**Causes and consequences of snake venom variation**

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**Abstract**

Snake venoms are mixtures of toxins that vary extensively between and within snake species. This variability has serious consequences for the management of the world’s 1.8 million annual snakebite victims. Advances in ‘omic’ technologies have empowered toxinologists to comprehensively characterise snake venom compositions, unravel the molecular mechanisms that underpin venom variation, and elucidate the ensuing functional consequences. In this review, we describe how such mechanistic processes have resulted in suites of toxin isoforms that cause diverse pathologies in human snakebite victims, and we detail how variation in venom composition can result in treatment failure. Finally, we outline current therapeutic approaches designed to circumvent venom variation and deliver next-generation treatments for the world’s most lethal neglected tropical disease.

**Snake venom and snakebite**

Venom is a remarkable evolutionary innovation found scattered across the animal tree of life [1]. Due to their diverse evolutionary histories and consequent variability, animal venoms have proven to be fascinating models for understanding a number of fundamental processes, including gene duplication, genotype-phenotype mapping, convergent evolution, and cell and tissue development [2-6], while the bioactivities of many toxins make them promising leads for the discovery of new human therapeutics [7]. The most well-studied venom systems are those of snakes. All “advanced snakes” (superfamily: Colubroidea) have a pair of homologous oral venom glands located behind the eye on either side of the upper jaw [8, 9]. These glands are connected to ducts that transfer the secreted venom to the base of morphologically diverse teeth that are often referred to as ‘fangs’. For many snakes, including those of greatest medical importance, these fangs are found at the front of the mouth, contain an enclosed venom canal, and are a highly efficient mechanism that facilitates the rapid injection of a bolus of venom. Although venomous snakes predominantly use their venom to assist with the acquisition of prey, they may also deploy it in defensive bites to deter potential predators and aggressors, including people.

The consequences of such human snakebites can be severe. Current estimates suggest that venomous snakes cause up to 138,000 deaths worldwide each year, and perhaps as many as 500,000 additional cases of venom-induced morbidity [10]. Snakebite envenomings predominantly affect the rural impoverished populations of the tropics, and consequently the World Health Organization (WHO) has listed snakebite as a priority **neglected tropical disease (NTD)** (see Glossary) [11]. The pathological effects of snakebite are diverse and can include neuromuscular paralysis (**neurotoxicity**), haemorrhage and coagulopathy (**haemotoxicity**), and/or local swelling, blistering, and tissue necrosis (**cytotoxicity**) around the bite site [10]. These highly variable clinical signs are a direct consequence of variation in the toxin components found in venom - such variation can be extensive, and occurs both inter- and intra-specifically [12-18]. This variation also has a direct impact on the efficacy of snakebite treatments (**antivenom**), resulting in different antivenoms having to be manufactured against the venoms of distinct snake species [19].

Despite such complexity, recent advances in ‘omic’ technologies (e.g. proteomics, transcriptomics) have enabled the rapid characterisation of the toxin components found in the venom of over 125 medically-relevant species [20]. Here, we outline how this data has transformed our understanding of the processes that have generated snake **venom variation**, and the consequences of such variation in the context of snakebite pathology and treatment. We also highlight how the rational application of venom composition data will enable the development of broadly effective therapies for snakebite envenoming, which is an essential step in mitigating the devastating effects this NTD inflicts upon the vulnerable victims of the tropics [21].

**Ecology drives inter- and intra-specific snake venom variation**

Venom is a functional trait used by one organism to interfere with the homeostatic processes of another, generally to facilitate feeding or deter predators or competitors [18]. Venom is therefore intrinsically ecological; a trait that mediates the outcome of interactions between two or more organisms [22]. “Venomous” is not synonymous with “dangerous”, and the majority of venomous organisms, including snakes, pose no threat to humans, either because they rarely, if ever, envenom humans, or because the consequences of envenoming are trivial. Indeed, venom is a widespread trait amongst the “advanced snakes”, but almost all “medically important” snakes (those capable of causing harm to humans via envenoming) are members of one of only three clades – the families **Elapidae** (cobras, mambas, sea snakes, taipans, and their relatives) and **Viperidae** (vipers and pit vipers, including adders, rattlesnakes, and their relatives), and the subfamily Atractaspidinae (mole vipers/stiletto snakes).

As venom is an ecologically important functional trait for venomous snakes, its composition and activity co-evolves with the physiology of the prey animals and, perhaps to a certain extent, the predators it is deployed against [23-27]. Although primates have been predators of snakes since time immemorial and envenoming of humans by snakes is almost exclusively defensive, it is unlikely that humans have exerted any major defensive selective pressure on snake venoms. Rather, human envenomings are best viewed as collateral damage of the chemical arms race taking place between venomous snakes and their (mammalian) prey.

Venom variation exists at multiple phylogenetic levels and is a consequence of both the contingent evolutionary histories of divergent lineages of venomous snakes and direct selection on the ecological deployment of specific toxins. At the deepest and most general level, the venoms of (e.g.) elapid and viperid snakes are different (see Box 1 for more details) – certain families of toxins have been recruited and utilised or have become central components of the venom of one lineage but not the other [28]. Similarly, broad differences can exist in venom compositions between genera within each family, and between species within each genus (e.g. [13, 20, 29]). This much has long been understood and is why a number of antivenom manufacturers have developed multiple products for use in a given region (e.g. viper- and elapid-specific antivenoms).

More recently, the extent of venom variation within species has begun to be recognised. Such variation exists between populations (i.e. regional variation) and between age/size classes [12, 14-17]. As venom is a dynamically evolving ecological trait, it stands to reason that whenever groups differ in their feeding ecology, there may be a corresponding difference in their venom composition. Juvenile snakes often consume different prey from adults of the same species, and may also exhibit different foraging strategies and prey-handling behaviour, e.g., juveniles may be nocturnal, whereas adults are more diurnal; juveniles may employ a bite-and-hold strategy, whereas adults may ‘bite and release’, etc. The dynamism of venom evolution, which has been documented at the molecular level (see below), is further evidenced by the existence of regional variation, which may be linked both to ecological variance amongst populations and to **neutral evolution**, which may be pervasive in venom systems and work in tandem with **positive selection** [30]. This dynamism itself generates another prediction based on evolutionary first principles – for a trait to evolve rapidly, there must be considerable heritable diversity *within* populations [31]. This prediction of variation in venom amongst adult members of a single population is only beginning to be investigated, but preliminary evidence suggests it will likely be confirmed [32, 33].

**The processes that underpin venom variation**

Venom toxin encoding genes originate from genes that code for endophysiological proteins (e.g., salivary, immunological and pancreatic proteins, etc) [34]. Numerous mechanisms have been proposed to explain the origin and diversification of toxins and, thereby, the evolution of venom variability across snakes. These include gene duplication, domain loss, evolutionary tinkering of expression levels, alternative- and trans-splicing, and rapid evolution under positive Darwinian selection [35] (Figure 1).

Gene duplication, whichplays a key role in the evolution of phenotypic complexity and functional innovation, has been implicated in the diversification of venom. Venom protein encoding genes are theorised to undergo extensive duplications and evolve under a ‘birth and death’ model of evolution [36]. According to this model, repeated duplication events lead to the origin of new copies, most of which undergo pseudogenization into dysfunctional forms over time, while some subsequently evolve novel functions and are retained [37]. Although some assumptions of the original “birth and death” model are questionable [38], gene duplication has led to the formation of **multi-locus toxin gene families** with extraordinary structural and functional diversity [2, 29, 39] (see below). Gene duplication does not always introduce novelty though, and can also underpin increased expression levels. Concerted evolution, in contrast to the ‘birth and death’ model, maintains high levels of sequence conservation amongst duplicates through recombination, as a strategy to increase expression levels of the encoded toxin [40]. Although concerted evolution is yet to be noted in snakes, recurrent snake venom gene duplications do facilitate increased expression of the encoded toxin types [41]. Since relative differences in the expression level of venom components can considerably alter the underlying toxicity [16], shifts in gene expression seem likely to also underpin evolutionary adaptations.

Stochastic degeneration of genes has also been reported to result in significant evolutionary consequences [42]. For example, several lineages of rattlesnakes have lost phospholipase A2 (PLA2) neurotoxin genes, which were once present in their common ancestor, and have shifted towards a more haemotoxic venom profile [2]. Partial degeneration of gene segments have also been documented in venom protein encoding genes. Domain losshas been shown to mediate toxin **neofunctionalization** (Figure 1), with notable examples including truncations of snake venom metalloproteinases (SVMP) that led to the origin of structurally-functionally diverse subclasses in Viperidae snakes [43, 44], and the evolution of potent neurotoxins from haemorrhagic precursors in the olive whip snake (*Psammophis mossambicus*) [45]. Recent studies have also highlighted the role of alternative- and trans-splicing in generating snake venom diversity (Figure 1). Genome-wide surveys of the transcriptional repertoire have led to the identification of alternative splicing in genes that encode SVMPs, snake venom serine proteases (SVSP) and vascular endothelial growth factors (VEGF), and trans-splicing in SVSP genes [42, 46].

Rapid evolution under positive selection has been widely documented to facilitate adaptations in the natural world. Many toxin-encoding genes are known to rapidly accumulate **non-synonymous substitutions** in their protein encoding regions, relative to **synonymous substitutions**. Three-finger toxins (3FTx), which are amongst the most gene-rich of the toxin superfamilies found in snake venoms, have predominantly evolved under the influence of positive selection, and this process has generated remarkable structural and functional diversity (see below). Similarly, many other toxin classes, including SVMP, PLA2 and cysteine-rich secretory proteins (CRISP) have experienced a significant influence of positive selection [43, 47-49].

While venom protein superfamilies are generally characterised by extreme conservation of structural residues, particularly disulfide bridge-forming cysteines that confer structural stability, they accumulate variations in other regions [50]. The acquisition of variation in surface-exposed regions and loops, for example, is known to facilitate the rapid diversification and neofunctionalization of toxins [51, 52]. Over evolutionary time, venom proteins accumulate variations in an episodic fashion. While **purifying selection** governs the conservation of structurally and functionally important residues in potent toxins, positive selection accelerates the rate of change mostly when significant shifts in ecology and environment are experienced [53]. In summary, although a number of mechanisms may contribute, the processes of gene duplication and positive selection appear to be the predominant mechanisms for generating diversity in snake venoms.

**Functional consequences of venom variation**

As a result of the various processes described above, most snake venoms contain high numbers of related toxin isoforms. Typically, the toxins encoded by such multi-locus gene families are the most abundant of those found in venom, and examples include the 3FTxs, PLA2s, SVMPs and SVSPs [20]. Notably, all of these toxin families exhibit evidence of multifunctionality [54]. The 3FTxs, which are dominant venom proteins in most elapid snake venoms, are a classic example of this. Here, gene duplication, coupled with accelerated evolution, has resulted in a suite of toxin isoforms, which share a structure consisting of multiple β-hairpin loops extending from a disulfide bond-stabilised hydrophobic core, but which also exhibit considerable variation in the protruding exposed loops that interact with target-site receptors (Figure 2A) [52]. Many 3FTxs exert neurotoxic effects by interacting with ion channel receptors, including nicotinic acetylcholine receptors, muscarinic receptors, potassium channels, calcium channels, and sodium channels (Figure 2B) [54]. The combined action of different 3FTxs found in the same venom likely results in additive or synergistic antagonising effects, and can cause neuromuscular paralysis and respiratory failure in envenomed snakebite victims [10]. However, other 3FTxs have dramatically distinct functional activities; including those that contribute to local tissue damage via direct cytotoxic effects, or those that interact with haemostatic components, such as Factor X and platelets [54].

Other venom toxin families exhibit similarly high degrees of functional diversity. These include diverse SVMP isoforms that act in concert to induce haemorrhage via the destruction of basement membrane components, while others cause coagulopathy via direct activation or cleavage of blood clotting factors [55]. Many other toxin families also contribute to systemic pathologies by acting synergistically on relevant physiological targets, such as certain PLA2 toxins that antagonise presynaptic potassium channels, or SVSPs that activate Factor V or degrade fibrinogen [54, 55].

Crucially, the presence, absence, and relative abundances of the numerous different toxin isoforms found in venom is highly variable across snake species. Thus, not every venom will have every functionally-diverse isoform from each toxin family. However, due to variable lineage-specific processes (e.g. gene duplication and loss, rates of evolution, expression level variations, etc), each species harbours its own mixture of toxins. Consequently, the ensuing pathologies observed following human snakebites are also highly variable. These can range from the predominant systemic neurotoxicity observed following bites by many elapid snakes (e.g. kraits, *Bungarus* spp.; mambas, *Dendroaspis* spp.), to particularly complex multi-pathological envenomings following bites by certain viperid snakes, like Russell’s vipers (*Daboia* spp.) [10, 56]. Perhaps the most extreme clinical examples of intra-specific venom variation are bites by the Mojave rattlesnake (*Crotalus scutulatus*) in the Southwestern USA, which result in considerably different pathologies (e.g. haemotoxic vs neurotoxic) at different ends of a cline covering only tens of miles [12, 57].

**Therapeutic consequences of venom variation**

Such functional variation makes snake venoms challenging drug targets, and has major consequences for the efficacy of snakebite treatments. Antivenoms are made by hyperimmunising animals (typically equines or ovines) over prolonged periods of time with venom from a number of snake species found in a particular geographical region, before purifying the resulting antibodies (immunoglobulin G or fragments thereof) and formulating them for intravenous delivery to snakebite victims [10]. Consequently, the specificity and efficacy of these therapeutics are inherently linked to those venoms used for immunisation, and toxin variation results in reduced recognition and neutralisation of toxins from different venoms [19]. In addition, different toxin classes have different levels of antigenicity, with low-molecular weight toxins generally being considered less immunogenic than their high-molecular weight counterparts. This leads to suboptimal antibody responses in the production animal and, therefore, limited efficacy of antivenoms against toxic, but non-immunogenic venom components [58, 59].

Despite these issues, there are some examples where antivenoms appear to exhibit cross-neutralising capabilities against distantly related snake venoms, particularly where species have broadly similar venom compositions as the result of shared ancestry [60], or coincidently as the result of convergent evolution of venom compositions [29]. However, venom variability often undermines cross-species efficacy, and can result in grave clinical consequences (Box 2). In sub-Saharan Africa, antivenom manufactured against the Indian saw-scaled viper (*Echis carinatus*) was used for treating bites by the congeneric West African saw-scaled viper (*E. ocellatus*). Due to variation in toxin constituents among saw-scaled vipers [13], these antivenoms proved to be highly ineffective, which resulted in case fatality rates increasing from <2% with species-appropriate antivenom to 10-12% [61, 62]. In South Asia, antivenom manufactured using Indian Russell’s viper (*D. russelii*) venom exhibits low neutralising potencies against venom from Bangladeshi populations of the same species, suggesting that perhaps 5-10 times the normal treatment dose might be needed for effective treatment [15]. Contrastingly, antivenom made in Thailand against the congeneric species *D. siamensis* appears to exhibit considerable cross-recognition of venoms from the same species found in distinct geographical locales [63]. In combination, these observations suggest that venom variation makes predictions of antivenom efficacy extremely problematic, although the application of ‘antivenomic’ approaches that quantify the depletion of chromatographically-separated and mass spectrometrically-identified venom toxins by antivenoms are gaining traction as a predictive technology to help address these challenges [64]. While such venom compositional data can undoubtedly help to rationally inform appropriate antivenom use, a severe lack of standardised efficacy data at both the preclinical and clinical level [65, 66] currently undermines the development of a robust framework for predicting cross-species antivenom efficacy.

Ultimately, venom variation necessitates the manufacture of many different antivenoms worldwide, each with a restricted geographical focus. This has led to a fragmented, largely unsustainable, market that has resulted in the commercial withdrawal and restricted availability of many antivenoms [67], despite these therapeutics being categorised as essential medicines by the World Health Organization (WHO). There is therefore an urgent, compelling need to design new snakebite therapeutics capable of circumventing the limitations associated with snake venom variation.

**Can novel snakebite therapeutics circumvent venom variation?**

Due to their animal origin, conventional antivenoms have many limitations, including undefined product compositions, batch-to-batch variation, a propensity to elicit adverse reactions in recipients, and typically limited cross-species efficacies due to venom variation [10, 68, 69]. However, the recent and widespread utilisation of ‘omic’ technologies has enabled antivenom researchers to better understand the composition and variability of snake venoms, which in turn has better informed the identification of toxins requiring neutralisation [70, 71].

**Next-generation antivenoms** currently under development encapsulate a range of different modalities, including monoclonal antibodies and antibody fragments, **nanobodies**, small molecule inhibitors, **aptamers** and **peptides**, metal ion chelators, and antivenoms manufactured using synthetic immunogens [72]. While the latter products are not fundamentally different from conventional antivenoms, as they are still derived from animal polyclonal antibodies [73], the other modalities are entirely different in their composition and manufacture. Although the manufacture of such next-generation antivenoms is not, in itself, dependent on venoms, antivenom formulation and dosing are highly dependent on knowledge of venom composition and toxicity for the indicated snake species.

This necessitates systematic research in snake genomics, (venom gland) transcriptomics, and (venom) proteomics, coupled with informative analyses of which toxins are of greatest pathological relevance. A successful example of this interdisciplinary approach was the demonstration that immunising horses with a recombinantly expressed short-chain ɑ-neurotoxin, designed as the consensus sequence of important 3FTx isoforms found in different elapid snake venoms, resulted in an experimental antivenom with broad *in vivo* neutralising capability against the neurotoxic effects of venoms from distinct elapid snake species [74].

Researchers are also using knowledge of venom composition to rationally select oligoclonal or monoclonal antibodies (or fragments thereof) as potential new snakebite therapeutics. For example, it was recently demonstrated that oligoclonal mixtures of recombinant immunoglobulin G antibodies could be used to neutralise the dendrotoxin-mediated *in vivo* neurotoxicity of black mamba (*D. polylepis*)venom [75]. Crucially, this antibody mixture was rationally designed based on prior proteomic and toxicity assessments of the venom [76], illustrating the importance of in-depth knowledge of venom composition in the development of future **recombinant antivenoms**.

In an attempt to circumvent venom variation by providing generic inhibition of specific toxin classes, researchers have also explored the utility of using small molecules as toxin inhibitors, with some notable successes against SVMP and PLA2 toxins. For example, it was recently reported that the metal ion chelator and licensed medicine 2,3-dimercapto-1-propanesulfonic acid (DMPS) provides *in vivo* protection against the local and systemic effects of the SVMP-rich venoms of saw-scaled vipers (*Echis* spp.) [77]. Researchers have also demonstrated the utility of the Phase 2-approved peptidomimetic small molecule SVMP inhibitors, batimastat and marimastat, which have been shown to broadly neutralize multiple viperid SVMPs both *in vitro* and *in vivo* [78-80]. Moreover, several recent studies have demonstrated the highly promising utility of a repurposed Phase 2-approved PLA2 inhibitor, varespladib, as a future snakebite therapeutic, as this molecule has been demonstrated to broadly neutralise PLA2-mediated pathologies caused by multiple different elapid and viperid venoms [81, 82]. Finally, it was recently described that a therapeutic combination of the SVMP inhibitor marimastat and the PLA2 inhibitor varespladib provide broad preclinical efficacy against lethality caused by a range of geographically-diverse viper venoms [80].

Ultimately, next-generation snakebite therapeutics may not necessarily be based on only one anti-toxin format (e.g. antibodies or small molecule inhibitors), but instead seem likely to be composite products comprising mixtures of different modalities to ensure breadth of toxin neutralisation across numerous distinct snake venoms (Figure 3) [80, 83, 84]. The recent gains described above demonstrate that this is likely achievable in the future as long as sufficient knowledge about venom composition and variation is at hand. This further emphasises the need for continued toxinological research into venom variation (see Outstanding Questions) and underlines the importance of bridging basic and applied sciences for the benefit of the world’s impoverished snakebite victims.

**Concluding Remarks**

Toxin-encoding genes are members of some of the most dynamically evolving gene families in nature, and detailed studies of their molecular evolution can yield knowledge that is broadly applicable to the deepest questions in biology, particularly those concerning the origins of novel functions [2-5]. Whilst this evolutionary dynamism makes toxins an attractive research subject for molecular biologists, it has led to the creation of a pharmacologically-diverse suite of toxic molecules that are the causative agents for the monumental clinical burden of snakebite envenoming observed today [10]. Venom variation, at both the inter- and intra-specific levels, results in diverse snakebite envenoming pathologies and presents a significant challenge to the development of broad-spectrum snakebite therapeutics [12-17]. Understanding the evolutionary processes generating this variation, and its functional and clinical consequences, is therefore of paramount importance. However, we still lack a comprehensive understanding of the interplay between predator and prey ecology influencing the evolution of venom variation, and a current lack of genomic resources for snakes hamper our interpretations of the varying roles that different molecular mechanisms of gene evolution play in this regard (see Outstanding Questions). Nonetheless, progress is being made through an interdisciplinary research framework underpinned by other ‘omic’ technologies, and combining perspectives and methods from evolutionary biology, immunology, and clinical toxinology. Current priorities for the application of this diverse data include robustly predicting the efficacy of existing antivenoms against untested snake species, and identifying those toxins, found amongst numerous diverse isoforms present across all medically important snakes, that are of greatest importance to neutralise. Despite these challenges, recent research efforts are already beginning to yield valuable insights that are now being applied to the design and development of next-generation snakebite therapeutics. Particularly promising approaches include the utilisation of monoclonal antibodies and repurposed small molecules that exhibit broad-spectrum neutralising capacities against taxonomically widespread and clinically relevant toxin families, such as 3FTxs, dendrotoxins, PLA2s, SVMPs, and SVSPs [75, 77-82]. Future broad-spectrum therapeutics will, thus, likely be developed by combining mixtures of these modalities in hybrid antivenom products. Much work remains to be done to strengthen our understanding of snake venoms as drug targets and to settle on the most optimal strategies for developing improved snakebite envenoming therapeutics, including identifying how many of these molecules are required to provide broad neutralisation of diverse snake venoms [68, 80, 83, 85]. However, recent achievements resulting from the mutually enlightening relationship between evolutionary and clinical toxinology provide a way forward that may, in the near future, help save many thousands of lives and ease the burden of morbidity caused by snakebite envenoming in the developing tropical world.

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**Text Boxes**

**Box 1. Toxin gene family expansion and coevolution with delivery mechanisms**

Venom composition varies as a consequence of contingent evolutionary history and direct functional selection. For example, specific families of toxin genes have undergone extensive expansion and neofunctionalization in specific snake lineages. Three-finger toxins (3FTxs) are a major component of the venom of elapid snakes and this gene family has expanded considerably via lineage-specific duplications, which have facilitated the emergence of multiple novel functions in addition to the ancestral activity of neurotoxic antagonism of nicotinic acetylcholine receptors [52, 86]. Contrastingly, in viperid snakes, it is the snake venom metalloproteinases (SVMP) [17] and group II phospholipase A2 (PLA2) [38] toxin gene families that have undergone lineage-specific expansions, again facilitating the emergence of multiple novel activities. The multiple duplication events within these gene families result in redundant arrays of genes that form neofunctionalization hotspots [2, 17, 44, 87, 88]. In both the elapid and viperid snake lineages, gene expansions and rapid sequence diversifications have occurred following the evolution of front-fanged, high-pressure delivery systems, highlighting the coevolutionary relationship that exists between toxin genes and venom delivery mechanisms [38, 52].

**Box 2. Timeworn Indian antivenoms**

Of the 300 species of Indian snakes described to date, 60 are capable of inflicting clinically significant envenomings in humans. However, the ‘big four’ - the Indian cobra (*Naja naja*), Russell’s viper (*Daboia russelii*), common krait (*Bungarus caeruleus*) and saw-scaled viper (*Echis carinatus*) - are responsible for the vast number of snakebite-related deaths and disabilities. While historically the Indian Government estimated snakebite mortality to be low (e.g. 948 deaths in 2017), a comprehensive survey in 2011 projected that the actual burden of snakebite equated to 46,000 deaths and 140,000 cases of morbidity - making India the world’s snakebite hotspot [89].

To combat snakebite in India, polyvalent antivenom is produced by six antivenom manufacturers against the venoms of the ‘big four’ snakes. However, nearly all antivenom manufacturers source venom from a single geographical population of these snakes, and thus there are grave concerns that intra-specific venom variation renders them less effective for treating bites in other parts of the country. Experimental evidence of this was recently demonstrated by an *in vitro* and *in vivo* evaluation of the efficacy of commercial antivenoms in countering the toxicities inflicted by north Indian population of *B. caeruleus* [16]. The tested commercial antivenom failed to meet the marketed neutralising claim of antivenom potency, highlighting the negative impact of geographic venom variability on snakebite therapy.

In addition to these concerns, it is apparent that many other snake species are capable of inflicting human mortality and morbidity in India. These ‘neglected many’ are common in certain parts of the country that lack their ‘big four’ congeners, and include various species of cobra (*N. kaouthia; N. oxiana*)*,* kraits (*B. sindanus*; *B. fasciatus; B. niger*), and vipers (*E. c. sochureki; Hypnale hypnale*). Despite being medically important, antivenoms are not manufactured against them, and thus clinicians are forced to rely on therapeutics designed to neutralise venom of the ‘big four’ snakes. This is, however, despite venom characterisation of the ‘neglected many’ revealing that their venom compositions and potencies are remarkably distinct [16]. *In vitro and in vivo* antivenom efficacy testing revealed that the existing Indian antivenoms have, at best, low efficacy against the venoms of the ‘neglected many’, and at worst, fail to neutralise venoms from certain populations of some of these species [16]. The likely consequences of this are treatment failure [90] and the delivery of dangerously high volumes of ineffective, yet financially costly, antivenom to impoverished snakebite victims.

**Figure legends**

**Figure 1. The molecular and evolutionary mechanisms that underpin the origin and diversification of snake venom toxins.** This figure depicts various evolutionary mechanisms that underpin the origin and diversification of snake venom coding genes. Here, introns are shown in grey, while exons are depicted in various colours. Following their origin from (endo)physiological homologues (P) via (1) duplication, snake venom coding genes (V) rapidly accumulate variation under the influence of (2) positive Darwinian selection. On rare occasions, this process results in (3) the origin of novel functions, while more commonly leads to (4) pseudogenization/degeneration. Snake venom diversity can also be generated via (5) alternative- and (6) trans-splicing, while increased expression can be achieved through (7) repeated gene duplications.

**Figure 2. The structural and functional diversity of three-finger toxins (3FTxs).** **A)** Structural model for a ‘typical’ 3FTx, the short-chain ɑ-neurotoxin cobrotoxin (PDB: 1COE, from *Naja atra*), highlighting the multiple β-hairpin loops extending from the disulfide bond-stabilised hydrophobic core. Disulfide bond numbers are coloured red. **B)** Via the processes of gene duplication and positive selection, 3FTxs have diversified from a plesiotypic form found in basal henophidian snakes (boas and pythons) into a paralogous suite of functionally diverse toxins in “advanced snakes”, many of which act on sites at the neuromuscular junction to cause neuromuscular paralysis. Homology models for various subclasses of 3FTx are displayed, with their variable disulfide bond numbers coloured red, and their differential sites of action at the neuromuscular junction shown. 1) Calliotoxin activates the voltage-gated sodium channel, Nav1.4; 2) Calciseptine selectively blocks L-type calcium channels; 3) Fasciculins exert inhibitory activities against acetylcholinesterase; 4) Muscarinic 3FTxs antagonise muscarinic acetylcholine receptors (mAChR); 5) Both short-chain (top) and long-chain (bottom) ɑ-neurotoxins antagonise a variety of different nicotinic acetylcholine receptor (nAChR) subtypes. Note that there are a number of other, functionally distinct, 3FTxs that are not shown here (e.g. cytotoxins, anticoagulant 3FTxs, etc).

**Figure 3. Conceptual representation of next-generation antivenoms as hybrid products comprising mixtures of antibodies, antibody fragments, and small molecule inhibitors.** These different modalities have different pharmacodynamics and pharmacokinetics, which may be suitable for neutralising different families of venom toxins with distinct functions and toxicokinetics. Note that this schematic is all encompassing and that future hybrid products seem likely to contain a small number of the different modalities presented, rather than all of them simultaneously. SVSP, snake venom serine proteases; 3FTx, three-finger toxins; PLA2, phospholipases A2; SVMP, snake venom metalloproteinases. Figure courtesy of: Tulika (Technical University of Denmark).

**Glossary**

**Antivenom:** Conventional snakebite therapies that consist of polyclonal antibodies purified from plasma/serum of equines/ovines hyperimmunised with snake venom/s.

**Aptamer:** An oligonucleotide that binds to a specific target molecule.

**Cytotoxicity:** The pathological consequences of venom cytotoxins acting predominantly at the region around the bite site, often resulting in swelling, blistering, and local tissue necrosis.

**Elapidae:** A widely distributed, medically important, family of front-fanged venomous snakes (“elapids”) that often cause systemic neurotoxicity, and which includes cobras, mambas, kraits, coral snakes, taipans, and relatives.

**Haemotoxicity:** The pathological consequences of venom haemotoxins acting on blood vessels and components of the coagulation cascade, often resulting in hypotension, systemic haemorrhage, and/or consumption coagulopathy.

**Multi-locus gene family:** A gene family consisting of multiple copies that have arisen due to recurrent tandem duplications of the ancestral gene.

**Nanobody:** A single-domain antibody, typically of camelid origin, consisting of a single monomeric variable antibody domain (antibody fragment). Similar to a whole antibody, a nanobody can bind selectively to a specific antigen.

**Neglected tropical diseases (NTDs):** A diverse group of communicable and non-communicable diseases that are common in low-income populations of Africa, Asia, and the Americas.

**Neofunctionalization:** The acquisition of a new protein function as the result of an accumulation of mutations in duplicated genes.

**Neurotoxicity:** The pathological consequences of venom neurotoxins acting on neuromuscular junctions, often resulting in descending neuromuscular paralysis and respiratory failure.

**Neutral evolution:** In molecular evolutionary terms, this is a mode of evolution where genes accumulate (nearly) equal proportions of synonymous (changes in nucleotides that do not alter the encoded amino acid residue) and non-synonymous (changes in nucleotides that alter the coded amino acid) substitutions.

**Next-generation antivenom:** Future snakebite therapies that may consist of recombinantly expressed monoclonal antibodies, antibody fragments, alternative binding proteins, and/or small molecule inhibitors, as well as plasma/serum-derived antivenoms manufactured with the use of recombinant toxins (or fragments thereof) as immunogens.

**Non-synonymous substitutions:** Mutations in the nucleotides that change the encoded amino acid

**Peptide:** A short chain of amino acids.

**Positive selection:** Also known as positive Darwinian selection, it is the force of natural selection that favours the accumulation of non-synonymous substitutions over synonymous substitutions, and results in the diversification of gene sequences across evolutionary time.

**Purifying selection:** A force of natural selection that favours the accumulation of synonymous substitutions over non-synonymous substitutions, resulting in the conservation of gene sequences across evolutionary time.

**Recombinant antivenom:** Antivenom based on recombinantly expressed antibodies or antibody fragments. Recombinant antivenoms can comprise either monoclonal, oligoclonal, or polyclonal antibodies.

**Synonymous substitutions:** Changes at the nucleotide level that do not alter the resulting amino acid

**Venom variation:** Relative differences in the composition and/or abundance of toxins in the venoms of closely/distantly related animals or their geographically disparate populations.

**Viperidae:** A widely distributed, medically important, family of front-fanged venomous snakes (“vipers”) that often cause systemic haemotoxicity, and which includes pit vipers, vipers, adders, and relatives.