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REVIEW

Proteomic analysis reveals geographic variation in venom composition of Russell's Viper in the Indian subcontinent: implications for clinical manifestations post-envenomation and antivenom treatment

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ABSTRACT

Introduction: The Russell's Viper (RV) (*Daboia russelii*), a category I medically important snake, is responsible for a significant level of morbidity and mortality in the Indian sub-continent.

Areas covered: The current review highlights the variation in RV venom (RVV) composition from different geographical locales on the Indian sub-continent, as revealed by biochemical and proteomic analyses. A comparison of these RVV proteomes revealed significant differences in the number of toxin isoforms and relative toxin abundances, highlighting the impact of geographic location on RVV composition. Antivenom efficacy studies have shown differential neutralization of toxicity and enzymatic activity of different RVV samples from the Indian sub-continent by commercial polyvalent antivenom (PAV). The proteome analysis has provided deeper insights into the variation of RVV composition leading to differences in antivenom efficacy and severity of clinical manifestations post RV-envenomation across the Indian sub-continent.

Expert commentary: Variation in RVV antigenicity due to geographical differences and poor recognition of low molecular mass (<20 kDa) RVV toxins by PAV are serious concerns for effective antivenom treatment against RV envenomation. Improvements in immunization protocols that take into account the poorly immunogenic components and geographic variation in RVV composition, can lead to better hospital management of RV bite patients.

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1. Epidemiology of snakebite, with special reference to Russell's Viper bites in tropical countries

Snake venoms contain a myriad of proteins and polypeptides that have diversified via accelerated evolution facilitated by positive Darwinian selection [1–3]. This often diverse suite of toxins aid in immobilizing, killing and digesting prey [4]. Bestowed with high affinity and selectivity, these biological toxins primarily affect the cardiovascular and neuromuscular systems, and blood coagulation of prey or victims [5–7]. Nevertheless, some of these medically important toxins of snake venom, following purification and extensive characterization, also hold promise as excellent candidates for producing lifesaving drugs [8–10].

Snake envenoming is an occupational health hazard that primarily affects rural agricultural workers in developing countries of Africa, Asia, Latin America, and Oceania [11]. Snakebite victims in these regions are subjected to high morbidity and mortality rates due to poor access to health services, and in many instances, a scarcity of antivenom, the only effective choice of treatment for snakebite [12]. In spite of the severe physical, psychological and socio-economic impacts of snakebite, this abundant health problem has received scant attention from regional and national health authorities, as well as research funding agencies throughout the world. Recently (2017), the World Health Organization (WHO) has again recognized snake envenoming as a neglected tropical disease (www.who.int/neglected_diseases/diseases/en). Subsequently, interest in understanding and addressing this grave problem is gradually emerging among health authorities, governmental and non-governmental organizations, antivenom manufacturers, and researchers across the globe.

Conservative estimates suggest globally at least 421,000 snakebites occur annually and approximately 20,000 of these result in death. The estimations of snakebite in India are even more poignant, with approximately 81,000 envenoming per year resulting in 11,000 deaths; these numbers are the highest for any country [13]. Mortality data from the Million Death Study (2001–03) conducted by the Registrar General of India and the Centre for Global Health Research provided the first-ever direct estimate of 45,900 annual snakebite deaths in India [14]. Nevertheless, accurate numbers of snake envenomings and fatalities are difficult to estimate due to inadequate reporting and record-keeping [13,15]. Further, occurrence and mortality is only part of the picture: a significant (but unknown) number of envenomings result in permanent morbidities, such as loss of limbs, digits and/or function, and these effects can debilitate a victim for life.

India is inhabited by more than 60 species of venomous snakes, and among them, the 'Big Four' snakes – Russell's Viper (*Daboia russelii*), Spectacled Cobra (*Naja naja*), Common Krait (*Bungarus caeruleus*), and Saw-scaled Viper (*Echis carinatus*) – are distributed throughout the country and are responsible for most cases of envenoming, morbidity and mortality [16,17]. In Asia, Russell's

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Viper bites account for a heavy toll of mortality, and this species is responsible for 70% and 40% of snakebite incidences in Myanmar and Sri Lanka (SL), respectively [18]. In western India (WI), these numbers are 20.8%, and the highest incidence of Russell's Viper envenomations has been recorded in eastern India (EI) [6,18,19]. However, snakebite data in India are fragmentary because a low proportion of snakebite patients are attended to at hospitals [18], so actual snakebite incidence is uncertain. Regardless, the Russell's Viper is a major source of snakebites and is considered as a category-I medically important snake in the Indian subcontinent.

2. The distinctive features of Russell's Viper

Russell's Viper (RV), or the Chain Viper is named after Dr. Patrick Russell, a Scottish surgeon and naturalist who described many of the Indian snakes. The genus name '*Daboia*' is adapted from a Hindi word in India that translates to 'that lies hidden' or 'the lurker'. The size of this snake varies from medium to large; RV can grow up to 180 cm and average about 100 cm, with keeled scales, a flattened and triangular head that is distinct from the neck and a blunt and rounded snout (Figure 1). The pattern consists of several distinctive bright chain patterns with dark, round to oblong blotches edged in black (and often white), and the body color is typically yellowish to brown; these typical features allow for Russell's Vipers to be positively identified relatively easily [17]. The underside is white in the western India, partly spotted in southeastern India and deeply spotted in the northeastern race of this species. Adults are usually slow and sluggish unless agitated, while the juveniles are very active. RV feed primarily on rodents, especially murid species, and they typically inhabit open, grassy or bushy areas but may also be encountered in forested plantations and farmland [17,20].

3. Geographic distribution and classification of Russell's Viper

RV is found over large parts of Asia; it is distributed throughout the Indian subcontinent, and much of SL, Pakistan, Myanmar, southern China, and Taiwan [21,22]. It is found up to 2756 m (9040 ft) above sea level [17]. It is abundant in the southern, western and eastern states of India but very rare in the Ganges Valley, Northern Bengal, and Assam. Generally, the distribution of RV is not restricted to a particular type of habitat; however, it has a tendency to avoid extensive forests. Its preferred habitat is the vicinity of the farmlands and rice fields where its prey such as rats and mice flourish, and this is the reason why rice farmers are the major victims of RV bites [6].

Based on the differences in coloration and markings, RV is classified into five regional subspecies: *Daboia russelii russelii* (India, Pakistan, Nepal, and Bangladesh), *Daboia russelii pulchella* (SL), *Daboia russelii siamensis* (Thailand, Myanmar and China), *Daboia russelii formosensis* (Taiwan), and *Daboia russelii limitis* (Indonesia) [23]. However, based on morphological characteristics and mitochondrial DNA analysis, Thorpe et al. (2007) classified Russell's Vipers into two species – *D. russelii* inhabiting the Indian sub-continent (Figure 1) and *D. siamensis* endemic to parts of southeast Asia (other than the Indian subcontinent), southern China, Indonesia, and Taiwan [24]. In 1996, Tsai et al. proposed two types of Russell's Vipers based on the presence of either Asparagine (Asn, N) or Serine (Ser, S) at the N-terminus of the venom phospholipase A_2 (PLA_2)

Figure 1. General distribution of the Russell's Viper (*Daboia russelii*) on the Indian sub-continent (adapted with permission from ©World Health Organization, 2009; retrieved from http://apps.who.int/bloodproducts/snakeantivenoms/database/Images/SnakesDistribution/Large/map_Daboia_russelii.pdf). **Inset**: photograph of an adult Russell's Viper (*Daboia russelii*). Photograph by A.K. Mukherjee, copyright 2018.

isoenzymes [25]; however, because of the rapid evolution and diversification of snake venom toxins, this may not be a good taxonomic parameter to classify RVs.

4. Biochemical analysis to reveal geographic variation in RVV composition

More than 90% of RVV is comprised of proteins and polypeptides, and it possesses a characteristic yellow color due to the presence of FAD, a cofactor of the L-amino acid oxidase enzymes. The pH of an aqueous solution of RVV is acidic (~5.8), while its specific gravity ranges from 1.03 to 1.07 [26,27].

Variation in venom composition is a ubiquitous phenomenon, and it is not surprising that it is evident in RVV as well. In 1988, Jayanthi and Gowda demonstrated geographic variation in RVV composition. They fractionated RVV samples from northern India (NI), WI, and southern India (SI) by cation-exchange chromatography and analyzed some biochemical and pharmacological properties of these fractions. SDS-PAGE analysis showed differences in protein banding patterns of RVV obtained from the three different regions of India; specifically, three protein bands corresponding to molecular weights of 66 kDa, 39 kDa, and 9 kDa were absent from SI RVV. Variation in venom composition was also evident from the number of peaks resolved and percent protein recovered from the cation-exchange chromatography of RVV samples. Further, NI and WI RVV samples were predominantly acidic protein fractions that demonstrated significantly higher PLA_2 activity compared to SI RVV, which was characterized as containing an abundance of basic PLA_2 fractions. In addition, SI RVV exhibited weak proteolytic and trypsin inhibitory activities compared to NI and WI RVV samples [28].

In another study, Mukherjee and colleagues (2000) analyzed the biochemical properties of crude EI RVV and its gel filtration fractions to shed light on its complexity. The EI RVV was characterized as possessing potent trypsin inhibitory, PLA_2 and pro-coagulant activities, and the first gel filtration peak (eluting in the void volume) contained maximum protein and carbohydrate content, along with the highest caseinolytic and pro-coagulant activities, suggesting a predominance of high molecular mass (>35 kDa) proteins in EI RVV. Although these preliminary studies were instrumental in understanding RVV complexity and compositional variation, information on the non-enzymatic, smaller toxin sub-proteome of this venom was yet to be described.

Recently, the enzymatic activities of RVV samples from Pakistan (P RVV) (captive specimen), WI, EI, and SI were assayed, their venom composition was determined by LC-MS/MS analysis (see below) and a correlation between geographic variation in venom composition and corresponding biochemical properties of RVV was presented [29–31]. A comparative analysis of the specific activity of enzymes present in RVV samples from these different geographic regions determined from the same laboratory is summarized in Table 1, which highlights the distinct variation in RVV composition across the Indian subcontinent.

Interestingly, although the relative cumulative abundance of SVMP and SVSP in WI and EI RVV samples was found to be comparable (32.0–33.7%) by proteomic analysis [30,32], the WI RVV sample demonstrated significantly higher ($p < 0.05$) fibrinogenolytic specific activity compared to EI RVV samples (Table 1). This finding indicates that it is not only the relative abundance of the protease enzymes found in RVV, but the potency or enzymatic strength (in terms of specific activity) of individual toxin may also determine the extent of pharmacological activity exhibited by RVV. Additionally, SVMPs have shown wide substrate specificity and all of them may not display fibrinogenolytic activity [33,34]; therefore, a direct correlation between fibrinogenolytic activity and content of SVMP may not always be expected. Nevertheless, P RVV (captive specimens) and SI RVV were characterized with the lowest fibrinogenolytic activity (Table 1), which is well-correlated with the proteomic analyses showing lower relative abundances of protease enzymes in these RVV samples (17.5–25.0%) [29,31].

Therefore, qualitative as well as quantitative differences in enzyme activity in RVV samples from different locales indicate

Table 1. A comparison of enzymatic activities displayed by RVV from different regions of the Indian sub-continent.

	Origin of RVV sample				
Enzymatic activity (U/mg)	WI [1]	EI (Burdwan) [2]	EI (Nadia) [3]	SI [4]	Pakistan (captive specimen) [5]
PLA_2 [a] ($\times 10^3$)	0.6 ± 0.03	0.8 ± 0.02	0.9 ± 0.02	1.1 ± 0.03	0.0078 ± 0.001*
SVMP [b]	0.15 ± 0.03	0.10 ± 0.021	0.07 ± 0.011	0.012 ± 0.01	0.2 ± 0.03
LAAO [c]	19.8 ± 0.92	26.7 ± 0.71	24.7 ± 0.6	105.9 ± 2.2	3.2 ± 0.5
Fibrinogenolytic [d]	9.8 ± 0.21	7.6 ± 0.13	5.4 ± 0.11	0.8 ± 0.02	1.5 ± 0.2
Fibrinolytic [d]	0.7 ± 0.04	0.5 ± 0.01	0.3 ± 0.01	0.9 ± 0.01	ND
ATPase [e] ($\times 10^3$)	4.5 ± 0.15	1.5 ± 0.05	1.9 ± 0.06	90.0 ± 20.0	4.1 ± 0.8
ADPase [e] ($\times 10^3$)	6.4 ± 0.25	2.4 ± 0.09	2.4 ± 0.05	180.0 ± 41.2	ND
AMPase [e] ($\times 10^4$)	1.7 ± 0.05	0.5 ± 0.02	0.4 ± 0.02	31.2 ± 8.80	1.2 ± 0.14
Hyaluronidase [f]	63.4 ± 2.11	1918.2 ± 64.1	1946.4 ± 56.3	126.0 ± 2.3	ND
PDE [g]	11.8 ± 0.08	4.5 ± 0.10	4.3 ± 0.12	4.7 ± 0.11	1.2 ± 0.10
TAME [h] ($\times 10^2$)	19.1 ± 0.8	3.4 ± 0.11	3.2 ± 0.09	1.6 ± 0.05	ND
BAEE [i] ($\times 10^2$)	2.8 ± 0.08	2.0 ± 0.07	2.0 ± 0.06	0.007 ± 0.04	19.1 ± 0.8

ND: not determined

Adapted from [1] Kalita et al., 2017 [30]; [2–3] Kalita et al. 2018 [32]; [4] Kalita et al., 2018 [31]; [5] Mukherjee et al., 2016 [29]

[1] One unit is defined as a decrease by 0.01 in absorbance at 740 nm after 10 min of incubation. * One unit of PLA_2 activity of Pakistan RVV is defined as nmol of 3-hydroxy-4-nitrobenzoic acid formed/min/mg protein. [b] One unit is defined as the change in absorbance at 450 nm per min at 37 °C. [c] One unit is defined as 1 nmol of kynurenic acid produced per min. [d] One unit is defined as 1.0 µg of tyrosine equivalent liberated per min per mL of enzyme. [e] One unit is defined as micromoles of Pi released per min. [f] One unit is defined as a 1% decrease in turbidity at 405 nm in comparison to control (100% turbidity). [g] One unit is defined as micromoles of *p*-nitrophenol released per min. [h] One unit is defined as an increase by 0.01 in absorbance at 254 nm during the first 5 min of the reaction at 37°C. [i] One unit is defined as an increase by 0.01 in absorbance at 244 nm during the first 10 min of the reaction at 37°C.

geographic variation in venom composition of RV. Further, the toxicity of venom samples depends on the qualitative and quantitative distribution of different enzymes and toxins in venom [6,35]; therefore, these disparities in enzymatic properties of RVV may also be responsible for the differences in severity of pathogenesis and clinical symptoms following RV envenomation.

5. Elucidation of geographic differences in proteome composition of RVV by tandem mass spectrometry analysis

With the advent of biochemical assays including purification and characterization of enzymes, RVV complexity was gradually determined [6,27,28,36,37]. However, this approach has a major limitation for the identification and quantification of the non-enzymatic and minor components of snake venom, which is often crucial because these components may dictate the differences in severity of pathogenesis and clinical symptoms following envenomation. Further, quantitative data on RVV proteomes across the country can be critical for the development of region-specific effective antivenoms. These constraints have now been overcome by the recent advancements in the field of mass spectrometry, the growth of robust database search algorithms, and the advent of powerful venom de-complexing strategies [38–40].

For proteomic analysis of snake venom, the choice of a particular workflow depends on several factors, such as aims and objective of the study, quantity of available starting material (snake venom), analytical facilities, and database status. Quantification of identified components through proteomic analysis (quantitative proteomics) is another important way to understand the variation in snake venom composition. However, without isotope labeling methods this can be very challenging; to address this limitation, toxinologists have represented the relative abundance of the venom proteome components by using various strategies. For example, determination of the area under RP-HPLC peaks at 215 nm (AUC) provides a surrogate measure of the peptide bonds [41], and therefore relative area provides a measure of relative abundance of specific components. MS-based label-free quantification strategies have become popular due to the relative ease of experimentation and the small amount of sample needed [29,30,32,42–46]. Nevertheless, the paucity of relevant entries in the target database is an inevitable drawback associated with shotgun mass spectrometry-based protein identification, and several important components of snake venoms, such as ATPase and ADPase enzymes, may be overlooked because they have yet to be documented in the databases [30–32,44].

The venom proteomes of *D. siamensis* from Myanmar, South China (Guangxi) and Taiwan, and *D. russelii* from SL, captive and wild specimens from P, WI, EI, and SI origin have now been deciphered; however, the proteomic workflows differed for different RVV samples [29–32,43,47–50]. For example, de-complexation of Myanmar RVV was accomplished by 2D SDS-PAGE analysis, whereas South China (Guangxi), Taiwan, and SL RVV samples were separated by 1D SDS-PAGE followed by in-gel trypsin digestion and MS/MS analysis [43,47,50]. Further, the RVV samples from P (captive specimen), WI, SI, and EI were fractionated by gel filtration chromatography; nevertheless P RVV (wild specimen) was de-complexed using RP-HPLC. These chromatographic fractions were subjected to in-solution trypsin digestion and MS/MS analysis [29–32,48,49]. Further, the proteome composition of SI RVV was expressed only in terms of number of proteins identified by LC-MS/MS analysis [48], while the relative abundance of different protein families in Myanmar RVV was not determined [47]. Quantitative information on snake venom proteomes is indispensable for correlation of severity of clinical manifestations with the corresponding variation in snake venom composition, and understanding intraspecific variation in RV venom is critical to understanding the varying clinical signs observed with snakebites. Therefore, the proteome composition of SI RVV was revisited in our laboratory with an aim to quantitate the toxins in this venom [31].

The proteomes of RVV samples from the Indian subcontinent were investigated using diverse de-complexation strategies, including 1D or 2D SDS-PAGE, gel filtration and/or ion-exchange chromatography followed by mass spectrometric analysis for protein identification. A schematic diagram of the proteomic workflow used for characterization of RVV from different geographic locations is depicted in Figure 2, and a summary of the proteome composition of RVV samples from different locations on the Indian sub-continent, Myanmar, South China (Guangxi) and Taiwan is shown in Table 2. Based on the above published proteomic reports on Indian RVV, it is evident that this venom is comprised of 40 to 75 proteins distributed over 13 to 15 enzymatic and non-enzymatic protein families (Table 2). The difference in number of proteins as well as protein families identified in RVV samples from different geographic locales of the Indian sub-continent underscores the level of variation in RVV composition as a function of geographical location. Nevertheless, the influence of different proteomic workflows, search engines, and protein identification criteria used to characterize these RVV samples cannot be completely ruled out for contributing to some of the observed compositional disparities. Because these were independent studies, directly comparing venom proteomes of these RVV samples from different geographic locations was challenging. However, the RVV proteomes from P (captive specimen), WI, EI, and SI were investigated by identical proteomic workflow in our laboratory. A detailed comparative analysis of the RVV proteomes from these different localities on the Indian sub-continent suggested that only 9 proteins (2 SVMPs, 2 SVSPs, and single isoforms each of NT, LAAO, NGF, VEGF, and CRISP) were found to be common (based on presence of homologous distinct peptides) to all RVV samples, whereas 22, 13, 19, 36, and 25 proteins were uniquely represented in EI RVV (Burdwan), EI RVV (Nadia), WI RVV, SI RVV, and P RVV proteomes, respectively (Figure 3(a)). The variation in relative abundance of different protein classes, enzymatic and non-enzymatic, of RVV from different locations is shown in Figure 3(b,c), respectively.

Recently, Faisal et al (2018) investigated the venom proteome of wild Pakistani RVV samples and observed striking variation in venom composition between samples from wild and captive RV [29,49]. This implies that venom variability is not only confined to geographical and phylogenetic factors but may also be noted within the same species of snake due to differences in conditions (wild or captive), feeding behavior and/or prey items that may alter the venom production [49].

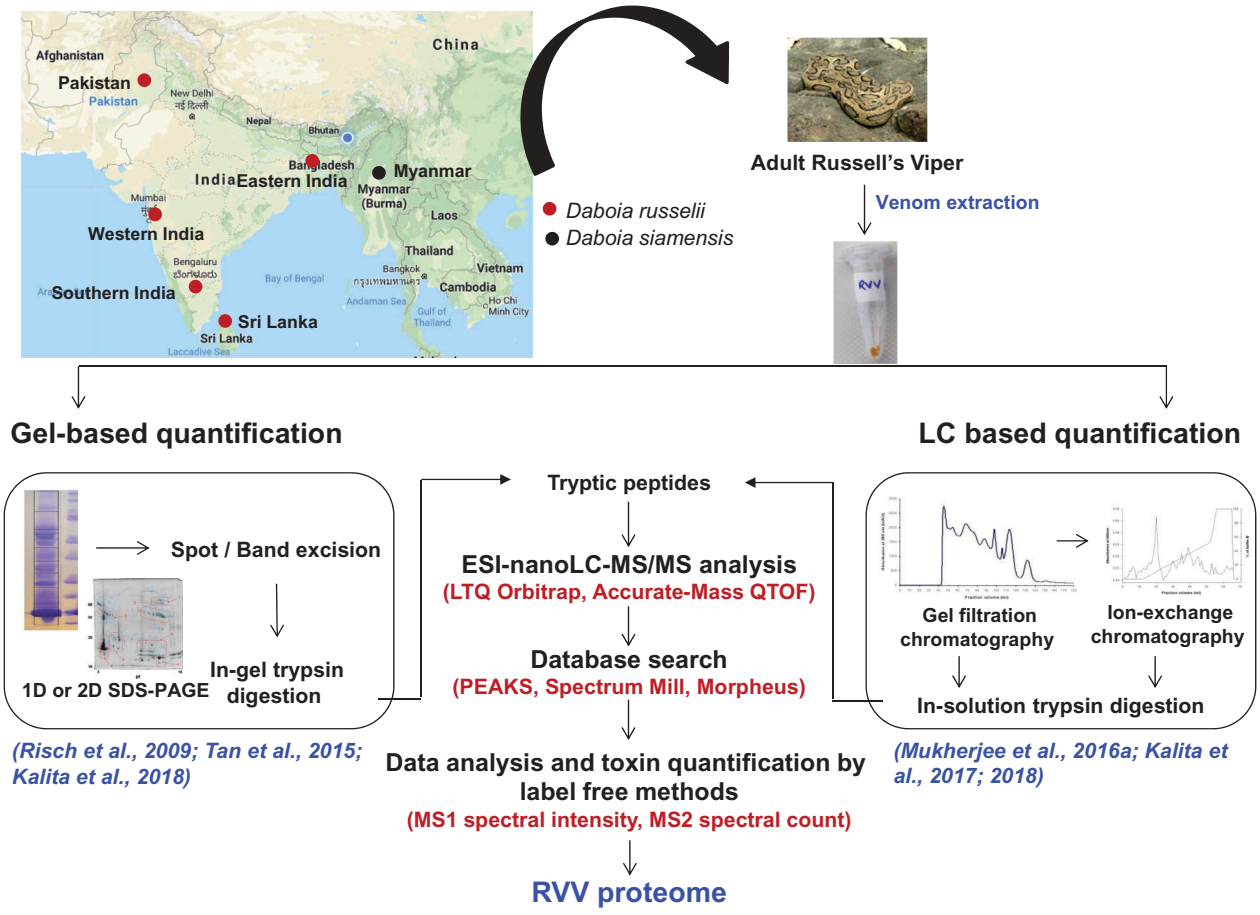

Figure 2. A schematic representation of the proteomic workflow to analyse RVV samples from different regions of the Indian sub-continent. Map of India is adapted from map data © 2018 google. Retrieved from https://www.google.com/maps/place/India/@28.1445493,73.5173445.

Notably, the occurrence of enzymatic proteins in RVV samples from SL, WI, EI, SI, and captive and wild specimens of P ranged from 56.5% to 75.2%, showing that irrespective of locale, venom of RV is predominated by enzymatic proteins.

5.1. Enzyme toxins in RVV

Previous studies showed that PLA_2s, the most abundant protein superfamily present in RVV, exhibit diverse pharmacological effects [36,52–54]. The relative abundance of PLA_2s among the RVV samples varies dramatically, and a striking difference was shown between RVV samples from SI and from wild specimens from Pakistan (Figure 3(b)) [49]. Further, a significant difference in the number of PLA_2 isoforms was also noted across the RVV samples (Table 3), indicating geographic variation in PLA_2 content and type. Another prominent difference was the occurrence of neurotoxic PLA_2 isoforms established by mass spectrometry analysis; EI RVV does not contain this subclass of PLA_2 toxins [32], but neurotoxic PLA_2s are reported in RVV samples from SL (>30%), SI (15.7%), WI (3.2%), and captive specimens of P (0.23%) [29–31,43].

Proteases including SVMPs and SVSPs are the second and third most abundant families of enzymatic proteins in RVV, respectively. In addition to affecting the hemostatic system in victims, SVMPs induce hemorrhage, necrosis and/or muscular degeneration, while SVSPs are closely associated with coagulopathies [37,55–59]. The relative abundance as well as number of isoforms of SVMP and SVSP in venom of RV from different locales of Indian subcontinent varies significantly (8.0–33.7%; Table 3, Figure 3(b)), suggesting variable extents of RV-induced hemostatic derangement in RV-envenomed victims from different locales of the Indian sub-continent. However, due to absence of clinical snakebite data from different regions of Indian sub-continent, the above supposition cannot be verified.

Snake venom LAAOs are high molecular weight (60–150 kDa) homodimeric enzymes that are reported to cause edema, platelet modulation, apoptosis, hemorrhagic effects, anticoagulant effects, and hemolysis [27,60,61]. The relative abundance of this class of proteins in RVV samples from captive and wild specimens of P, WI and EI is relatively low (0.3–1.6%); however, LAAOs occur in significantly higher amounts in RVV samples from SL (5.2%) and SI (7.5%) (Figure 3(b)).

NT and PDE are thermolabile, high molecular weight RVV components that can cleave a wide range of nucleotide molecules and they may play an important role in affecting blood coagulation and modulation of platelet function [62–64]. The relative abundances of NT and PDE were found to be comparable among different RVV samples [29,30,43,49]. The relative abundance of minor enzymatic classes of RVV, such as glutaminyl cyclase (GC), phospholipase B (PLB), aminopeptidase (APase), and carboxypeptidase (CP), is comparable in RVV

Table 2. A summary of the proteomic studies on RVV from different geographical locations of the Indian sub-continent, Myanmar, South China, and Taiwan. The relative abundances of predominant enzymatic and non-enzymatic families in respective RVV samples are shown in parenthesis.

Geographic origin of RVV sample	Venom de-complexation strategy	MS/MS search engine	Number of Proteins identified	Protein classes identified	Predominant enzymatic and non-enzymatic families (% relative abundance)	References
Myanmar	2D SDS-PAGE	MASCOT	55	SVSP, SVMP, PLA_2, LAAO, VEGF, snaclec	NA	[47]
Sri Lanka	1D SDS-PAGE	Agilent Spectrum Mill MS Proteomics Workbench software package	41	SVSP, SVMP, PLA_2, NT, PDE, LAAO, PLB, KSPI, snaclec, NGF, CRISP	PLA_2 (35.0%) and snaclec (22.4%)	[43]
Southern India	Gel filtration chromatography	Proteome Discoverer 3.1	63	SVSP, SVMP, PLA_2, NT, PDE, LAAO, KSPI, VEGF, snaclec, NGF, Dis, CRISP	PLA_2 (23.8%) and CRISP (11.0%)	[48]
Pakistan (captive specimens)	Gel filtration chromatography	PEAKS 7.0	75	SVSP, SVMP, PLA_2, NT, PDE, LAAO, AMT, Hya, KSPI, VEGF, snaclec, NGF, Dis, CRISP	PLA_2 (32.8%) and KSPI (28.4%)	[29]
Western India	Gel filtration followed by ion-exchange chromatography	Proteome Discoverer 3.1	55	SVSP, SVMP, PLA_2, NT, PDE, LAAO, PLB, KSPI, VEGF, snaclec, NGF, Dis, CRISP	PLA_2 (32.5%) and KSPI (12.5%)	[30]
Burdwan (Eastern India)	Gel filtration chromatography	PEAKS 8.5	73	SVSP, SVMP, PLA_2, NT, PDE, LAAO, APase, GC, Hya, KSPI, VEGF, snaclec, NGF, Dis, CRISP	PLA_2 (22.2%) and KSPI (20.3%)	[32]
Nadia (Eastern India)	Gel filtration chromatography	PEAKS 8.5	69	SVSP, SVMP, PLA_2, NT, PDE, LAAO, PLB, GC, Hya, KSPI, VEGF, snaclec, NGF, Dis, CRISP	PLA_2 (21.5%) and KSPI (22.9%)	[32]
Southern India	1D SDS-PAGE	Morpheus	66	SVSP, SVMP, PLA_2, NT, PDE, LAAO, PLB, GC, CP, KSPI, VEGF, snaclec, NGF, CRISP	PLA_2 (43.6%) and snaclec (14.6%)	[31]
Pakistan (wild specimens)	Reversed phase-high performance liquid chromatography	Agilent Spectrum Mill MS Proteomics Workbench software package	55	SVSP, SVMP, PLA_2, NT, PDE, LAAO, KSPI, VEGF, snaclec, NGF, CRISP	PLA_2 (63.8%) and KSPI (16.0%)	[49]
South China (Guangxi)	1D SDS-PAGE	Agilent Spectrum Mill MS Proteomics Workbench software package	47	SVSP, SVMP, PLA_2, NT, PDE, LAAO, KSPI, VEGF, snaclec, NGF, CRISP, APase	PLA_2 (22.2%) and KSPI (23.2%)	[50]
Taiwan	1D SDS-PAGE	Agilent Spectrum Mill MS Proteomics Workbench software package	28	SVSP, SVMP, PLA_2, PDE, KSPI, VEGF, snaclec, NGF, APase	PLA_2 (24.5%) and KSPI (28.2%)	[50]

NA – not available

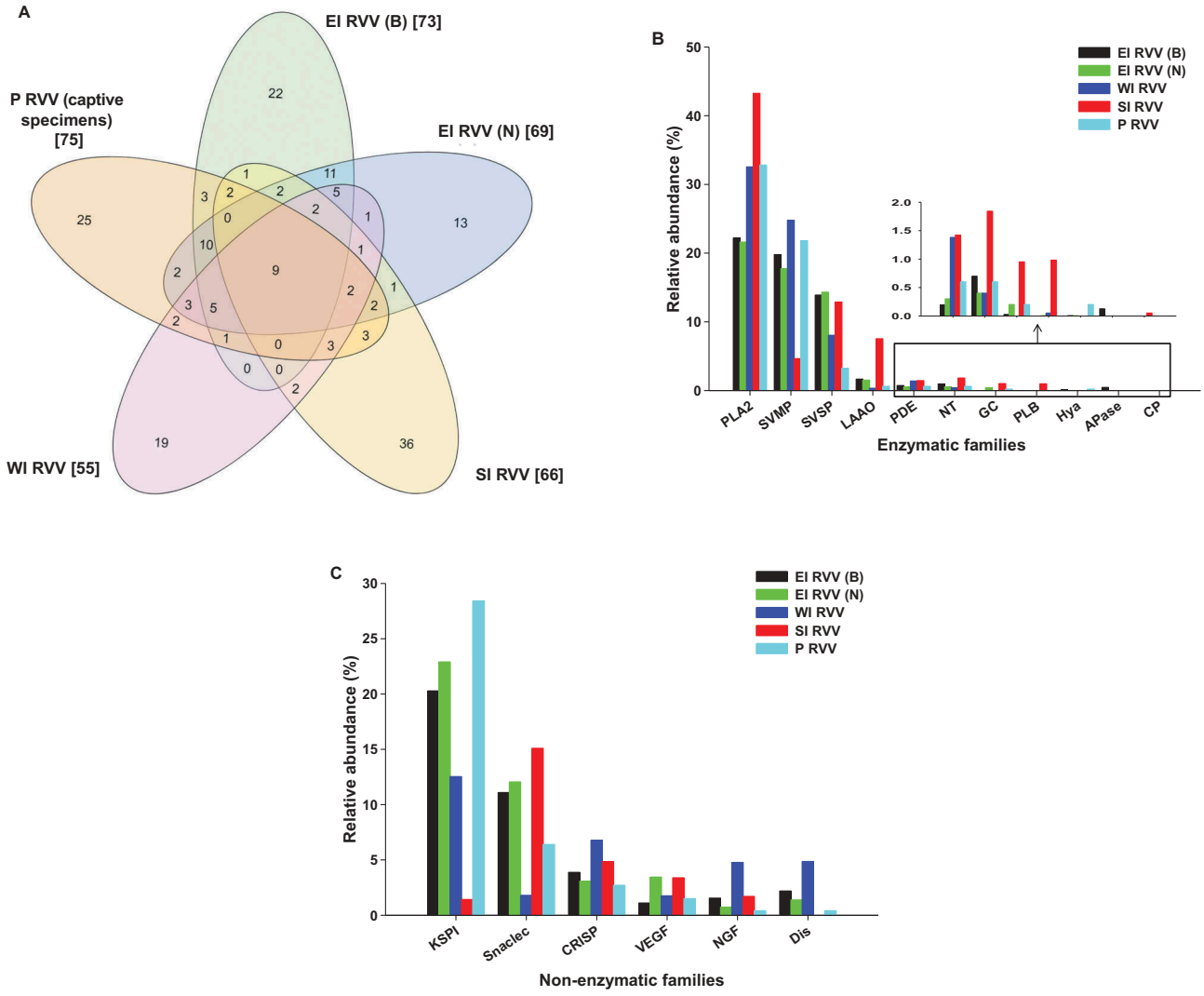

Figure 3. (a)A Venn diagram representing the distribution of common and unique proteins/toxins among RVV samples from different parts of India and Pakistan. The Venn diagram was generated using InteractiVenn [51]. The numbers in parentheses indicate the total number of proteins identified in the respective RVV proteomes. Variation in relative abundance of (**b**) enzymatic proteins and (**c**) non-enzymatic proteins in RVV samples from Pakistan (P RVV), western India (WI RVV), southern India (SI RVV), and Burdwan [EI RVV (B)] and Nadia [EI RVV (N)] districts representing EI RVV samples.

samples throughout the Indian sub-continent (Figure 3(b)); however, proteomic analysis could not detect some of these enzymes in all RVV samples, perhaps consistent with their probable roles as housekeeping proteins, rather than venom toxins. Additionally, because of their extremely low contribution to total venom composition (<1%), variation in the relative abundance of these enzymes in a particular RVV sample is not likely to influence the toxicity of venom.

5.2. Nonenzyme toxins in RVV

KSPI and snaclec were the major non-enzymatic proteins identified in all the RVV samples across the Indian sub-continent, albeit with variable relative abundances (Table 2). They have been demonstrated experimentally to inhibit serine proteases, block ion-channels, and act on coagulation, fibrinolysis, and inflammation [65–67]. Further, a KSPI characterized from Pakistani RVV (named Rusvikunin) was found to form a protein complex or interact with other components of RVV to induce neurotoxicity in experimental mice models [66,68]. The number of KSPI isoforms and their relative abundance were also found to vary among the RVV samples from different locations on the Indian sub-continent (Figure 3(c)), Table 3).

Non-enzymatic snaclecs are structurally characterized as αβ heterodimers that are reported to target blood coagulation factors, cell membranes, and platelet receptors, thereby contributing to haemostatic imbalances in victims [69,70]. Like KSPI, the relative abundance of this pharmacologically active RVV toxin was also found to differ greatly, from 1.8% in WI RVV to 22.4% in SL RVV (Figure 3(c)).

CRISP, VEGF, NGF, and disintegrins are the less abundant non-enzymatic proteins in RVV, and their relative abundance also differs in RVV samples across different regions of the Indian sub-continent (Figure 3(c)). Although the precise pharmacological roles of these components are unknown, some of them are believed to interfere with the hemostatic system of victims [71–74].

Table 3. Number of toxin isoforms identified in RVV samples from different geographical locations of the Indian sub-continent.

Protein family	Southern India [1]	Sri Lanka [2]	Pakistan (captive specimens) [3]	Western India [4]	Burdwan [5]	Nadia [6]	Southern India [7]	Pakistan (wild specimens) [8]
PLA_2	15	4	17	17	21	12	10	11
SVMP	6	5	13	5	10	13	4	3
SVSP	11	12	9	6	9	15	18	9
LAAO	5	4	2	2	1	2	9	3
PDE	2	1	2	1	1	1	2	3
NT	3	2	2	2	1	1	2	1
Hya	ND	ND	1	ND	1	1	1	ND
PLB	ND	1	ND	1	ND	1	1	ND
GC	ND	ND	ND	ND	1	1	1	ND
AMT	ND	ND	1	ND	ND	ND	ND	ND
Apase	ND	ND	ND	ND	1	ND	ND	ND
KSPI	3	2	8	8	6	5	2	10
Snaclec	5	6	11	7	13	12	11	8
CRISP	7	1	3	2	3	2	2	3
VEGF	2	ND	3	2	2	1	2	2
NGF	1	3	1	1	2	1	1	2
Dis	2	ND	1	1	1	1	ND	ND
UP	1	ND	1	ND	ND	ND	ND	ND
Total	**63**	**41**	**75**	**55**	**73**	**69**	**66**	**55**

ND: not detected by LC-MS/MS analysis
Adapted from [1] Sharma et al., 2015 [48]; [2] Tan et al., 2015 [43]; [3] Mukherjee et al., 2016 [29]; [4] Kalita et al., 2017 [30]; [5–6] Kalita et al. 2018 [32]; [7] Kalita et al., 2018 [31]; [8] Faisal et al., 2018 [49]

6. Pharmacology and clinical manifestations of envenomations by RV and their correlation with RVV proteome composition

An adult RV possesses approximately 200–225 mg of venom in its glands, and so bites to prey or a victim can result in large amounts of venom being injected [53]. The LD_{50} of RVV (in mice) ranges from 0.7 (*i.v.*) to 10 mg/kg (*i.p.*) depending upon the geographic source of the venom; EI RVV was found to be the most lethal compared to RVV samples from western, southern and northern India [6,28,75]. This indicates that in addition to geographic differences in RVV composition, acute toxicity of venom also varies across the Indian sub-continent. The variation in pharmacological properties exhibited by RVV from different parts of India is well corroborated by the proteomic findings. For example, the lower pro-coagulant and fibrin(ogen)olytic activity displayed by SI RVV, compared to RVV samples from P (captive specimens), WI and EI, was consistent with the lower cumulative abundance of SVMP and SVSP in the former RVV [29–32]. The exceptionally low relative abundance of KSPI in SI RVV (compared to EI, WI, and P RVV) was well correlated with its low trypsin inhibitory activity [28,31]. Further, platelet aggregation by RVV components such as snaclec, LAAO, and SVTLE is yet another mechanism of provoking hemostatic disturbance in prey/victims [76–78]. The cumulative relative abundance of these components in SI RVV (23.7%) surpassed that of WI (2.4%) and EI RVV (13.6–13.8%) samples, thus explaining the greater platelet aggregation activity demonstrated by SI RVV [30–32]. Proteomic analyses of these venoms provided sufficient evidence to account for the observed differences in pharmacological property exhibited by RVV samples from different localities on the Indian sub-continent.

The common clinical features of RV bites, such as rapid swelling and extreme pain of the bitten body part, local ecchymosis and hemorrhage (due to disruption of integrity of capillary blood vessels and subsequent capillary leakage syndrome), and intense blebs over the affected extremities [6,19,79] are primarily caused by the abundant SVMPs in RVV [80–82]. Therefore, the variation in SVMPs observed among the RVV samples from different localities (Figure 3(b)) is expected to result in different levels of severity of SVMP-induced toxicity, and clinical manifestations in RV bite patients across the Indian sub-continent are likewise expected to be variable. Largely due to the activities of SVSPs, RVV initially affects the vascular system by provoking hemostatic disturbances, including rapid thrombosis and hypofibrinogenemia that ultimately results in consumptive coagulopathy and incoagulable blood [19]. This results from the concerted action of the serine proteases and some metalloproteases (FX activator) that activate prothrombin, Factor X and V, and fibrin(ogen)olytic enzymes that catalyze hydrolysis of fibrinogen and/or fibrin [37,83,84]. Subsequently, abundant anti-coagulant RVV proteins such as PLA_2s, KSPIs, and snaclecs exert anti-coagulant action by inhibiting various blood factors such as thrombin, and/or Factor Xa, further promoting incoagulable blood [36,67,70]. As with other toxin families, the relative abundance of these hemostatically active components also varies dramatically among RVV samples (Figure 3(b,c)), again highlighting the impact of geographic location on the severity of pathogenesis of RV envenomation.

Intravascular hemolysis and related complications observed in RV-envenomed patients primarily result from the action of PLA_2 isoenzymes that can cause lysis of phospholipids of erythrocyte membranes, leaving the cells vulnerable to dissolution [53]. Notably, the RVV samples from WI, EI, and SI demonstrated different extents of indirect hemolysis, which is correlated with the relative abundance of PLA_2 isoenzymes. Variation in this pharmacological property of RVV may also lead to different degrees of intravascular hemolysis and bleeding complications in RV-envenomed patients from different regions.

Acute kidney injury (previously termed as acute renal failure or ARF) is a persistent clinical manifestation observed in RV-envenomed patients throughout the Indian sub-continent.

RVV factor X activators, LAAO, and PLA_2 isoforms are the likely components responsible for RVV-induced acute kidney injury [56,85,86], and significant variation in the amounts of these components in RVV, as observed by proteomic analyses, may lead to differences in the severity of RVV-induced acute kidney injury in different regions. However, due to a lack of clinical data on snakebite from different regions of Indian subcontinent, this presumption cannot yet be verified. Additionally, acute kidney injury has also been reported to be caused by reduced blood flow to the kidneys following intravascular coagulopathy and hemodynamic alteration, which is a secondary effect [87]. Another component, VEGF, may also exacerbate kidney injury; it exhibits potent hypotension and enhancement of vascular permeability activities, thereby resulting in overall bleeding complications [73]. Unlike some venom components, VEGF levels in the RVV samples from different localities were similar (Figure 3(c)).

In addition to the common clinical symptoms mentioned above, RV bite patients from SI, SL, and occasionally from WI are also reported to show neuroparalytic symptoms such as ptosis, bulbar palsy, inter-nuclear ophthalmoplegia, and respiratory paralysis due to pre-synaptic neuromuscular block [19,79,88,89]. While the neurological symptoms are quite severe in SL RV-envenomed patients, they are moderate in SI and very rarely reported in WI. These differences in severity of neurological symptoms can be explained on the basis of variation in the relative abundances of neurotoxic PLA_2 isoforms in RVV samples from SL (>30%), SI (15.7%) and WI (3.2%) [30,31,43].

The proteomic analyses of RVV from different regions of the Indian sub-continent have enabled us to document significant levels of variation in venom composition of this medically relevant snake. Further, as discussed above, the proteomic findings were consistent with the clinical manifestations of RV envenomation reported across the country.

7. Potency of commercial polyvalent antivenom in the treatment of RV-envenomed patient in Indian sub-continent

Parenteral administration of animal-derived antivenom is the single well-substantiated choice of treatment for snake envenomation. Due to the frequency and severity of envenomation by the 'Big Four' snakes (Indian Cobra, Indian Common Krait, Russell's Viper, and Saw-scaled Viper), several antivenom manufacturing companies in India produce polyvalent antivenom (PAV) against a cocktail of venoms of these four species. However, safety and efficacy of equine antivenom are of immense concern for successful hospital management of bite victims [12,15,90]. Notably, variation in snake venom antigenicity due to geographical differences is another important consideration in antivenom design. It has been reported that the Indian PAV is raised primarily against venoms collected from snakes inhabiting one small area around Mahabalipuram in Tamil Nadu by the Irula Snake Catchers' Industrial Cooperative Society (ISCICS), and there are clinical reports stating the inefficacy of Indian PAV against snakebite in areas distant from the source of immunizing venoms [15]. In particular, the Indian PAV is reported to be less effective in the treatment of *D. russelii* envenoming in Maharashtra and northern Kerala [15].

Enzyme-linked immunosorbent assay (ELISA) and immunoblotting (IB) have been widely used to determine the immunoreactivity of venom components against commercial PAV [29,30,49]. ELISA and IB analyses of P (wild and captive specimens), WI, EI, and SI crude RVV and/or gel filtrations fractions unequivocally pointed out the poor immunogenicity of low molecular mass components (<20 kDa) of these venoms [29–32,49]. Further, the poor immuno-recognition of low molecular mass RVV proteins may also be due to the swamping of abundant, more highly immunogenic high molecular mass RVV proteins as well as 'dilution' due to the presence of antibodies against venoms of the other three species of Big Four snakes present in PAV. In addition, the cross-reactivity of P RVV (wild and captive specimens) towards Indian PAV was significantly lower compared to RVV samples from other parts of India [29,49]. Western blot analysis also demonstrated that SI RVV, compared to RVV samples from WI, and EI exhibited better cross-reactivity against Indian PAVs [30–32]. As discussed, most of the commercial antivenom manufacturing companies procure snake venom from ISCICS for raising equine antivenom [15], which likely explains the observed differential cross-reactivity of commercial PAVs against RVV samples from different locations. Therefore, there is urgency in developing improved immunization schemes that include venom collected from wide geographic locations in order to render PAV effective throughout the country. Alternatively, efforts can also be made to develop region-specific and species-specific antivenom for better treatment of RV bite patients. Studies from our lab have demonstrated that monovalent antivenom (MAV, raised against RVV) exhibited better cross-reactivity than PAV against P and WI RVV gel filtration fractions, thereby indicating that MAV is a better choice of antivenom treatment against RV bites [29,30]. However, due to a lack of reliable snakebite detection kits in India and its neighboring countries, the offending species of snake cannot be identified with certainty; therefore, physicians prefer administration of PAV for treating snakebite.

ELISA and IB methods cannot appropriately pin-point the poorly immunogenic component(s) of RVV. To overcome these limitations, antivenomics analysis to assess the binding efficacy of antivenom has become the preferred method of determination [31,32,44,91,92]. Identification of poorly immunogenic components of venom can provide valuable insights for designing immunizing protocols specifically against these toxins so as to develop improved antivenom that will deliver more extensive protection against all venom toxins. Using an antivenomics approach, the major PAV unbound toxins in WI, EI, and SI RVV were identified as PLA_2s and KSPI. In addition, PAVs also lacked sufficient antibodies against proteases (SVMP and SVSP) of EI RVV [30–32]. Further, using an HPLC/ELISA-based antivenomics approach, Faisal et al. (2018) reported that HPLC fractions of P RVV (wild specimens) containing SVMP, SVSP, snaclec, LAAO, PDE, and 5′-NT exhibited better immuno-recognition by Indian PAV compared to low molecular mass RVV toxins such as KSPI, VEGF and PLA_2 [49]. The above poorly

immunogenic components are hemostatically active toxins in RVV that play a pivotal role in RVV-induced toxicity and exhibit diverse pharmacological effects in bite victim. Therefore, poor recognition of these RVV toxins by commercial PAVs is likely the cause of poorly effective antivenom therapy.

Because enzymes have a profound role in pharmacological effects of venoms, particularly those of the family Viperidae, another *in vitro* method for testing the efficacy of PAV is the determination of the neutralization potency of PAV against a wide array of enzymatic activities and pharmacological properties of RVV. Interestingly, the neutralization potency of PAVs toward various properties of WI, EI and SI RVV were found to vary significantly. The enzymatic activity and pharmacological properties of SI RVV were neutralized by PAVs with exceptions of PLA_2 and indirect hemolytic activity, while the PAVs are extremely poor in neutralizing several enzymes such as fibrin (ogen)olytic, TAME, BAEE, and pro-coagulant properties of WI and EI RVV [29–32]. This discrepancy in neutralization potency of PAVs against venom of same species of snake further demonstrates the role of geographic location and its influence on RVV composition, leading to differential efficacy of the same antivenom against different populations of RV. Further, the better neutralization of SI RVV toxins compared to those from EI and WI correlates well with the findings of the ELISA as well as antivenomics studies. Nevertheless, assessment of neutralization of venom lethality in animal models still remains the gold standard for determining the preclinical efficacy of PAV. However, the above *in vitro* tests can minimize the numbers of animals needed for these protection assays. A recent proteomic study assessed the lethality neutralization of Pakistan RVV (wild specimens) by Indian PAV (VINS) in a mouse model, and their findings suggested that the antivenom neutralized the RVV-induced toxicity with moderate efficacy [49]. However, such lethality neutralization studies on RVV samples from other parts of the Indian sub-continent against commercial PAV are yet to be documented.

8. Expert commentary

During the last quarter of the twentieth century, biochemical and pharmacological characterization, and purification of several proteins from RVV have provided valuable information to understand the complexity of RVV. Nevertheless, a detailed profile on the non-enzymatic sub-proteome as well the minor RVV components was still warranted. The recent technological advancements in the field of mass spectrometry coupled to robust database search algorithms, and the advent of powerful protein separation strategies (fast protein liquid chromatography, RP-HPLC, 2D SDS-PAGE) have led to accelerated knowledge on venom composition and its clinical correlations. The introduction of 'omics' technologies, by the integration of genomics, transcriptomics and proteomics, in the early twenty-first century have provided comprehensive perspectives in molecular toxinology. Successful treatment of snake envenoming relies on the ability of antivenoms to recognize and neutralize the venom toxins. However, due to the extensive complexity and variability of snake venoms, antivenoms often fail to provide extensive protection to bite victims. In addition, antivenoms have become either scarce or expensive in the low and middle-income countries where snake envenoming is a frequently encountered medical threat. Another key concern is the selection and design of venom mixtures for equine immunization so that the antivenoms are effective in a wide geographic area where they are commercially distributed. This important issue can be addressed by proteomic and antivenomics analyses of venoms and antivenoms that can guide in selecting the best combination of venoms/toxins for antivenom production.

The application of proteomic tools in venom research has provided deeper insights into the variation of RVV composition from different locations across the Indian sub-continent. These variations are responsible for the observed differences in antivenom efficacy and the severity of clinical manifestations post RV-envenomation. Further, antivenomics studies of different RVV samples using commercial PAVs have shed light into the identity of the poorly immunogenic sub-proteome toxins of RVV, and this information is of prime relevance for the improved production and clinical use of antivenom to treat RV envenomation in the Indian sub-continent. Due to the extensive variation in RVV composition, there is a need for design improvements by creating immunization protocols that take into account geographic variation in composition and help mitigate the toxic effects of low molecular mass, poorly immunogenic RVV components, leading to the development of region-specific antivenom for better hospital management of RV bite patients. Further, a collaborative venture among the epidemiologists, clinicians, and toxinologists towards the understanding of snake envenoming and its treatment is crucial to fight back this global health issue.

9. Five-year view

Although the proteomic profiles of RVV from different geographical locations of the Indian sub-continent have been documented; however, the composition of RVV from Nepal, Bhutan, and Bangladesh are yet to be deciphered. Since the antivenoms manufactured in India are commercially distributed to these neighboring countries, there is a need to expand the proteomic and antivenomics studies on RVV samples from these geographic locations of the Indian sub-continent, as regional variation in composition is expected. Furthermore, RVV and PAV cross-reactivity can also be analyzed by a third generation antivenomics approach [93] to get a more vivid picture of the maximal binding capacity of antivenoms toward different RVV components and to quantify the RVV-specific antibodies present in commercial antivenoms. Genomic and transcriptomic studies on RVV to complement the proteomic findings are also warranted, and coupled with proteomic studies, a very deep understanding of intraspecific compositional variation of RVV across the entire Indian sub-continent is possible. Additionally, this integrated view of RVV composition will aid in understanding the transcriptional and translational control mechanisms that may play a crucial role in generation of geographically distinct venoms of RV.

Immunological profiling of RVV against commercial Indian PAV clearly suggests that a shift from the conventional PAVs towards toxin- and region-specific antivenoms will be the better choice for improved clinical management of RV bite patients.

Therefore, it is expected that in the next five years, Indian governmental regulators will recommend new specifications for production of antivenom in the Indian sub-continent.

Although snakebite victims receiving timely treatment have a much greater probability of surviving, they may be left with permanent physical disabilities, primarily caused by severe local necrosis. Therefore, in addition to improvement of current antivenom therapy, exploration of ways for counteracting these locally acting toxins is also an important future aspect for toxinological research. Further, spreading general awareness among rural communities regarding snakes and how to avoid them, educating clinicians via workshops, and persuading the rural population to move snakebite victims to hospitals are all crucial to solve the neglected tropical disease problem of snakebite.

Key issues

- Highest incidences of snake envenoming and mortality cases are reported from India, and Russell's Viper is responsible for a significant number of snakebite cases in the Indian sub-continent.
- Tandem mass spectrometry coupled to efficient database search algorithms has been instrumental to decipher the complexity of snake venom proteomes.
- The proteomic analyses of RVV from different regions of the Indian sub-continent have documented significant levels of variation in venom composition of this medically relevant snake.
- Variation in RVV composition is accountable for the differences and severity of RV envenomation across the Indian sub-continent.
- The hallmarks of clinical manifestations of RV envenomation, such as coagulopathy, edema, hematuria, systemic bleeding, hematemesis, intravascular hemolysis, hypotension, and acute renal failure, correlated well with regional differences in the proteome composition of RVV.
- The efficacy of antivenom to treat snake envenoming depends on its ability to neutralize and reverse the toxic effects of the venom toxins.
- Differential neutralization of toxicity and enzymatic activity of RVV samples from different areas of the Indian subcontinent by commercial PAV, and poor recognition of low molecular mass (<20 kDa) RVV toxins such as PLA_2 and KSPI by PAVs, are the most serious concerns for effective antivenom treatment against RV envenomation.

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