharashtra); **(b)** local swelling and blistering (Kerala); **(c)** chemosis, a sign of generalized increase in capillary permeability; **(d)** bilateral ptosis, external ophthalmoplegia and facial paralysis; **(e)** chronic pan-hypopituitarism (loss of secondary sexual hair and gynaecomastia) following a bite 3 years previously (Kerala).

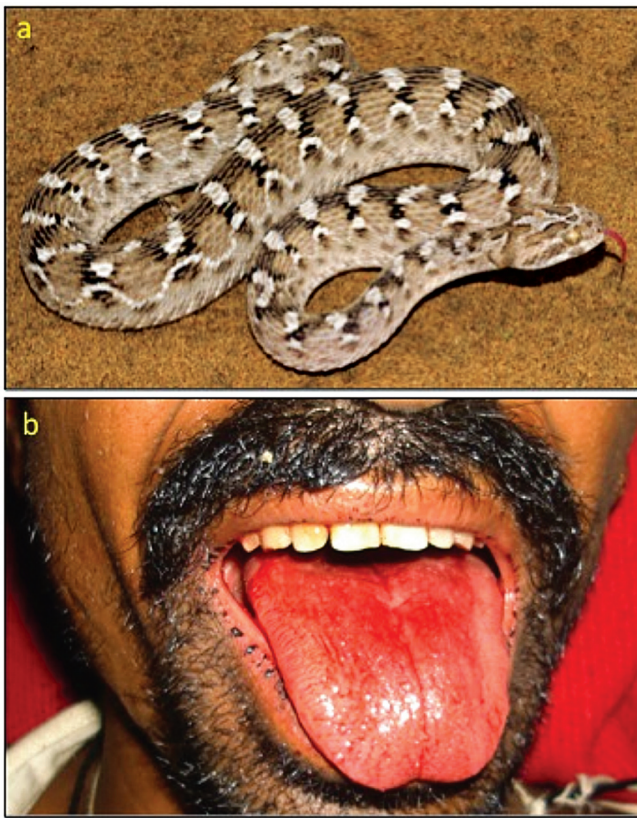

Fig. 4. Envenoming by northern saw-scaled viper (*Echis carinatus sochureki*): **(a)** the snake (specimen from Bikaner, Rajasthan; **(b)** Bleeding from the gums (Bikaner) (courtesy of Dr PD Tanwar).

disability. Over the past century, venoms of only these four species have been used by Indian manufacturers to raise polyvalent antivenoms (also called anti-snake venoms, ASV, or anti-snakebite serums) for national use. However, there have been increasing concerns about the design of these antivenoms, due in part to recognition that many more species of venomous snakes are implicated in envenoming across India and also to ongoing concerns about the safety and effectiveness of products that have gained a reputation for being poorly manufactured in some cases, and poorly effective in published clinical studies of snakebites[32].

Which species are responsible for snakebite envenoming in India?

Apart from the widely distributed "big four", there are a number of other species occurring in localized areas of the country that are now known to be capable of causing severe envenoming[32]:

Monocellate cobra (*N. kaouthia*) - occurs in north-eastern India, in many parts of which it causes more bites than *N. naja* with equally severe consequences.

Central Asian or Oxus cobra (*N. oxiana*) - is confined to the far north (Kashmir and Himachal Pradesh) where it is rare. No bites have been reported in India but it has proved dangerous in other parts of its range.

Kraits (*Bungarus*) - taxonomic revisions have elevated two former sub-species to the status of full species. Wall's krait (*Bungarus walli*) occurs in the north-east and the Sind krait (*B. sindanus*) in the west. *Bungarus sindanus* has caused life-threatening envenoming in Maharashtra[33] and Rajastan (Dr PD Tanwar, personal communication, February 2011). *Bungarus walli* has caused fatalities in Nepal and Bangladesh (unpublished data of Aniruddha Ghose, Md A. Faiz, Sharma SK, Harris JB, Kuch U, Warrell DA in preparation). The greater black krait (*B. niger*) occurs in the far north-east of India. In Bangladesh, it has caused fatal envenoming through generalized rhabdomyolysis and acute kidney injury (AKI), as well as by more familiar paralytic effects[34].

King cobra (*Ophiophagus hannah*) - occurs in the Western Ghats and north-east India. It is the largest and potentially the most dangerous of all venomous snakes but is rare and elusive, causing very few bites and deaths[35].

Levantine or snub-nosed viper (*Macrovipera lebetina*) - this large and dangerous species is confined to Jammu and Kashmir at altitudes above 900 m and has caused only one recorded bite in India[35].

Pit-vipers (Crotalinae) - among the 15 Indian species, the hump-nosed pit-viper (*Hypnale hypnale*) (Fig. 5a) of the south-west coast and Western Ghats, where it has been misidentified as *E. carinatus,* has caused local necrosis, coagulopathy, bleeding and AKI[37]. The Himalayan pit-viper (*Gloydius himalayanus*) of the western Himalayas, bamboo pit-viper (*Trimeresurus gramineus*) of the Western and Eastern Ghats, large-scaled pit-viper (*Peltopelor macrolepis*) of the south-west[37], mountain pit-viper (*Ovophis monticola*) of north and north-east and northern white-lipped green pit-viper (*Cryptelytrops septentrionalis*), red-tailed pit-viper (*C. erythrurus*) and some other arboreal green pit-vipers of the north-east, can cause local swelling, coagulopathy, bruising and bleeding. Envenoming by the Malabar pit-viper (*Trimeresurus malabaricus*) of the Western Ghats[38] can cause local necrosis. The venoms of none of these species are covered by Indian polyvalent antivenoms.

Sea-snakes (Hydrophiinae, Laticaudinae) - there have been a few reported cases of sea snake bites around the coast of India[39] (Fig. 6).

Fig. 5. Envenoming by hump-nosed viper (*Hypnale hypnale*): **(a)** the snake (specimen from Thattekkad, Kerala); **(b)** marked local swelling after a bite on the foot (Angamaly, Kerala).

Fig. 6. Beaked sea snake (*Enhydrina schistosa*): the snake (courtesy of Mark O'Shea).

Clinical phenotypes of envenoming by Indian snakes

Elapid envenoming is characterized by neurotoxicity, first evident as ptosis and external ophthalmoplegia (Fig. 1b) then involving muscles innervated by the cranial nerves in a descending pattern followed by the axial muscles of the trunk, resulting in respiratory paralysis and finally, if respiration is supported, generalized flaccid paralysis, partially sparing only the extremities. Krait bites are almost always inflicted on people sleeping indoors on the ground. Local envenoming is absent or minimal (Fig. 1c) and there is usually no local pain. However, severe crescendo abdominal pain may be a presenting symptom. Destructive, irreversible neurotoxicity that does not respond to antivenom is a feature of krait envenoming. Cobra bite envenoming causes, in addition, local envenoming: pain, swelling, regional lymphadenopathy, blistering and necrosis (Fig. 2b). Russell's viper bite envenoming causes marked local envenoming (Fig. 3b) and a variety of systemic manifestations: shock, coagulopathy, bleeding and AKI. In some parts of the country such as Kerala, there is generally increased capillary permeability (Fig. 3c). In others, such as Tamil Nadu, there is neuro-myotoxicity (Fig. 3d). The risk of anterior pituitary infarction resulting in fatal acute pituitary adrenal failure or chronic pan-hypopituitarism occurs in southern India. Saw-scaled viper bites are usually less severe, lacking the risk of AKI, permeability and pituitary infarction but associated with local necrosis, coagulopathy and bleeding (Fig. 4b). Hump-nosed viper bites are associated with local envenoming (Fig. 5b), coagulopathy, bleeding, microangiopathic haemolysis and AKI. Bites by other Indian pit-vipers can cause local envenoming resulting in necrosis, coagulopathy, bleeding rarely if ever in AKI. Generalised rhabdomyolysis causing myalgia followed by myoglobinuria and AKI is a feature of sea snakebite envenoming.

Is Indian polyvalent antivenom equally effective in treating bites by the "big four" species throughout the whole of India?

The phenomenon of intra-species variation in venom composition has been well documented in the cases of many species of viperid and elapid snakes[40,41]. Geographical diversity in venom antigenicity is the most important consideration in antivenom design. In India, there is evidence of such variation in the venoms of *N. naja* and *D. russelii*[42,43] and yet 80 per cent of the venom currently used to raise Indian polyvalent antivenoms is collected by the Irula cooperative from snakes inhabiting one small area around Mahabalipuram in Tamil Nadu (*http://www.madrascrocodilebank.org/cms/conservation-and-research-2/the-irulas/*). There is growing clinical suspicion that Indian antivenom may be less effective in some areas distant from the source of immunizing venoms. In Rajasthan, envenoming by the larger northern sub-species of the saw-scaled viper (*Echis carinatus sochureki*) requires larger doses of antivenom than for the smaller southern sub-species (*E. c. carinatus*)[44]. In Maharashtra and northern Kerala, antivenom appears to be less effective in the treatment of *D. russelii* envenoming. For example, in Kozhikode, treatment with generally accepted initial doses of 10-20 vials of polyvalent antivenom within a few hours of the bite failed to prevent development of the syndrome of generalized increase in capillary permeability (capillary leak syndrome) (Dr Remi Thomas - personal communication, February 2012).

Should the classic Indian polyvalent antivenoms be redesigned to provide broader cover?

Because of geographical intra-species variation in venom composition and hence immunogenicity, the current practice of using venom from restricted geographical source(s) may have the result that envenoming even by the traditional 'big four' species may not be adequately treated by Indian polyvalent antivenoms. Ideally, venom pools including samples from a wide geographical range should be used for immunization. The range of species against which polyvalent antivenom is effective should also be expanded to cover some of the additional species. In a recent study, two Indian polyvalent antivenoms manufactured by Vins Bioproducts and Bharat Serums and Vaccines showed different levels of neutralization for both Indian and south-east Asian *Naja* and *Bungarus* venoms, indicating that polyvalent antivenoms produced in India, using the same source of venom, should not be assumed to be identical in their therapeutic potential[45]. In reconsidering their future strategy for antivenom design, the Indian authorities might decide to continue with one polyvalent antivenom with expanded range to cover the whole country or perhaps three new regional antivenoms. For example, NE (North-East) antivenom might include, in addition to the big four, *N. kaouthia, B. walli*, *B. niger* and one of the pit-vipers among its immunizing venoms; NW (North-West) - *N. oxiana, B. sindanus, E. c. sochureki* and *M. lebetina*; SW (South-West)- *B. sindanus*, *H. hypnale*, *T. malabaricus* and *T. gramineus*.

Toolbox for the preclinical assessment of antivenom efficacy

An ideal therapeutic antivenom should contain antibodies that can neutralize the widest possible range of medically important toxins to give the widest polyspecific coverage, using as few specific venoms as possible in its production. The large variation in the composition of snake venoms, both at the inter- and intra- species levels, has implications for the design of venom mixtures for immunization of animals for antivenom production and in the analysis of antivenom neutralizing efficacy at the preclinical level. The introduction of a new antivenom in a particular geographical setting should be preceded by a rigorous analysis of its preclinical efficacy against the venoms of the most relevant snakes in the region. The single most important test to assess neutralization of venoms by antivenoms is the mouse lethality test but, depending on the toxicological profile of the venom being tested, additional assays should also be included.

Neutralization of lethality assay

Lethality is the single most important effect to be tested when analysing venom toxicity and its neutralization by antivenoms. Thus, the gold standard for the assessment of preclinical antivenom efficacy is the neutralization of lethal activity of venoms[46]. First, the overall toxicity of venom, the median lethal dose (LD_{50}), is determined, usually using the mouse model. To this end, serial doses of venom, diluted in saline solution, are injected in mice of a determined weight, either by the intravenous (i.v.) or the intraperitoneal (i.p.) routes. Deaths occurring during a predefined time span (24 or 48 h) are recorded and the LD_{50} is estimated by statistical methods such as Spearman-Karber[47], probits[48] or non-parametric procedures. LD_{50} corresponds to the venom dose which induces death in 50 per cent of the injected mice; it can be expressed

as µg venom per mouse or as µg venom per gram of animal weight. For the neutralization assays, a fixed dose of venom, known as the 'challenge dose', which corresponds to a defined number of LD_{50}s (usually from 3 to 6 depending on the laboratory), is selected. This fixed venom solution is mixed with various dilutions of the antivenom, in order to achieve several antivenom/venom ratios, and the mixtures are incubated (usually for 30 min at 37°C). Control samples include venom incubated with saline solution instead of antivenom. Aliquots of the mixtures are then injected into mice and deaths are recorded. Neutralization is expressed as the median effective dose (ED_{50}), *i.e.* the antivenom/venom ratio in which 50 per cent of the injected mice survive. ED_{50} can be expressed in various ways, which varies depending on the laboratory, *i.e.* milligram of venom neutralized per millilitre antivenom, millilitre antivenom required to neutralize one milligram venom, or number of LD_{50}s of venom neutralized per millilitre antivenom. The assessment of the ability of antivenoms to neutralize lethality is routinely performed in the manufacturers' quality control laboratories and by national regulatory agencies, as part of regular analyses of the antivenoms being manufactured, purchased and distributed. Unfortunately, some countries rely mostly on data reported by the manufacturers, rather than on control exerted by regulatory agencies.

Towards a more complete assessment of the preclinical efficacy of antivenoms

The neutralization of venom lethality is, and will remain, the gold standard in the preclinical testing of antivenom efficacy[46]. However, the study of the biochemical and toxicological complexity of snake venoms has shown clearly that, besides lethality, the venoms of many species induce additional toxic activities that play a key role in the pathophysiology of human envenoming. For example, envenomings by viperid snakes in many regions around the world are characterized by complex local tissue damage (myonecrosis, dermo-necrosis, haemorrhage, blistering) and by systemic disturbances (haemorrhage, coagulopathy, cardiovascular shock, acute kidney injury). Therefore, it has been argued that a more detailed analysis of the preclinical efficacy of antivenoms should encompass, in addition to lethality, the neutralization of these other clinically relevant effects[46,49,50]. Several simple *in vivo* and *in vitro* laboratory assays have been developed for the quantitative assessment of haemorrhagic, myotoxic, dermo-necrotic, coagulant, and defibrinogenating activities, among others[51-53]. Thus, although the routine quality control of antivenoms involves the neutralization of lethal activity, when a new antivenom is being developed, or when an existing antivenom is introduced to a new geographical setting, a comprehensive preclinical analysis of neutralizing efficacy should be performed against the most relevant toxic effects of the most important snake venoms in that particular region. This allows a rigorous testing of whether the antivenom is effective, not only against venom-induced lethality, but also against other clinically-relevant effects. A brief description of the fundamentals of these additional tests follows:

Haemorrhagic activity: The most widely used method is based on the intradermal injection of venom solutions followed, several hours later, by the measurement of the area of the haemorrhagic spot in the inner side of the skin. The original method was described in rabbits[54], and has been adapted for use in rats[51] and mice[55]. Venom activity is expressed as the minimum haemorrhagic dose (MHD), which corresponds to the dose of venom that induces a haemorrhagic halo of 10 mm diameter[55]. More recently, the analysis of systemic haemorrhage has been performed by the study of pulmonary haemorrhage. Mice were injected i.v. with venom and, one hour later, the animals were sacrificed and the thoracic cavity exposed for observation of haemorrhagic spots on the surface of the lungs. The minimum pulmonary haemorrhagic dose (MPHD) corresponds to the lowest amount of venom that induces haemorrhagic spots in the lungs of all mice injected[56].

Myotoxic activity: Venom-induced skeletal muscle necrosis can be assessed by histological examination of muscle tissue injected with venom. Mice receive an intramuscular injection of venom solution, for example, in the gastrocnemius muscle, are sacrificed after 24 h, and the injected muscle dissected out, placed in fixative solution (*e.g.* formalin), and processed for histological analysis. The number of necrotic cells and the total number of muscle cells are quantified by microscopic assessment, and the myotoxic effect is expressed as the necrotic index, *i.e.* the ratio of necrotic muscle fibres to total muscle fibres[57]. Venom activity can be expressed as the dose inducing a necrotic index of 0.5. Since histological analysis is time consuming and not available in many laboratories, an alternative and highly convenient test is based on the quantification of the plasma activity of the enzyme creatine kinase (CK), which is present in large amounts in muscle tissue and is released when the plasma membrane of muscle cells is disrupted by the action of venom myotoxins[52]. The

minimum myotoxic dose (MMD) is defined as the dose of venom that increases the plasma CK activity four times as compared to mice injected with saline solution[58].

Dermo-necrotic activity: This effect is assessed in either rats or mice by carrying out intradermal injections of venom solutions, followed by the measurement of the necrotic area in the inner side of the skin 72 hour after venom injection[51].

Coagulant activity: *In vitro* coagulant activity of venoms is assessed by the addition of various doses of venom to samples of citrated human plasma, followed by the determination of clotting time. Activity is expressed as the minimum coagulant dose (MCD), defined as the dose of venom that induces clotting in 60 sec[51,53]. For assessing thrombin-like activity of venoms, a similar test is performed on fibrinogen solutions instead of plasma[51].

Defibrinogenating activity: It is assessed in rats or mice by intravenous injection of venom solutions. After a defined period of time, a blood sample is collected, placed in a glass tube, and incubated at room temperature for observation of clot formation. Activity is expressed as the minimum defibrinogenating dose (MDD), defined as the dose of venom that induces incoagulability in all animals injected[51,53].

***Other tests*:** In addition to the most frequently performed tests described above, other toxic activities can be also assessed, such as oedema-forming activity[59], neurotoxic activity using nerve-muscle preparations *ex vivo*[60], and thrombocytopenic effect[61]. Likewise, the analysis of neutralization of venom enzyme activities has been also investigated, such as neutralization of proteinase, phospholipase A_2 and hyaluronidase activities[55,62,63]. As in the case of neutralization of lethality, a 'challenge dose' of venom is selected and a solution of a fixed dose of venom is mixed with various dilutions of antivenom, followed by incubation (30 min at 37ºC). Aliquots of the mixtures are then tested in the corresponding assay systems described; controls of venom solutions incubated with saline solution instead of antivenom are included. Neutralization is expressed as ED_{50}, *i.e.* the antivenom/venom ratio at which the effect of venom is neutralized to a 50 per cent[64]. In the case of coagulant and defibrinogenating activities, neutralization is expressed as effective dose (ED), defined as the antivenom/venom ratio at which the clotting time of plasma is prolonged three times when compared to plasma incubated with venom alone (for coagulant activity) or as the antivenom/venom ratio at which blood clots in all animals injected (for defibrinogenating effect)[53].

Selection of tests for preclinical evaluation of antivenoms should be based on the toxicological profile of venoms

Since snake venoms are highly variable in their toxicological profiles, the selection of laboratory tests to be implemented for the preclinical evaluation of antivenom efficacy should be based on the toxicological profile of venoms and on the predominant clinical manifestations of envenomings. The following are some examples:

(i) Venoms of elapid snakes exerting predominantly neurotoxic effect: The venoms of a large variety of elapid snakes exert, both at the clinical and the experimental levels, a predominant neurotoxic effect, based on the action of either presynaptically-acting phospholipase A_2 (PLA_2s), such as β-bungarotoxin from species of *Bungarus*, or post-synaptically-acting neurotoxins of the 'three finger family', known as α-neurotoxins, which bind with strong affinity to the nicotinic cholinergic receptor at the motor end-plate. These neurotoxins act at the peripheral nervous system and generate flaccid paralysis which, when affects respiratory muscles, might result in respiratory paralysis and death[65]. The preclinical efficacy of antivenoms against these venoms can be assessed by the neutralization of lethal effect (the ED_{50} test), since the end result of neurotoxicity is death. Thus, in the cases of venoms of many species of cobras (*Naja* sp.) and kraits (*Bungarus* sp.), neutralization of lethality suffices to test the preclinical efficacy of antivenoms[45].

(ii) Venoms of viperid snakes: Despite presenting significant variations in venom composition and activities between and within species, viperid venoms are generally characterized for inducing local tissue damage (*i.e.* haemorrhage, myonecrosis and oedema) and systemic alterations associated with haemorrhage, coagulopathy, cardiovascular disturbances and renal damage[65]. Therefore, the preclinical assessment of antivenoms against viperid venoms should include the analysis of lethal, haemorrhagic, myotoxic, coagulant, and defibrinogenating activities of venoms[66]. This would be the case of the venoms of *Echis* sp., *Daboia* sp. and *Hypnale* sp. in the Indian subcontinent.

(iii) Other venoms: Venoms of a number of spitting cobras in Africa and Asia induce predominantly a local necrotising effect in humans[65,67]. In these cases,

antivenoms should be assessed for their capacity to neutralize lethality and dermo-necrosis[68]. Sea snake venoms induce, in addition to neurotoxic effect, a systemic myotoxic action which might result in rhabdomyolysis. Hence, antivenoms should be tested for the neutralization of lethal and myotoxic activities[66]. On the other hand, the venoms of some Australian land elapids induce neurotoxicity, myotoxicity and coagulopathy; consequently, preclinical evaluation of antivenoms should include the neutralization of these effects[69].

When an antivenom, which is being developed or introduced to a new geographical setting for the first time, is shown to be effective at the preclinical level, subsequent routine quality control of its efficacy can be based on the neutralization of lethality only.

The issue of venom variability and the preparation of venom pools

A key aspect in the preclinical assessment of antivenom efficacy is the quality and representativeness of the venoms used to test neutralization. There may be a large intraspecific variability in venom composition, especially in species of wide geographical distribution, such as *Daboia russelii* in Asia[43], *Bitis arietans* in sub-Saharan Africa[70] and *Bothrops atrox* in South America[71]. In these and in similar cases, it is necessary to ensure that the venoms utilized correspond to representative pools prepared from specimens distributed in different geographical locations[40,46]. These venom pools should be prepared from specimens acquired from right across the range of the species, that have been correctly identified, since the traceability of the venoms for preclinical testing is of paramount relevance. Ideally, each country should have snake collections and laboratories for maintenance of snakes and for venom collection and storage, following appropriate standardised protocols[46] and, the preclinical assessment of antivenom efficacy should be carried out with venoms from specimens collected in the region where this antivenom is intended to be used. This task demands national and international cooperation from health authorities, National Regulatory Agencies, independent scientists and manufacturers to ensure the qualification of snake collections and venom sources and the adequacy of selected preclinical tests for the species venoms concerned. Furthermore, protocols for quality control of venoms used in antivenom production and evaluation should be also implemented according to the national and international standards[46].

The need for the development of *in vitro* tests for assessing antivenom preclinical efficacy

Many of the tests used for the preclinical evaluation of antivenoms involve the use of experimental animals, mostly mice. Since venoms induce pain and other effects in these animals, there is a growing concern about this issue and interest in finding suitable surrogate *in vitro* tests that could be used in the evaluation of antivenoms. One of the main drawbacks for achieving this goal has to do with the great biochemical and toxicological complexity of venoms, in which several relevant effects are induced by different venom components[66]. Despite this limitation, some advances have been achieved, such as determining procoagulant activity *in vitro*[40], and using enzyme immunoassays whose results correlate with the neutralization of lethality[72-74]. However, compared to the murine ED_{50} assay, the neuromuscular preparation assay requires a high degree of skill and experience to perform it well, and the technique is not easily reproducible from one laboratory to another. In addition, the use of chicken embryos at a stage prior to the development of pain sensitivity has been proposed as an alternative to rodent tests for assessing lethal and haemorrhagic activities of viperid venoms[75]. The search for alternative *in vitro* tests for assessing the neutralizing ability of antivenoms is an area of research and development that deserves urgent attention. In particular, at the start of the 21st century, developments in proteomics-centred approaches for assessing the detailed composition of venoms (venomics) and the immunochemical profile of antivenoms (antivenomics) are likely to revolutionise the design and pre-clinical assessment of antivenoms, especially in predicting paraspecific neutralization. The operational principles of these approaches are discussed below.

International cooperative projects should be implemented to assess the preclinical efficacy of antivenoms on a worldwide basis

The analysis of the neutralizing ability of antivenoms at the preclinical level has been the focus of a number of collaborative research projects in Latin America. As a consequence, a large body of evidence has accumulated and, on this basis, the public health authorities in the region are able to make informed decisions on antivenom acquisition and deployment in several countries. Examples are studies performed in Argentina[76], Brasil[77,78], Perú[58], Colombia[79], Ecuador[80], Costa Rica[55,77] and Guatemala[81]. Recently, a large collaborative regional project evaluated several

antivenoms against the venoms of the medically most important *Bothrops* species in the region[82]. In contrast, studies on the preclinical efficacy of antivenoms in Africa and Asia have been scarce and there is a large gap in our knowledge of the cross-neutralization of viperid and elapid snake venoms by antivenoms used in these continents. For instance, several antivenoms are distributed in sub-Saharan Africa, but only a few studies have reported data on their preclinical neutralizing profile[83-86]; moreover, venoms from snakes of many African countries have not been tested for their neutralization by antivenoms. This situation jeopardises efforts to bring antivenoms to large areas of Africa and Asia since the introduction of antivenoms for clinical use without preclinical information on their efficacy, is not recommended.

The solution to this problem demands concerted international efforts involving diverse partners, including herpetologists, toxinologists, antivenom manufacturers, epidemiologists and public health authorities, among others. The development and strengthening of local groups, in universities and ministries of health in developing countries, in the preclinical testing of antivenoms are of paramount importance and should be promoted through national and international workshops and training programmes. Likewise, the establishment of snake collection facilities and laboratories responsible for obtaining venoms are required to ensure the proper identification and quality assurance of venoms to be used in preclinical testing. Research laboratories working on venom proteomics and other classical and "omics" aspects of toxinology are essential to generate detailed knowledge of venom composition and variability.

The Global Snakebite Initiative (GSI, *http://www.snakebiteinitiative.org*), with the support of the International Society on Toxinology (IST, *http://www.toxinology.org*), has proposed an international strategy to improve our knowledge of the composition of the medically most important venoms, as well as of the ability of currently available antivenoms to neutralize these venoms at the preclinical level[7]. This knowledge may pave the way for the design of novel, knowledge-based immunizing mixtures with the aim of generating polyspecific antivenoms of broad efficacy, particularly in sub-Saharan Africa and Asia. Already, GSI has developed protocols for Indian researchers to enable them to collect very high quality venom samples in the field, and return these to the laboratory for analysis without degradation. Efforts are now focusing on ensuring that these venoms are used in well-designed, relevant assays to measure preclinical effectiveness of current Indian antivenoms. A pilot study, examining venom variation in Russell's vipers from different locations in India, and the ability of commercial antivenoms to neutralize all of the venoms has been designed and local researchers have begun collecting the venom samples (personal communication of Chellam R, Martin G, Whitaker R, *et al*, Madras Crocodile Bank Trust).

Proteomic tools for exploring the venom proteome

Snake venomics: Research on venoms has been continuously enhanced by advances in technology. Advances in instrumentation and high-throughput "omics" methodologies have fuelled an expansion of the scope of biological studies from simple biochemical analyses involving a few molecules at a time to the systematic study of whole genomes, transcriptomes, and proteomes. Specifically, the last decade has witnessed the development of techniques and strategies for assessing the toxin composition of snake venoms ("venomics"), directly (through proteomics-centred approaches)[8,87,88] or indirectly (via high-throughput venom gland transcriptomics and bioinformatic analysis)[89-91] in a relatively rapid and cost-effective manner. Bottom-up proteomics remains the workhorse for venomic analysis. Standard approaches include the tryptic digestion of the proteins into peptides, previously separated through single- or multi-dimensional protocols combining electrophoretic and chromatographic techniques[87,88]. These are then sequentially isolated in the mass spectrometer and sequenced by analysis of their fragmentation pattern. Parent proteins can then be identified by database searching strategies[92]. Proteomics-centred venomics requires homologous searchable databases for exploiting fully its analytical capabilities[93-96]. However, the slow progress in the generation of genomic and transcriptomic databases precludes the application of automated high-throughput proteomic approaches to study venoms. In addition, in the absence of reliable automatic *de novo* sequencing mass spectrometric algorithms, snake venom proteome investigation remains a laborious task. Recent contributions to the literature of venom proteomics emphasised that sample de-complexation before mass spectrometry represented the best approach for maximizing proteome coverage[97]. Fractionation via multi-dimensional techniques and strategies for the depletion of high abundance proteins, as well as the enrichment of extremely low abundance

proteins to mine the venom proteome more effectively, have been investigated[98,99]. Clearly, if sufficient pre-mass spectrometric and mass spectrometric efforts are applied, thorough proteomic coverage can be achieved by an experienced researcher.

Our snake venomics approach[7-9,100] (Fig. 7) includes an initial step of fractionation of the crude venom by reverse-phase (RP) HPLC followed by the initial characterization of each protein fraction by a combination of *N*-terminal sequencing, SDS-PAGE, and mass spectrometric determination of the molecular masses, and eventually the cysteine (-SH and S-S) content, of the isolated components. RP-HPLC allows the quantitative recovery of all venom components present in the molecular mass range of 7–150 kDa that can be separated by conventional 2DE. Further, 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 separation. Monitoring the eluate at the absorption wavelength of the peptide bond (190-230 nm) represents a reliable method for quantifying the relative abundance of the different venom components in the reverse-phase chromatogram[8,9]. In-gel digestion, peptide mass fingerprinting, and *de novo* peptide sequencing by collision-induced dissociation (CID)-tandem mass spectrometry (MS/MS) coupled to database search by BLAST analysis allow the unambiguous assignment to known or unknown protein families of all venom toxin bands visualized in a Coomassie blue-stained SDS-polyacrylamide gel. Sequence coverage becomes an important issue to differentiate between the different classes of multi-domain proteins, such as the haemorrhagic Zn^{2+}-dependent metalloproteinases (SVMP), whose pharmacological activities are modulated by their distinct domain structures[101].

Venomics analyses involving nearly 100 taxa have revealed that snake venoms are mixtures of pharmacologically active proteins and peptides synthesized from several tens to a few hundred of unique gene products[89-91], which, however, belong to only a handful (<13) of toxin families[8-10,87,88]. A major goal of bottom-up venomics is to measure the full chemical space within which the pathological effects of snakebite envenoming are manifested. Another prime challenge of venomics is the formulation of rigorous biological hypotheses based on the information gathered by the discovery-based proteomics platform. In this respect, it is worth recalling the words of the prominent geneticist and evolutionary biologist, Theodosius G. Dobzhansky: "*Nothing in biology makes sense except in the light of evolution*"[102]. Understanding the molecular mechanisms and evolutionary trends that underlie inter- and intra-specific venom variation provides insights into snakebite pathology[103-110]. The occurrence of variability in the biochemical composition of venoms and in the symptomatology after envenomation by snakes from different geographical locations and age has long been appreciated by herpetologists and toxinologists[38,111], although detailed comparative analyses were scarce in the literature before 2008. A specific question that we have assessed in recent years by venomics includes the occurrence and onset of geographical and ontogenetic variations in *Bothrops asper*[107], *B. atrox*[71], the *Crotalus durissus* complex[105,112], *C. tigris*[113], *C. scutulatus*[106], *Sistrurus miliarius barbouri*[114], and across genus *Lachesis*[115]. Emerging views of these, and other continuing studies indicate that *(i)* geographical variability is a general phenomenon and phenotypic variation across the population encompasses two distinct but overlapping causal factors: individual and ontogenetic variations. The occurrence of intraspecific individual allopatric variability highlights the concept that a species should be considered as a group of meta-populations; *(ii)* ontogenetic changes follow genus-specific trends[71,107,112-115]; and *(iii)* within a species range, paedomorphic and ontogenetic venom phenotypes often occur in geographically differentiated areas[71,107,116,117].

Translational venomics: antivenomics

A robust knowledge of the toxin composition of venoms and the recognition of convergent and divergent trends along venom evolution is of fundamental importance in the selection of species and specimens for the manufacture of improved therapeutic antivenoms. This is particularly relevant for highly adaptable and widely distributed species, in which allopatric venom variability represents a source of diversity of the pathological effects of envenoming. To help antivenom design and to assess the range of possible clinical application of currently commercial or experimental mono- and polyspecific antivenoms, we have developed, since 2008, "antivenomics"[118]. Antivenomics is a proteomics-based protocol to quantify the extent of cross-reactivity of antivenoms against homologous and heterologous venoms[110,119,120]. First generation antivenomics consisted of the immuno-depletion of toxins upon incubation of whole venom with antivenom followed by the addition of a secondary antibody. Antigen-antibody complexes immuno-depleted from the reaction mixture contain the toxins against which

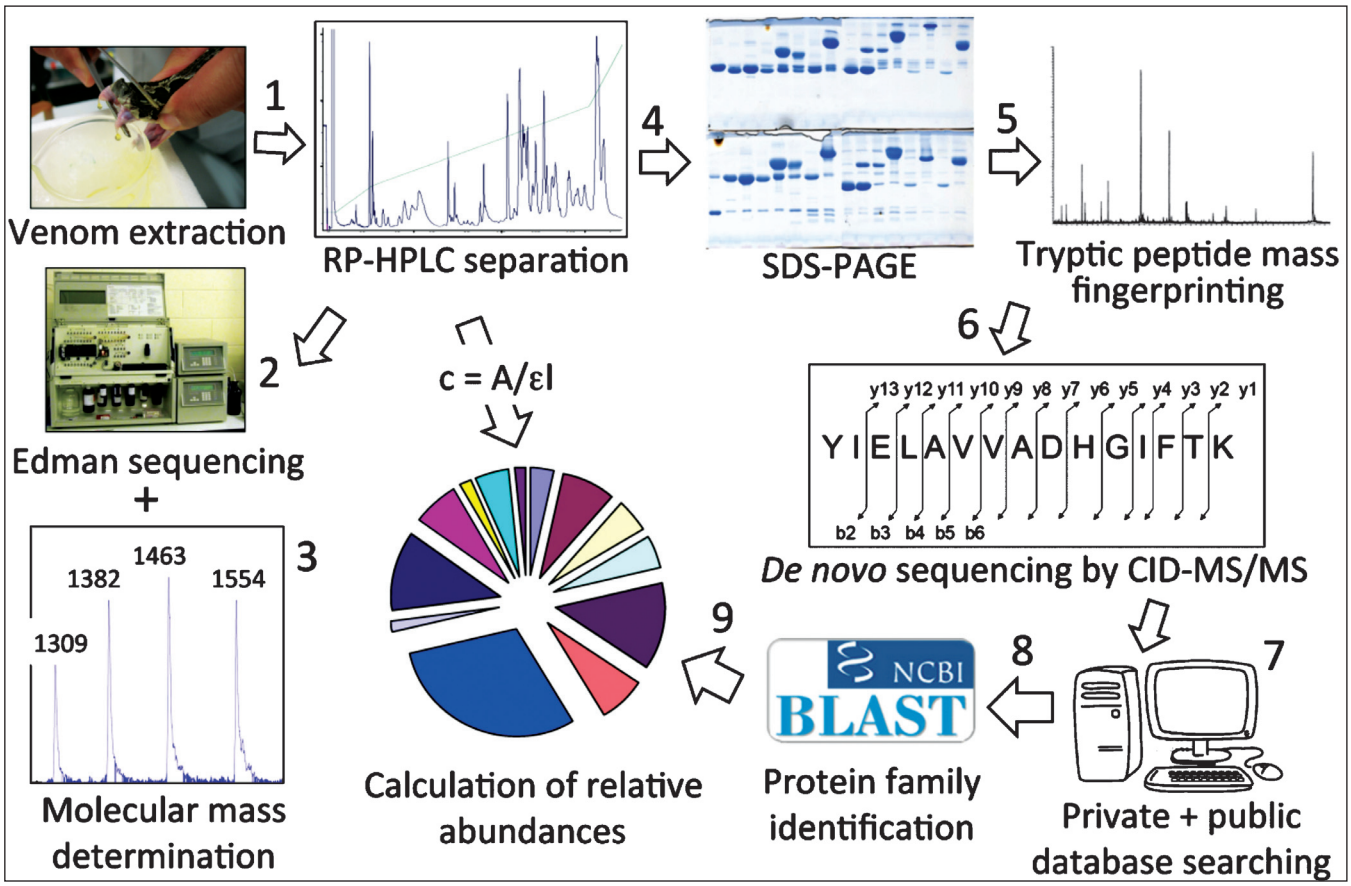

Fig. 7. Venomics workflow. The bottom-up venomics approach includes an initial step of fractionation of the crude venom by reverse-phase (RP) HPLC **(1)** followed by the initial characterisation of each protein fraction by means of N-terminal sequencing **(2)**, mass determination by MALDI- or ESI-MS **(3)**, and SDS-PAGE **(4)**. Tryptic peptides generated by in-gel digestion of electrophoretic bands are subjected to peptide mass fingerprinting **(5)**, and *de novo* peptide sequencing by collision-induced dissociation (CID)-tandem mass spectrometry (MS/MS) **(6)**. MS/MS-derived amino acid sequences are assigned to toxins/toxin families by database searching **(7)** and BLAST analysis **(8)**. Structural assignments and the relative abundance of the venom's toxin families are integrated into a pie chart **(9)**. Pie charts offer a simple way of displaying the toxin profile of a venom and represent an intuitive method for comparing quantitative data across different taxa.

antibodies in the antivenom are directed. By contrast, venom components that remain in the supernatant are those which failed to raise antibodies in the antivenom, or which triggered the production of low-affinity antibodies. These components can be easily identified by comparison of reverse-phase HPLC separation of the non-precipitated fraction with the HPLC pattern of the whole venom previously characterized by venomics. Second generation antivenomics[119] is an affinity chromatography protocol to investigate the immuno-capturing ability of immobilized $F(ab')_2$[120,121] or IgG[123]antibody molecules followed by the proteomic characterization of the bound and the non-bound venom components (Fig. 8). Antivenomics provides qualitative and quantitative information on both the set of toxins bearing antivenom-recognized epitopes and those toxins exhibiting poor immunoreactivity. Its ease of use, reproducibility, sensitivity, and low cost suggest the possibility that antivenomics might supplant the use of immunoassays and Western blots, the most popular techniques for assessing the immunoreactivity of antibodies. However, the immuno-chemical detection of blotted proteins provides a Yes/No response: a given protein is either recognized or not by the antivenom, and it is essentially a non-quantitative technique.

Despite its recent introduction, the usefulness and validity of antivenomics to complement the *in vivo* standard preclinical assays of neutralization of lethality and toxic activities by antivenoms has been extensively demonstrated[68,105,120-123]. Antivenomics thus provides a ground for rationalizing the paraspecificity of antivenoms thereby expanding their potential clinical range.

Current venomics analyses of Indian snakes of medical relevance

The common krait (*Bungarus caeruleus*) (Fig. 1a), the Indian cobra (*Naja naja*) (Fig. 2a), Russell's viper (*Daboia russelii*) (Fig. 3a), and the saw-scaled viper (*Echis carinatus*) (Fig. 4a), collectively called the "Big 4", are considered to be the major endemic venomous snakes responsible for fatality in the Indian subcontinent[124]. The efficient clinical management of snakebites requires timely administration of appropriate antivenoms that neutralize the numerous toxins contained in the snake venom. Antivenoms against the "big four", produced by several Indian firms (*http://apps.who.int/bloodproducts/snakeantivenoms/database/default.htm*) are available. However, detailed studies on the immunoreactive properties of these polyvalent antivenoms have not been performed. To complicate matters, the emergence of the hump-nosed pit viper (*Hypnale hypnale*) (Fig. 5a) and other species as snakes of medical significance in south-western India[36,124] , has rendered the "Big 4" obsolete and incomplete.

Snake venomics of the eastern Russell's viper (*Daboia siamensis*) and its relation to pharmacological activities have been addressed by Risch and coworkers[125]. *D. siamensis* is found in Burma, Thailand, Cambodia, parts of China, Taiwan and Indonesia[126,127]. The clinical manifestations of Russell's viper bite

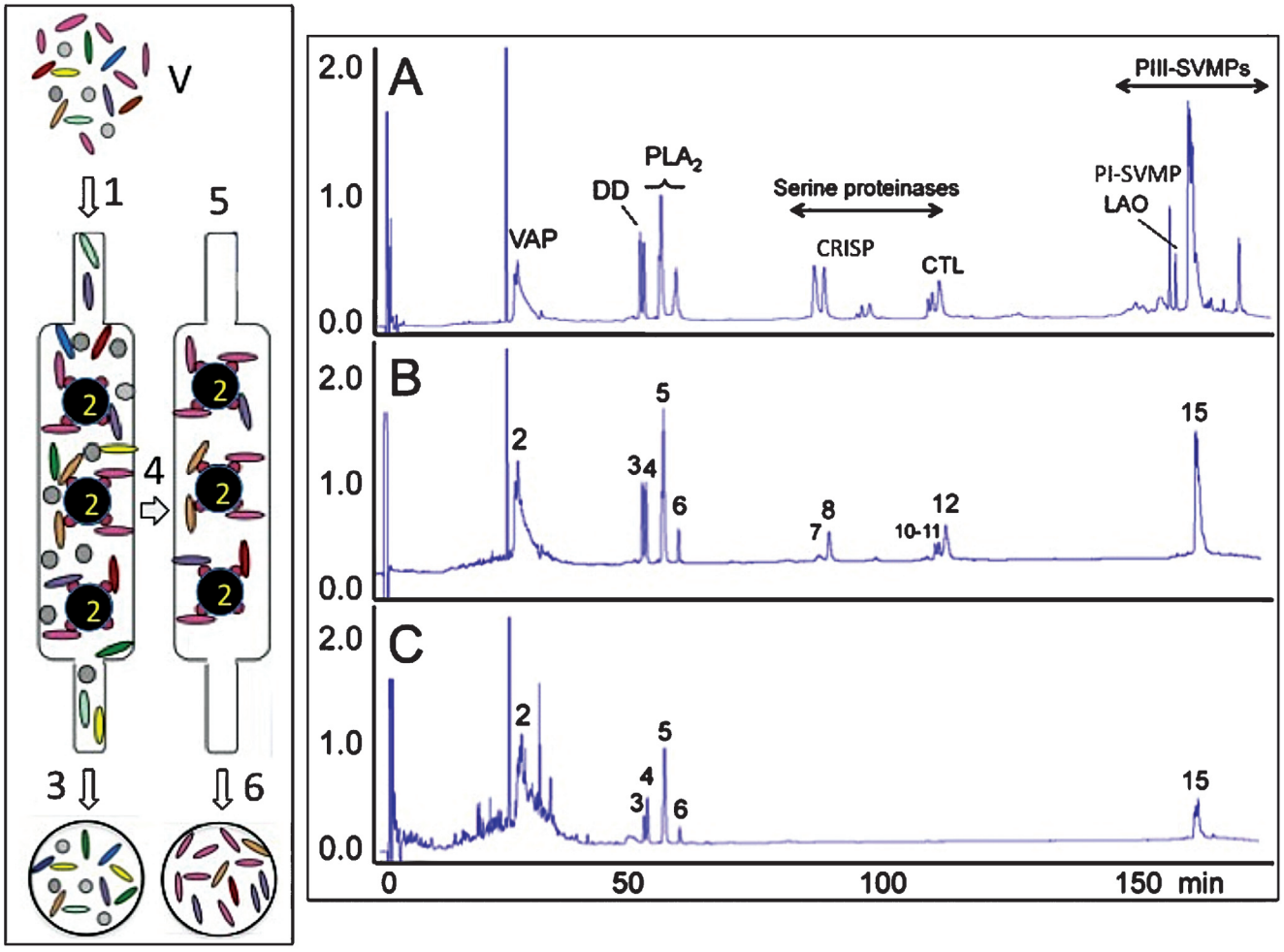

Fig. 8. Antivenomics workflow. Left panel, whole venome (V) is applied to an immunoaffinity column (1) packed with antivenom antibodies immobilized onto Sepharose beads (2). After eluting the non-binding fraction (3), the column is thoroughly washed with equilibration buffer (4) and the immunocaptured proteins eluted (6) with elution buffer (5). Right display, panels A-C show, respectively, reverse-phase chromatographic separations of the components of whole venom, the fraction retained and subsequently recovered from the antivenom affinity matrix, and the non-immunocaptured venom fraction. Proteins within the immunocaptured and the flow-through fractions are identified by the venomics approach schematically displayed in Fig.7.

vary from one region to another within Southeast Asia, including India[43,128-130]. Some studies on the biochemical properties of Russell's viper venom from eastern India that correlate with the distinct clinico-pathological manifestation in Russell's viper bites have been reported[130]. In particular, non-enzymatic peptides present in *Daboia russelii* (Eastern region) venom, and which render commercial polyvalent antivenom ineffective, have been detected[41]. The identity of these venom components has not been determined. In addition, there is a dearth of knowledge about the qualitative and quantitative protein composition of *D. russelii* venom across the Indian peninsula.

The Indian cobra, *Naja naja*, and the common krait, *Bungarus caeruleus* (Elapidae) cause severe neuromuscular paralysis as a result of the blockade of neuromuscular transmission. Toxins from cobra venom predominantly act postsynaptically, whereas those from krait venom mainly act presynaptically[131]. The common krait is regarded as the most dangerous species of venomous snake in the Indian subcontinent. Chemical and pharmacological characterization of toxic polypeptides from the venom of *B. caeruleus* have been long reported[131,132]. The major neurotoxic component of krait venom is β-bungarotoxin, a heterodimeric basic PLA_2 which has high affinity for presynaptic neuromuscular receptors[133]. However, a global picture of the toxin composition and geographical variability of *B. caeruleus* venom is lacking. Similarly, although there are 32 UniProtKB/TrEMBL entries of Indian cobra venom toxins, the relative contribution of these proteins to the whole venom remains undisclosed. Furthermore, regional variation in venom composition and toxicity has also been described for Indian *N. naja*[134,135]: eastern *N. naja* venom is neurotoxic (being PLA_2 molecules largely responsible for the toxicity) and procoagulant (*in vitro*, not clinically), whereas western region venom is myotoxic and anticoagulant (*in vitro*, not clinically)[132]. Region-specific neutralization of Indian cobra (*Naja naja*) venom by polyclonal antibody raised against the eastern venom has been published[42]. However, the identity of the geographically variable molecules and the immunoreactivity pattern of available antivenoms towards regional venoms deserve future studies.

A large proportion of the 71 *E. carinatus* venom protein sequences available in the UniProtKB/TrEMBL database (*http://www.expasy.org*) has been derived by venom gland transcriptome surveys using cDNA libraries constructed from ten wild-caught specimens of *E. carinatus sochureki* from Sharjah, UAE[136,137]. Their expression and relative abundance in the venom have not been addressed. The phylogeny of the medically important, and taxonomically unresolved, viper genus *Echis* has been investigated by Pook and colleagues[138]. The results showed that the populations of the genus fall into four main clades: the *Echis carinatus*, *E. coloratus*, *E. ocellatus* and *E. pyramidum* groups. The *E. carinatus* group includes haplotypes from India (*E. c. carinatus*), Pakistan and the northeastern Arabian Peninsula (*E. c. sochureki*) that form a cluster without clear phylogeographical structure. More basal sister haplotypes originate from western and southern India. Whether *E. c. sochureki* should be recognised as distinct from southern Indian *E. c. carinatus* at species or subspecies level remains an open question. Although the low divergence between northwestern Indian, Pakistani, northeastern Arabian and Central Asian *E. carinatus* populations indicated that these haplotypes first diverged approximately 0.9 Mya[138], the link between the transcriptomic data gathered from the *E. c. sochureki* specimens from Sharjah, UAE and the venom proteome composition of the Indian populations of *E. c. carinatus* (South Indian saw-scaled viper; peninsular India) remains obscure.

Snakes of the genus *Hypnale* are of local medical importance in Sri Lanka and southwestern India and pathological effects of the venom of Sri Lankan *Hypnale* in animals was the subject of some early studies[139]. Recently, Maduwage and colleagues[140] have investigated the biochemical and pharmacological properties of the venoms from the three *Hypnale* species in Sri Lanka. Cytotoxicity appeared to be the most potent effect of these venoms, which had similar reverse-phase chromatographic toxin profiles. Indian polyvalent antivenom raised against *Naja naja*, *Daboia russellii*, *Bungarus caeruleus* and *Echis carinatus* did not neutralize the venom effects[140]. Kumar and Sabitha[141] have also reported that available Indian anti-snake venom in central Kerala does not cover envenomation by *H. hypnale*. Cross-neutralization *in vitro* and in a rodent model of *H. hypnale* venom by polyvalent and monovalent antivenoms against Malayan pit viper (*Calloselasma rhodostoma*) venom, a sister taxon of *H. hypnale*[142], has been documented[143]. Establishing the molecular basis for this finding requires detailed venomics and antivenomics investigation.

Remarks and perspectives

An essential defining characteristic for a biological hypothesis is its ability to generate testable predictions. Developments in venomics and antivenomics are likely

to revolutionize the design and preclinical assessment of antivenoms by bringing to bear technologies that enable us to peel back the layers of imprecision that exist in current methodologies, and get to heart of the problem by being able to design and test new antivenom preparations developed using these dynamic platforms. Antivenomics represents a knowledge-based approach to help to design improved venom-based immunogen mixtures, and to predict paraspecific neutralization by antivenom preparations to the level of species-specific toxins. In India, antivenom production began at the Central Research Institute, Kasauli during the 1920s. There are now several producers manufacturing Indian polyvalent antivenoms, all of which follow a standard set out in the Indian Pharmacopoeia requiring products to neutralize 0.6 mg/ml *Naja naja* venom, 0.6 mg/ml *Daboia russelii* venom, 0.45 mg/ml *Bungarus caeruleus* venom and 0.45 mg/ml *Echis carinatus* venom. This potency is much lower than the standards that applied during the early 1950s when Indian antivenoms were required to neutralize 4.0 mg/ml *Daboia russelii* venom and 2.0 mg/ml *Naja naja* venom[144]. This low potency demands that patients be given initial doses of at least 10 vials. In some cases, considerably larger doses are administered. A study in Chandigarh found that patients received an average of 51.2 vials (512 ml) of Indian polyvalent antivenom for bites by *Naja* sp., and *Bungarus* sp., and an average of 32 vials (320 ml) to treat *Daboia russelii* or *Echis carinatus* envenoming. At the extreme, some patients received as many as 130 vials (1.3 litres) for bites by Russell's vipers; while up to 190 vials (1.9 litres) were administered after a neurotoxic snakebite[145]. Such high doses of immunoglobulin carry the risk of inducing severe dose-related adverse reactions, including anaphylaxis[146-148]. Clearly, new strategies are needed to meet the challenge of neutralizing large injected doses of venom without compromising patient safety. Production of higher potency antivenoms will be required. Some of the snake species that occur in India produce large amounts of venom; Asian cobras (*Naja* sp.) have yields ranging from 58-742 mg[149], kraits can produce 8-60 mg[150] and Russell's vipers venom yields a range from 21-268 mg[151]. A new antivenom, recently produced in Costa Rica for the treatment of Papuan taipan (*Oxyuranus scutellatus*) envenoming in Papua New Guinea, has addressed the problem of balancing antivenom potency and dose against the average venom yield (120 mg) of the species involved. A single 40 ml vial of antivenom, with a potency standard of at least 3 mg/ml and total protein of just 4.59±0.9 g/dl, should allow an initial treatment dose of 1 vial to neutralize at least 120 mg *O. scutellatus* venom[69]. Modern proteomic and antivenomic methods have been employed in the characterization of this venom and in the preclinical evaluation of this new antivenom. Such an approach could easily be used to design and produce a high potency polyvalent antivenom for use in India. The new antivenom could be evaluated preclinically and then subjected to clinical trial. This evidence might persuade Indian regulators to adopt new specifications for antivenom in the Indian Pharmacopoeia.

Acknowledgment

Funding for the projects (to JJC) was provided by grant BFU2007-61563 and BFU2010-17373 from the Ministerios de Educación y Ciencia and Ciencia é Innovación, Madrid; CRUSA-CSIC (2007CR0004 and 2009CR0021); and CYTED (206AC0281). Travelling between Spain and Costa Rica was financed by Acciones Integradas 2006CR0010 between CSIC and the University of Costa Rica (UCR).

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