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Proteomics, functional characterization and antivenom neutralization of the venom of Pakistani Russell's viper (*Daboia russelii*) from the wild

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ABSTRACT

The venom proteome of wild Pakistani Russell's viper (*Daboia russelii*) was investigated through nano-ESI-LCMS/MS of the reverse-phase HPLC fractions. A total of 54 venom proteins were identified and clustered into 11 protein families. Phospholipase A_2 (PLA_2, 63.8%) and Kunitz-type serine protease inhibitor (KSPI, 16.0%) were most abundant, followed by snake venom serine protease (SVSP, 5.5%, mainly Factor V activating enzyme), vascular endothelial growth factor (VEGF, 4.3%), snake venom metalloproteinase (SVMP, 2.5%, mainly Factor X activating enzyme) and phosphodiesterase (PDE, 2.5%). Other minor proteins include cysteine-rich secretory protein (CRiSP), snake venom C-type lectin/lectin-like protein (snaclec), nerve growth factor, L-amino acid oxidase and 5′-nucleotidase. PLA_2, KSPI, SVSP, snaclec and SVMP are hemotoxic proteins in the venom. The study indicated substantial venom variation in *D. russelii* venoms of different locales, including 3 Pakistani specimens kept in the USA. The venom exhibited potent procoagulant activity on human plasma (minimum clotting dose = 14.5 ng/ml) and high lethality (rodent LD_{50} = 0.19 μg/g) but lacked hemorrhagic effect locally. The Indian VINS Polyvalent Antivenom bound the venom immunologically in a concentration-dependent manner. It moderately neutralized the venom procoagulant and lethal effects (normalized potency against lethality = 2.7 mg venom neutralized per g antivenom).

Biological significance: Comprehensive venom proteomes of *D. russelii* from different locales will facilitate better understanding of the geographical variability of the venom in both qualitative and quantitative terms. This is essential to provide scientific basis for the interpretation of differences in the clinical presentation of Russell's viper envenomation. The study revealed a unique venom proteome of the Pakistani *D. russelii* from the wild (Indus Delta), in which PLA_2 predominated (~60% of total venom proteins). The finding unveiled remarkable differences in the venom compositions between the wild (present study) and the captive specimens reported previously. The integration of toxicity tests enabled the correlation of the venom proteome with the envenoming pathophysiology, where the venom showed potent lethality mediated through coagulopathic activity. The Indian VINS Polyvalent Antivenom (VPAV) showed binding activity toward the venom protein antigens; however the immunorecognition of small proteins and PLA_2-dominating fractions was low to moderate. Consistently, the antivenom neutralized the toxicity of the wild Pakistani Russell's viper venom at moderate efficacies. Our results suggest that it may be possible to enhance the Indian antivenom potency against the Pakistani viper venom by the inclusion of venoms from a wider geographical range including that from Pakistan into the immunogen formulation.

1. Introduction

The Russell's viper (family Viperidae, subfamily Viperinae, genus *Daboia*) is a medically important snake in South Asia and most parts of Southeast Asia [1,2]. The true viper, together with *Naja naja* (Indian cobra), *Bungarus caeruleus* (Indian krait) and *Echis carinatus* (saw-scaled viper) constitute 'The Big Four' or the four most medically important species of venomous snakes in the Indian subcontinent, including Pakistan. Snakebite cases in Pakistan are prevalent in the active agricultural areas as around the Indus Valley (Punjab) and the Indus Delta (Sindh) [3]. Of the Big Four in the region, the Russell's viper is considered as one of the most commonly encountered snakes. It is typically

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found in the rice fields and villages and propagates very fast in the farming areas where rodent preys are abundant.

Envenomation by Russell's viper (*Daboia* sp.) can lead to diverse pathological events including systemic coagulopathy, pituitary infarct, neurotoxicity, acute kidney injury, hemorrhages and tissue necrosis [4–9]. The clinical presentations of Russell's viper envenomation are known to vary across geographical regions; for instance, besides coagulopathy and renal failure, envenoming cases in Sri Lanka were reported to show prominent neurotoxicity and myotoxicity [5,10], while envenoming cases from Myanmar were largely hemorrhagic with chronic complication of pituitary infarct [11]. Recent phylogenetic analysis indicated that the Russell's vipers constitute two distinct species, *i.e. Daboia russelii* in the South Asia (west to the Bay of Bengal) and *Daboia siamensis* in the Southeast Asia (east to the Bay of Bengal), however, the variation in the clinical effects of envenoming does not necessarily conform to the systematics [12]. Instead, the variation (clinical presentation) appears to reflect potential venom variability of Russell's vipers from different geographical locales [13–16]. The phenomenon highlights the importance of comprehensive knowledge on venom composition and pathophysiological action of the snake venom, as this has an important ramification on the production and the use of antivenom in the region. This is because antivenoms are typically raised against venom from snakes of a particular geographical origin, and very often, these snakes are kept under captivity or are of captive-bred strains, whereas snake envenomation is largely inflicted by wild snakes in various places. The variation in snake venom composition may be accompanied by different protein antigenicity that leads to suboptimal immunoreactivity and weak neutralization by clinically used antivenoms [17,18]. Currently, the treatment of *D. russelii* envenoming in Pakistan relies mainly on the polyvalent antivenom imported from India (*e.g.* products of VINS or Bharat) which are raised against the Big Four snake venoms of Indian origin [19]. A recent study revealed that the Indian antivenom was reactive and effective *in vitro* against the procoagulant effect of Pakistani Russell's viper venom (unspecified locale origin), but the *in vivo* neutralization of the venom remains to be further investigated. In addition, the venom tested in the previous study was sourced from 3 captive specimens housed in a facility in the USA (Kentucky Reptile Zoo) [20], hence it is relevant and important to also study the local specimens, in particular those from the wild, since long captivity may be a contributing factor to intraspecific snake venom variation [21–23].

With the advent of proteomics, the venom compositions of Russell's vipers from several locales have been profiled, including that of *D. russelii siamensis* (proper nomenclature: *D. siamensis*) from Myanmar and *D. russelii* from India, Pakistan and Sri Lanka [20,24,25]. The reported venom profiles varied to some extent among the studies, with notable differences in terms of the protein type/family detected and the protein abundance quantitated. The observation was likely reflective of true geographical venom variability, although it could also be partly confounded by the proteomic or analytical method. Knowledge on the venom composition of Russell's viper venom especially that of authenticated specimen from known locality, is very crucial to improve our understanding of the venom complexity and variability. Furthermore, when coupled with functional studies, the knowledge will contribute toward elucidating the clinical pathophysiology of Russell's viper envenoming and how antivenom production and use can be improved.

In this study, we aimed to investigate the venom proteome of wild Pakistani Russell's vipers through a protein decomplexation approach using reverse-phase high performance liquid chromatography (RP-HPLC) followed by nano-electrospray ionization (ESI) liquid chromatography tandem mass spectrometry (nano-ESI-LCMS/MS) of the digested peptides. The specimens were wild adult snakes collected in Pakistan (specifically the Indus Delta). The preliminary findings indicated substantial variations from the venom proteome reported recently for the Pakistani *D. russelii* of a different source, which was a venom sample of 3 captive snakes from a serpentarium in the USA [20]. In addition, we also examined the effectiveness of the commonly used India-manufactured polyvalent antivenom in neutralizing the procoagulant and lethal effects of the venom. It is hoped that the findings will shed light on the venom variability of Russell's viper, thus propelling the improvement of antivenom production and use in the region. The proteomic results will also enrich the database for comparison to venom proteomes of Russell's vipers of various geographical regions.

2. Material and methods

2.1. Venoms and antivenom

Daboia russelii (Indus Delta, Pakistan) venom was milked and pooled from > 10 adult snakes authenticated by author NQ. The venom was lyophilized and stored at −20 °C prior to use. The antivenom used was Indian VINS Polyvalent Antivenom (VPAV, Batch no:01AS12041, expiry date: March 2016, used before expiry) reconstituted in 10 ml of sterile water prior to use. This antivenom was obtained from the sera of horses hyperimmunized against venoms of the Indian “Big Four”: *Naja naja* (Indian cobra), *Bungarus caerelus* (common krait), *Daboia russelii* (Russell's viper), and *Echis carinatus* (saw-scaled viper).

2.2. Mice and ethics clearance

The albino ICR strain (20–25 g) mice were supplied by the Animal Experimental Unit, University of Malaya. Animal studies protocol was designed based on the Council for International Organizations of Medical Sciences (CIOMS) guidelines on animal experimentation [26]. The protocol was approved by the Faculty of Medicine Institutional Animal Care and Use Committee of the University of Malaya (Ref: 2014–09-11/PHAR/R/TCH).

2.3. Materials

Ammonium bicarbonate, dithiothreitol (DTT) and iodoacetamide were purchased from Sigma-Aldrich (USA). Mass-spectrometry grade trypsin protease, Spectra™ Multicolor Broad Range Protein Ladder (10 to 260 kDa), and HPLC grade solvents used in the studies were purchased from Thermo Scientific™ Pierce™ (USA). Millipore ZipTip® C_{18} Pipette Tips were obtained from Merck (USA). Other chemicals and reagents of analytical grade were purchased from Sigma-Aldrich (USA).

2.4. C_{18} reverse-phase HPLC of the Pakistani D. russelii venom

The lyophilized venom (3 mg) was reconstituted in 0.1% trifluoroacetic acid (TFA) and centrifuged at 10,000*g* for 12 min at 4 °C. The supernatant was then fractionated by a LiChrospher® WP 300 C_{18} reverse-phase column (250 mm × 4.6 mm, 100 Å) using a Shimadzu LC-20AD HPLC system. Elution was carried out at a flow rate of 1 ml/min [27], using a linear gradient of 0.1% TFA in water (Solvent A) and 0.1% TFA in 100% acetonitrile (Solvent B) (0–5% B for 10 min, followed by 5–30% B over 30 min, 30–55% B over 150 min and 50–70% B over 170 min). Protein elution was monitored at 215 nm. The eluted fractions were subjected to SDS-PAGE and separately analyzed by nano-ESI-LCMS/MS shotgun proteomic analysis after in-solution tryptic digestion.

2.5. SDS PAGE of venom and HPLC fractions

SDS-PAGE was conducted according to Laemmli [28]. The Prestained Protein Ladder (10–260 kDa) was used for calibration. The crude venom (50 μg) and HPLC-eluted protein fractions were individually loaded onto a 15% gel and electrophoresis was performed under reducing condition at 90 V for 2 h. Proteins were visualized using Coomassie Brilliant Blue R-250. The relative density of protein band

was analyzed using myImage Analysis software (Thermo Scientific).

2.6. Nano-electrospray ionization-liquid chromatography and tandem mass spectrometry (nano-ESI-LCMS/MS)

The protein fractions obtained from RP-HPLC were subjected to reduction by DTT, alkylation by iodoacetamide, and then digestion by trypsin [29]. The trypsin-digested peptides were desalted with Millipore ZipTip® C_{18} Pipette Tips (Merck, USA) and subjected to nano-ESI-LCMS/MS analysis, using an Agilent 1260 Infinity Nanoflow LC system (Agilent, Santa Clara, CA, USA) connected to Accurate-Mass Q-TOF 6550 series with a nano-electrospray ionization source. Samples were reconstituted in 7 µl of 0.1% formic acid in water and loaded onto HPLC Large-Capacity Chip Column Zorbax 300-SB-C18 (160 nL enrichment column, 75 µm × 150 mm analytical column and 5 µm particles) (Agilent, Santa Clara, CA, USA). Injection volume was adjusted to 1 µl per sample and the sample was eluted at a flow rate of 0.4 µl/min, with linear gradient of 5–7% of solvent B (0.1% formic acid in 100% acetonitrile). Drying gas flow was 11 l/min and drying gas temperature was 290 °C. Fragmentor voltage was set to 175 V and capillary voltage, 1800 V. Mass spectra was acquired using Mass Hunter acquisition software (Agilent, Santa Clara, CA, USA) in a MS/MS mode with a MS scan range of 200–3000 *m/z* and MS/MS scan range of 50–3200 m/z. Data were extracted with MH^+ mass range between 50 and 3200 Da and processed with Agilent Spectrum Mill MS Proteomics Workbench software package version B.04.00 against merged database incorporating non-redundant NCBI database of Serpentes (taxid:8570) and in-house venom-gland transcript database. Carbamidomethylation was specified as a fixed modification while oxidized methionine as a variable modification. Protein identifications were validated with the following filters: protein score > 20, peptides score > 10 and scored peak intensity (SPI) > 70%. Only results with "Distinct Peptide" identification of two or greater than two are considered significant. Identified proteins were filtered to achieve false discovery rate (FDR) < 1% for the peptide-spectrum matches. Protein abundance was estimated based on the area under the curve of HPLC fractions, and normalized by the relative peptide intensity of respective protein as reported previously [27]. For example, the formula used to estimate the relative abundance of protein x in HPLC-fraction y is given as follows:

$$\text{Protein X abundance in fraction Y} = \frac{\text{Mean spectral intensity of protein X in faction Y}}{\text{Total spectral intensity of all proteins in fraction Y}} \times \text{Area under curve of fraction Y (\%)}$$

2.7. Biological and toxic activities of D. russelii venom

2.7.1. Determination of procoagulant activity

Procoagulant activity of the venom was determined using human citrated plasma as substrate. Venom samples (100 µl, diluted in normal saline to various concentrations) were loaded into microplate wells. Citrated human plasma (100 µl), containing 40 µl 0.4 M $CaCl_2$/ml, was then added simultaneously to each well using a multichannel pipette. Coagulation activity was measured using the turbidimetric method [30], where the formation of clots was monitored at 405 nm (absorbance) every 30 s at 37 °C for 15 min, using the Tecan M1000Pro Multimode plate reader (TECAN, Switzerland). The clotting time was determined as the time when the absorbance was 0.02 U greater than the mean of the first two absorbance measurement. The minimum clotting dose (MCD) was defined as the venom concentration that induced substrate coagulation in 3 min.

2.7.2. Screening of venom hemorrhagic activity

The venom (5, 10 and 20 µg) was dissolved in 50 µl saline and injected intradermally into the back of isoflurane-sedated mice (n = 2 per dose). After 90 min, the mice were euthanized with CO_2 overdose and the back skin was exposed for inspection of hemorrhagic lesion.

2.7.3. Determination of venom lethality

The venom was dissolved in saline to varying concentrations in a total volume of 100 µl, and injected intravenously *via* caudal vein into mice (20–25 g). The mice were allowed access to food and water *ad libitum*. The survival ratio was recorded after 24 h and the LD_{50} was calculated using the Probit analysis method [31].

2.8. Immunological binding assay of venom

The immunological binding activities between antivenom and venom antigens were tested with an indirect enzyme-linked immunosorbent assay (ELISA) modified from Tan et al. [32]. Immunomicroplate wells were pre-coated overnight at 4 °C with 10 ng of venoms (Pakistani *D. russelii*, Indian *D. russelii*, or *C. rhodostoma*). The venoms of Indian *D. russelii* and *C. rhodostoma* served as positive and negative reference controls, respectively. The venoms were then discarded, the wells were flicked dried and washed four times with phosphate-buffered saline containing 0.5% Tween®20 (PBST). Antivenom (VPAV) was prepared at 20 mg/ml protein concentration, and diluted by 1:300 to 1:24300 in PBST to a total volume of 100 µl. The antivenom in various dilutions were added into venom-coated well, followed by incubation for 1 h at room temperature. The wells were washed four times with PBST before adding horseradish peroxidase-conjugated anti-horse-IgG (Jackson ImmunoResearch Inc., USA) in PBST (1,8000), followed by incubation at room temperature for another hour. The wells were then washed four times with PBST before adding the freshly prepared substrate (100 µl of 0.5 mg/ml *o*-phenylenediamine and 0.006% hydrogen peroxide in 0.1 M citrate-phosphate buffer, pH 5.0). The plate was left for 30 min at room temperature in the dark for enzymatic reaction to take place. The reaction was then terminated by adding 50 µl of 12.5% sulfuric acid per well, and the absorbance was read at 492 nm against a blank using a Tecan M1000Pro Multimode plate reader (TECAN, Switzerland). Immunological binding activity to venom was measured in absorbance unit against the varying dilutions of antivenom. The absorbance values are expressed in mean ± S.E.M. of triplicate experiments. The median effective concentration (EC_{50}) for the antivenom binding activity was defined as the concentration of antivenom that produced 50% binding of venom antigens (corresponding to half maximum absorbance).

2.9. HPLC/ELISA-based immunoprofiling of venom

The assay was performed for assessing the immunorecognition of HPLC-eluted venom components by antivenom. The HPLC-eluted venom proteins were freeze-dried and reconstituted in water. Ten nanogram of venom proteins from each eluted fraction was further dissolved in coating buffer to 100 µl, followed by overnight coating on the immuno-microplate wells. The subsequent steps were similar to the indirect ELISA outlined in Section 2.8, except that the dilution of the antivenom (VPAV) was standardized at 1:2700 after optimization.

2.10. Neutralization of venom procoagulant effect by VINS polyvalent antivenom (VPAV)

For *in vitro* procoagulant neutralization, the venom samples at a dose of 2 MCD were pre-incubated with various dilutions of VPAV at 37 °C for 30 min (the volume of venom-antivenom mixture was 50 µl). Citrated human plasma (100 µl) containing 40 µl 0.4 M $CaCl_2$/ml was then added simultaneously into each well using a multichannel pipette. The clotting time was determined as the time when the absorbance was 0.02 U greater than the mean of the first two absorbance measurement. The effective dose (ED) of VPAV neutralizing the procoagulant effect was defined as the ratio of µg venom/µl antivenom that prolonged the

clotting time of the citrated human plasma three times that of the control (2 MCD of venom, without antivenom).

2.11. Neutralization of venom lethality by antivenom

The potency of antivenom neutralization of venom lethality was determined using an immunocomplexation assay as described in Tan et al. [33]. A challenge dose of the venom (5 LD_{50}) was preincubated with various dilutions of VPAV at 37 °C for 30 min for immunocomplexation reaction. Following brief centrifugation, the supernatant mixture was then injected intravenously into the mice *via* the caudal vein. The mice were monitored with free access to food and water. The number of survival was recorded after 24 h. Neutralizing potency of the antivenom was expressed as ED_{50} (the volume of antivenom in μl that gave 50% survival rate) and ER_{50} (the ratio of venom amount to ED_{50} when there was 50% survival rate) as well as "neutralization potency" (P, the amount of venom completely neutralized per unit volume of antivenom) [29,34]. The potency was then divided by the protein content of the antivenom to yield the "normalized potency" (n-P, the amount of venom completely neutralized per unit mass of antivenom proteins). The ED_{50} and the 95% C.I. of the *in vivo* neutralization of lethality were calculated by Probit analysis [31].

3. Results

3.1. The venom proteome of Pakistani D. russelii

Fig. 1 shows the reverse-phase HPLC profile of the Pakistani *D. russelii* venom and the SDS-PAGE of the fractions as well as the crude venom under reducing conditions. The venom was resolved into 15 HPLC fractions, and the proteins identified through LCMS/MS were assigned by the respective fractions as shown in Table 1. Mass spectrometric data and tryptic peptide sequences matched to the database were provided in Supplementary Table S1. A large amount of proteins with low molecular weight below 20 kDa was present on the SDS-PAGE profile, especially from fractions 9 to 12 (~60% of total gel band density). Altogether, a total of 54 distinct proteins belonging to 11 different protein families were identified (Table 2). The most abundant protein was phospholipase A_2 (PLA_2, 63.8%), which were eluted mainly through fractions 9–12 (AUC ~60%) with intense staining around 12–15 kDa of molecular weight range on the gel. Consistently, the crude venom protein band between 13 and 15 kDa (typical PLA_2 molecular weight) showed a high relative density estimated to be 62.3%. Kunitz-type serine protease inhibitor (KSPI, 16.0%) constituted the second most abundant protein in the venom. There were four other protein families with relative abundances > 2% each, including snake venom serine protease (SVSP, 5.5%), vascular endothelial growth factor (VEGF, 4.3%), snake venom metalloproteinase (SVMP, 2.5%) and phosphodiesterase (PDE, 2.5%). Other protein families of lower abundances were cysteine-rich secretory protein (CRiSP, 1.3%), snaclec (C-type lectin/lectin-like proteins, 1.3%), venom nerve growth factor (VNGF, 1.1%), L-amino acid oxidase (LAAO, 0.8%), 5′-nucleotidase (5′-NUC, 0.1%), and approximately 0.7% of unidentified proteins (Fig. 2). The majority of the Pakistani *D. russelii* venom proteins identified were annotated to proteins of Russell's viper (*D. russelii* or *D. siamensis*) in the database during bioinformatics analysis (Table 2).

3.2. Procoagulant effect of the venom and its neutralization by antivenom

The Pakistani *D. russelii* venom exhibited dose-dependent procoagulant activity on human citrated plasma, with a minimal coagulant dose (MCD) of 14.5 ng/ml. The procoagulation effect of the venom at 2 MCD was neutralized by VPAV with an effective dose (ED) of 0.64 μg/μl (Table 3).

3.3. Screening of hemorrhagic effect of the venom

The venom when inoculated intradermally did not elicit significant skin hemorrhage over 90 min up to the dose of 20 μg venom. The dermis examined did not have obvious signs of vascular congestion or blood exudation (image not shown).

3.4. Lethality of the venom and its neutralization by antivenom

Table 4 shows the parameters of the venom lethality and its neutralization using VPAV. The intravenous LD_{50} of the venom was determined to be 0.19 μg/g and the Indian polyvalent antivenom neutralized it with a moderate ED_{50} of 78.29 μl (against 5 LD_{50}) or a normalized potency of 2.7 mg/g antivenom.

3.5. Immunological binding of venom-antivenom antigens and HPLC/ELISA-based immunoprofiling of venom

The immunological binding activities of VPAV toward the antigens of *D. russelii* venom are shown in Fig. 3a. VPAV exhibited a concentration-dependent increase in binding activity toward the Indian and Pakistani *D. russelii* venom antigens. The antivenom bound more effectively toward the venom of Indian origin (positive control) compared with that of the Pakistani origin. The half effective concentrations (EC_{50}) of VPAV binding were 6.77 ± 0.29 μg/ml and 3.40 ± 0.06 μg/ml for the Pakistani and the Indian venom samples, respectively. Meanwhile, VPAV binding to *C. rhodostoma* venom proteins (negative control) was weak throughout and was negligible at high dilutions of the antivenom.

Fig. 3b shows the immunoreactivity of the venom protein fractions toward VPAV. Fractions P1-P11 showed low immuno-binding activities with absorbance ranging from below 0.1 to 0.3. The immunoreactivity increased in fractions P12, P13, P14 and P15 (0.6–2.0 absorbance unit), with P14 exhibiting the strongest binding activity with VPAV.

4. Discussion

The venomics of the wild Pakistani *D. russelii* from the Indus Delta revealed a venom profile in which PLA_2 predominated (63.8% of total venom proteins), a unique feature that is distinct from previously reported quantitative venom proteomes for the Sri Lankan (~35%) [25], western Indian (~32%) [24] and Pakistani (~33%, unspecified Pakistan locale, captive specimens) [20] origins. The SVMP content was, however, low in the venom (~2.5%) compared with the reported 22% in the captive Pakistani *D. russelii* venom [20], the western Indian (25%) [24] and the Sri Lankan (6%) [25] venom samples. In terms of SVSP, while the Sri Lankan *D. russelii* venom had a higher abundance (~16%), the western Indian and the two Pakistani samples (one from this study) showed a lower proportion of SVSP (3.2–8%). These are the major viperid enzymes commonly involved in the pathogenesis of hemotoxic envenomation (particularly coagulopathic and hemorrhagic complications) [35], and the present study of wild Pakistani specimens implies that the venom composition variability is not confined only to geographical and phylogenetic factors but may be noted even within the same species of Pakistani origin (formerly known as *D. russelii sochureki*). The origin of the observed variation is unclear, but it may be postulated that living in the wild or living in captivity could have conditioned or altered the venom production and toxin composition in adaptation to the change in feeding behavior and prey items. The proteomic differences need to be interpreted in the light of the pathophysiological activities of the venom, and importantly the therapeutic responses to antivenom as the compositional variability may affect the immunoreactivity of the venom proteins.

Coagulopathy is the established key clinical feature of *D. russelii* envenoming [7,36,37], and PLA_2 is among the coagulopathic toxins known for Russell's viper venom. Previous proteomic studies have

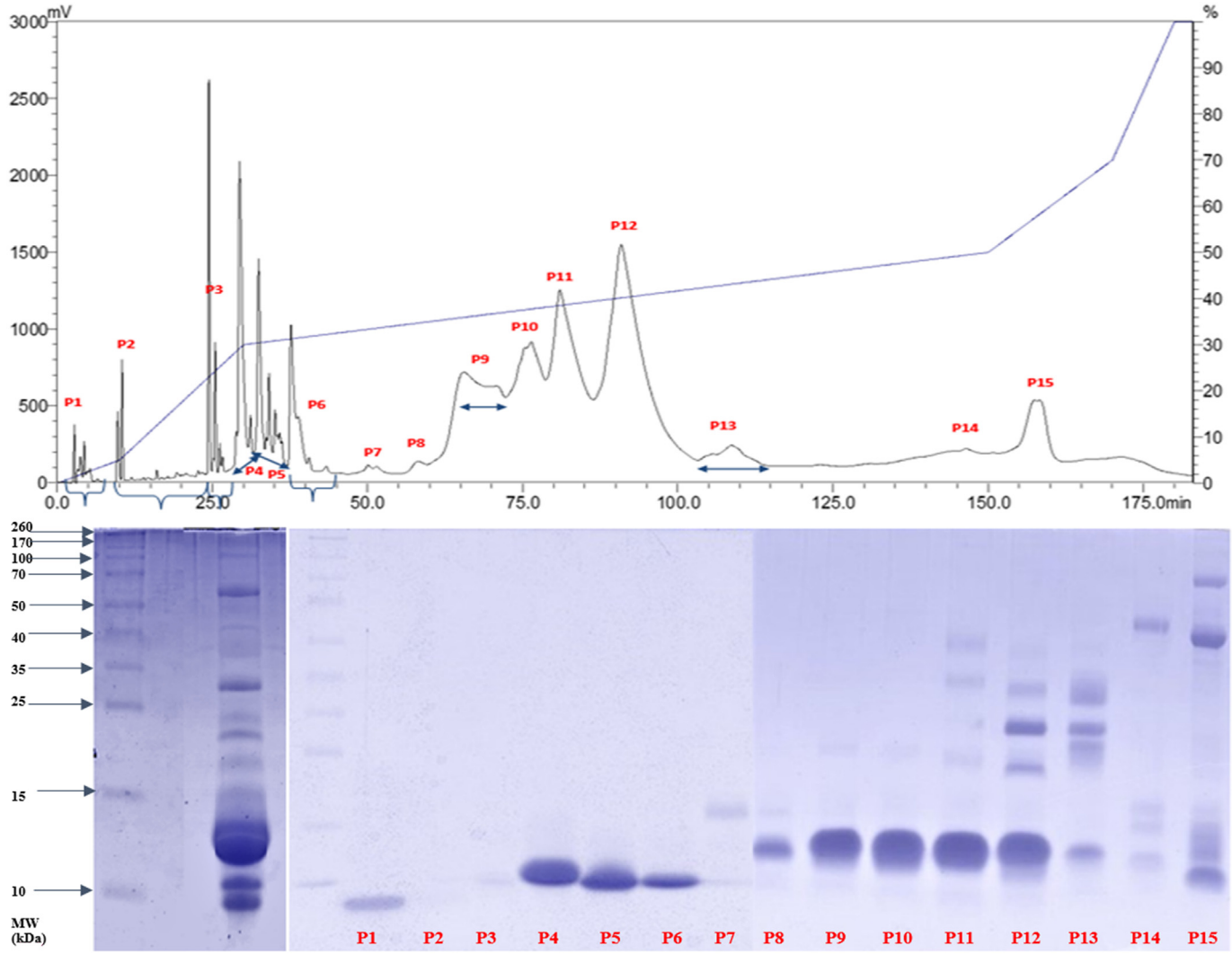

Fig. 1. Decomplexation of the wild Pakistani *Daboia russelii* venom proteins. Upper panel: C_{18} reverse-phase high performance liquid chromatography (RP-HPLC) of *Daboia russelii* venom (3 mg). Lower panel: 15% SDS-PAGE of the crude venom (next to protein ladder) and HPLC venom fractions under reducing conditions.

shown that PLA_2 constitutes the major toxin component in most Russell's viper venoms [20,24,25], while it has also been reported that snake venom PLA_2 can exhibit a variety of pharmacological activities [38]. In the present study, most of the PLA_2 forms detected were annotated to PLA_2 of Russell's viper, with the dominant form being homologous to the basic PLA_2 RVV-VD (P81458, 26%) and acidic PLA_2-RV-7 (14%), based on similarity in conserved sequences and motifs of the proteins. Phospholipase A_2 RVV-VD (a class II anticoagulant toxin) has a low enzymatic activity but is a strong anticoagulant [39]. The active site conveying the anticoagulancy consists of a negatively charged part of glutamic acid residue ($Glu^{52/53}$), together with a positively charged ridge of lysine [40], corresponding to one of the tryptic peptides detected for the protein in this study (^{42}RCCFVHDCCYEKV^{54}). Recent research studies have also shown that the acidic PLA_2 of Pakistani Russell's viper exhibited anticoagulant activity [41,42], and this may be applied to the abundant PLA_2 currently identified in the Pakistani *D. russelii* venom from the wild. Individually, the PLA_2 RV-7 protein was reported earlier to be non-toxic with low enzymatic activity but could potentiate presynaptic neurotoxicity through dimerization with other PLA_2 components in the eastern Russell's viper (*D. siamensis*) venom, although neurotoxicity is not a commonly observed clinical feature of Russell's viper envenoming in the east [39]. The same applies to the presence of several other PLA_2 forms in the present study. When tested *in vitro*, many of these PLA_2 exhibited neurotoxic activity at varying degrees [43]; however, neuromuscular paralysis induced by Russell's viper bites in the northern India and Pakistan, as well as in Southeast Asia is not commonly reported [12,44]. The neurotoxicity induced by *D. siamensis* PLA_2 in laboratory animals hence probably reflects the complex interactions between toxins and the neurons of different specificity in animals. It is possible that the natural prey such as rodents appear to be more susceptible to the PLA_2 neurotoxins than human beings are [45].

KSPIs are low molecular weight proteins (~6–7 kDa) present commonly in viperid venoms including that of *D. russelii*. The biological function of this toxin group was thought to be primarily disrupting blood coagulation and blood pressure regulation, a strategy employed by the vipers in ambush to prey upon mammals, as the resulting hemostatic derangement and hemodynamic instability lead to subjugation or death of the prey [46]. Mukherjee et al. [47] reported a KSPI termed Rusvikunin with anticoagulant and antiplasmin activities from *D. russelii* venom, supporting that the protease inhibitor may play a pathological role in envenomation where it contributes to the overall systemic coagulopathy. From the application perspective, the protein may be targeted in drug discovery as a functional treatment or prophylaxis for cardiovascular disorders. The major KSPI subtype detected in the present study highly resembles DrKln-I (H6VC05) which is a potent inhibitor of activated protein C, and it has a synergistic action with the snake venom metalloproteinase RVV-X (Q7LZ61) in promoting

Table 1
Protein assignment of *Daboia russelii* of Pakistan (*DrP*) venom by fractions of reversed-phase HPLC. Data were obtained by ESI-LCMS/MC analysis of in-solution digested peptides.

Fraction	Protein ID	Protein name[a]	Database accession/species name	Protein score	Distinct peptides matched[b]	Relative abundance (%)
1	–	–	–	–	–	0.7%
3	*DrP 1*	Kunitz-type serine protease inhibitor DrKIn-I	H6VC05 *(D. r. russelii)*	38.28	2	1.8%
	DrP 2	Kunitz-type serine protease inhibitor C6	A8Y7N9 *(D. siamensis)*	30.26	1	0.8%
4	*DrP 1*	Kunitz-type serine protease inhibitor DrKIn-I	H6VC05 *(D. r. russelii)*	38.74	2	3.8%
	DrP 2	Kunitz-type serine protease inhibitor C6	A8Y7N9 *(D. siamensis)*	31.25	1	1.1%
5	*DrP 1*	Kunitz-type serine protease inhibitor DrKIn-I	H6VC05 *(D. r. russelii)*	23.17	1	0.2%
	DrP 2	Kunitz-type serine protease inhibitor C6	A8Y7N9 *(D. siamensis)*	27.00	1	0.1%
	DrP 3	Trypsin inhibitor-4 precursor	A8Y7N7 *(D. siamensis)*	151.11	7	0.5%
	DrP 4	Kunitz-type serine protease inhibitor 2	P00990 *(D. siamensis)*	62.23	3	1.5%
	DrP 5	Kunitz-type serine protease inhibitor DrKIn-II	H6VC06 *(D. r. russelii)*	47.24	2	0.5%
	DrP 6	Kunitz-type serine protease inhibitor C8	P85039 *(D. siamensis)*	43.54	2	1.5%
	DrP 7	Protease inhibitor 4	Unigene17386_DRSL *(D. r. russelii)*	42.65	2	0.4%
6	*DrP 4*	Kunitz-type serine protease inhibitor 2	P00990 *(D. siamensis)*	65.03	4	2.2%
	DrP 8	Factor X activator heavy chain	K9JAW0 *(D. r. russelii)*	50.77	3	0.1%
	DrP 7	Protease inhibitor 4	Unigene17386_DRSL *(D. r. russelii)*	40.24	2	0.3%
	DrP 9	Basic phospholipase A_2 Drk-b2	A8CG90 *(D. r. russelii)*	36.15	2	0.1%
	DrP 1	Kunitz-type serine protease inhibitor DrKIn-I	H6VC05 *(D. r. russelii)*	34.31	2	0.2%
	DrP 10	Snake venom vascular endothelial growth factor	Unigene30051_DRSL *(D. russelii)*	25.33	1	0.6%
	DrP 11	Snake venom vascular endothelial growth factor toxin VR-1	P67861 *(D. r. russelii)*	20.97	1	0.5%
7	*DrP 12*	Venom nerve growth factor	P30894 *(D. r. russelii)*	135.01	8	0.4%
	DrP 13	Venom nerve growth factor	V9I168 *(V. ursinii)*	87.86	5	0.7%
	DrP 14	Basic phospholipase A_2 RVV-VD	P81458 *(D. r. russelii)*	89.71	6	0.1%
	DrP 4	Kunitz-type serine protease inhibitor 2	P00990 *(D. siamensis)*	74.24	4	~0.1%
	DrP 1	Kunitz-type serine protease inhibitor DrKIn-I	H6VC05 *(D. r. russelii)*	72.46	4	0.1%
	DrP 15	Basic phospholipase A_2	B3RFI7 *(D. r. limitis)*	54.44	3	~0.1%
	DrP 16	Kunitz-type protease inhibitor	H9BFA3 *(D. russelii)*	40.92	2	~0.1%
	DrP 17	Acidic phospholipase A_2 daboiatoxin B chain	Q7T3T5 *(D. siamensis)*	32.42	2	~0.01%
	DrP 18	Kunitz-type serine protease inhibitor 2	Q2ES49 *(D. r. russelii)*	26.89	2	0.1%
8	*DrP 14*	Basic phospholipase A_2 RVV-VD	P81458 *(D. r. russelii)*	182.80	10	0.1%
	DrP 10	Snake venom vascular endothelial growth factor	Unigene30051_DRSL *(D. russelii)*	94.16	5	0.2%
	DrP 11	Snake venom vascular endothelial growth factor toxin VR-1	P67861 *(D. r. russelii)*	93.55	5	0.4%
	DrP 12	Venom nerve growth factor	P30894 *(D. r. russelii)*	70.28	4	~0.1%
	DrP 1	Kunitz-type serine protease inhibitor DrKIn-I	H6VC05 *(D. r. russelii)*	59.57	3	~0.1%
	DrP 19	Kunitz-type serine protease inhibitor B6	A8Y7P6 *(D. siamensis)*	56.35	3	0.1%
	DrP 16	Kunitz-type protease inhibitor	H9BFA3 *(D. russelii)*	58.31	3	~0.1%
	DrP 4	Kunitz-type serine protease inhibitor 2	P00990 *(D. siamensis)*	57.82	3	~0.1%
	DrP 5	Kunitz-type serine protease inhibitor DrKIn-II	H6VC06 *(D. r. russelii)*	46.03	2	~0.1%
	DrP 15	Basic phospholipase A_2	B3RFI7 *(D. r. limitis)*	37.32	2	~0.01%
9	*DrP 14*	Basic phospholipase A_2 RVV-VD	P81458 *(D. r. russelii)*	223.81	12	11.8%
	DrP 10	Snake venom vascular endothelial growth factor	Unigene30051_DRSL *(D. russelii)*	41.29	2	0.7%
	DrP 11	Snake venom vascular endothelial growth factor toxin VR-1	P67861 *(D. r. russelii)*	42.99	2	1.5%
	DrP 1	Kunitz-type serine protease inhibitor DrKIn-I	H6VC05 *(D. r. russelii)*	30.51	2	0.2%
10	*DrP 14*	Basic phospholipase A_2 RVV-VD	P81458 *(D. r. russelii)*	209.22	12	5.6%
	DrP 15	Basic phospholipase A_2	B3RFI7 *(D. r. limitis)*	145.57	8	4.6%
	DrP 20	Ammodytin I1 (A) variant	Q6A3M8 *(V. a. ruffoi)*	36.64	2	0.5%
	DrP 21	Acidic phospholipase A_2 Drk-a2	A8CG87 *(D. r. russelii)*	30.34	2	0.2%
	DrP 11	Snake venom vascular endothelial growth factor toxin VR-1	P67861 *(D. r. russelii)*	21.31	1	0.3%
11	*DrP 14*	Basic phospholipase A_2 RVV-VD	P81458 *(D. r. russelii)*	196.58	11	5.1%
	DrP 9	Basic phospholipase A_2 Drk-b2	A8CG90 *(D. r. russelii)*	90.36	5	2.5%
	DrP 22	Phospholipase A_2 (EC 3.1.1.4)	B2YHV1 *(D. russelii)*	112.77	6	7.6%
	DrP 1	Kunitz-type serine protease inhibitor DrKIn-I	H6VC05 (*D. r. russelii*)	31.74	2	0.2%

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Table 1 (*continued*)

Fraction	Protein ID	Protein name[a]	Database accession/species name	Protein score	Distinct peptides matched[b]	Relative abundance (%)
12	*DrP 14*	Basic phospholipase A_2 RVV-VD	P81458 *(D. r. russelii)*	220.00	13	1.8%
	DrP 9	Basic phospholipase A_2 Drk-b2	A8CG90 *(D. r. russelii)*	93.55	6	0.6%
	DrP 15	Basic phospholipase A_2	B3RFI7 *(D. r. limitis)*	77.23	4	0.3%
	DrP 1	Kunitz-type serine protease inhibitor DrKIn-I	H6VC05 *(D. r. russelii)*	40.28	2	0.1%
	DrP 23	Phospholipase A_2-IV	Q2ES51 *(D. r. russelii)*	62.47	4	3.0%
	DrP 24	Acidic phospholipase A_2 RV-7	P31100 *(D. russelii)*	163.64	8	13.3%
	DrP 25	Acidic phospholipase A_2 Drk-a1	A8CG86 *(D. r. russelii)*	69.25	4	2.9%
	DrP 26	Factor V activator RVV-V gamma	P18965 *(D. siamensis)*	160.22	10	1.1%
	DrP 27	Beta-fibrinogenase-like	E5L0E4 *(D. siamensis)*	109.03	7	0.4%
	DrP 28	Serine protease VLSP-3	E0Y420 *(M. lebetina)*	69.68	4	0.3%
	DrP 29	Ancrod-like protein	A1E236 *(C. rhodostoma)*	38.16	2	0.2%
	DrP 30	Cysteine-rich secretory protein Da-CRPa	F2Q6G0 *(D. acutus)*	81.28	4	0.6%
	DrP 31	Venom serine proteinase-like protein 2	Q9PT40 *(M. lebetina)*	70.65	4	0.3%
	DrP 32	Cysteine-rich secretory protein Dr-CRPK	CL3658.Contig2_DRSL *(D. russelii)*	64.68	4	0.5%
	DrP 33	Vipera russelli proteinase RVV-V homolog 2	P86531 *(D. r. russelii)*	26.91	1	1.2%
13	*DrP 34*	Factor V activator RVV-V alpha	P18964 *(D. siamensis)*	210.74	12	0.3%
	DrP 35	Serine protease VLSP-1	CL2958.Contig6_DRSL *(D. russelii)*	148.20	7	1.0%
	DrP 14	Basic phospholipase A_2 RVV-VD	P81458 *(D. r. russelii)*	133.75	8	0.3%
	DrP 23	Phospholipase A_2-IV	Q2ES51 *(D. r. russelii)*	62.73	4	0.5%
	DrP 36	Alpha-fibrinogenase-like	E5L0E3 *(D. siamensis)*	131.15	9	0.2%
	DrP 31	Venom serine proteinase-like protein 2	Q9PT40 *(M. lebetina)*	60.03	4	0.1%
	DrP 8	Factor X activator heavy chain	K9JAW0 *(D. r. russelii)*	90.29	5	~0.1%
	DrP 37	Coagulation factor X activating enzyme heavy chain	Q7LZ61 *(D. siamensis)*	45.26	3	0.1%
	DrP 15	Basic phospholipase A_2	B3RFI7 *(D. r. limitis)*	84.40	4	0.1%
	DrP 24	Acidic phospholipase A_2 RV-7	P31100 *(D. russelii)*	82.15	4	0.5%
	DrP 38	Chain A, Crystal Structure Of Rv4RV7 COMPLEX	CL3663.Contig1_DRSL *(D. russelii)*	77.23	4	0.5%
	DrP 25	Acidic phospholipase A_2 Drk-a1	A8CG86 *(D. r. russelii)*	70.66	4	0.4%
	DrP 11	Snake venom vascular endothelial growth factor toxin VR-1	P67861 *(D. r. russelii)*	51.35	3	0.1%
	DrP 1	Kunitz-type serine protease inhibitor DrKIn-I	H6VC05 *(D. r. russelii)*	35.76	2	0.1%
	DrP 32	Cysteine-rich secretory protein Dr-CRPK	CL3658.Contig2_DRSL *(D. russelii)*	32.56	2	~0.1%
	DrP 30	Cysteine-rich secretory protein Da-CRPa	F2Q6G0 *(D. acutus)*	32.56	2	~0.1%
	DrP 39	L-amino-acid oxidase	G8XQX1 *(D. r. russelii)*	31.28	2	~0.1%
	DrP 33	Vipera russelli proteinase RVV-V homolog 2	P86531 *(D. r. russelii)*	26.62	1	0.3%
14	*DrP 8*	Factor X activator heavy chain	K9JAW0 *(D. r. russelii)*	389.03	22	0.5%
	DrP 37	RVV-X-heavy chain	Q7LZ61 *(D. russelii)*	309.64	17	0.5%
	DrP 14	Basic phospholipase A_2 RVV-VD	P81458 *(D. r. russelii)*	82.50	5	0.3%
	DrP 26	Factor V activator RVV-V gamma	P18965 *(D. siamensis)*	48.33	3	~0.1%
	DrP 40	Coagulation factor X activating enzyme light chain	Q4PRD1 *(D. siamensis)*	34.76	2	~0.1%
	DrP 41	Dabocetin alpha subunit	K9JBU0 *(D. r. russelii)*	32.72	2	~0.1%
	DrP 1	Kunitz-type serine protease inhibitor DrKIn-I	H6VC05 *(D. r. russelii)*	32.40	2	~0.1%
	DrP 42	Dabocetin beta subunit	Unigene920_DRSL *(D. r. russelii)*	27.84	2	~0.1%
	DrP 24	Acidic phospholipase A_2 RV-7	P31100 *(D. russelii)*	23.77	1	~0.1%
	DrP 43	Factor X activator light chain 2	CL1101.Contig1_DRSL *(D. siamensis)*	22.28	2	0.1%

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Table 1 (*continued*)

Fraction	Protein ID	Protein name[a]	Database accession/species name	Protein score	Distinct peptides matched[b]	Relative abundance (%)
15	*DrP 44*	Phosphodiesterase 1	CL3655.Contig2_DRSL *(D. russelii)*	491.49	27	0.2%
	DrP 45	Phosphodiesterase	U3TBJ5 *(O. okinavensis)*	168.67	10	0.4%
	DrP 46	Phosphodiesterase 1	Unigene21602_TW *(T. wagleri)*	27.74	2	1.9%
	DrP 39	L-amino-acid oxidase	G8XQX1 *(D. r. russelii)*	394.67	20	0.3%
	DrP 47	L-amino-acid oxidase	Q4F867 *(D. siamensis)*	290.25	14	0.3%
	DrP 48	L-amino acid oxidase Bs29	A0A024BTN9 *(B. schlegelii)*	52.32	3	0.1%
	DrP 8	Factor X activator heavy chain	K9JAW0 *(D. r. russelii)*	267.85	15	0.7%
	DrP 37	RVV-X-heavy chain	Q7LZ61 *(D. russelii)*	171.54	10	0.6%
	DrP 49	Snake venom 5′-nucleotidase	CL3322.Contig1_DRSL *(D. russelii)*	247.59	14	0.1%
	DrP 50	P68 alpha subunit	K9JDF2 *(D. r. limitis)*	140.13	8	0.5%
	DrP 14	Basic phospholipase A_2 RVV-VD	P81458 *(D. r. russelii)*	95.81	6	0.8%
	DrP 23	Phospholipase A_2-IV	Q2ES51 *(D. r. russelii)*	25.49	2	~0.1%
	DrP 26	Factor V activator RVV-V gamma	P18965 *(D. siamensis)*	82.35	5	0.1%
	DrP 35	Serine protease VLSP	CL2958.Contig6_DRSL *(D. russelii)*	49.80	3	~0.1%
	DrP 40	Coagulation factor X activating enzyme light chain	Q4PRD1 *(D. siamensis)*	60.15	3	0.2%
	DrP 51	Snaclec 3	Q4PRD0 *(D. siamensis)*	36.89	2	0.4%
	DrP 15	Basic phospholipase A_2	B3RFI7 *(D. r. limitis)*	55.76	3	~0.1%
	DrP 52	Cysteine-rich secretory protein Dr-CRPB	F2Q6F3 *(D. russelii)*	39.48	2	~0.1%
	DrP 32	Cysteine-rich secretory protein Dr-CRPK	CL3658.Contig2_DRSL *(D. russelii)*	35.01	2	0.1%
	DrP 24	Acidic phospholipase A_2 RV-7	P31100 *(D. russelii)*	37.79	2	~0.1%
	DrP 1	Kunitz-type serine protease inhibitor DrKIn-I	H6VC05 *(D. r. russelii)*	35.06	2	0.1%
	DrP 53	Snaclec agglucetin subunit alpha-1	Q8JIV9 *(D. acutus)*	30.11	2	~0.1%
	DrP 54	Agglucetin-alpha 2 subunit precursor	Q8AYA5 *(D. acutus)*	20.39	1	~0.1%

D. r. russelii: Daboia russelii russelii; D. siamensis: Daboia siamensis; D. r. limitis: Daboia russelii limitis; V. ursinii: Vipera ursinii; V. a. ruffoi: Vipera ammodytes ruffoi; M. lebetina: Macrovipera lebetina C. rhodostoma: Calleoselasma rhodostoma; D. acutus: Deinagkistrodon acutus; O. okinavensis: Ovophis okinavensis; T. wagleri: Tropidolaemus wagleri; B. schlegelii: Bothriechis schlegelii.

[a] Refers to protein names taken from protein sequence database based on homology match in BLAST and UniProt search.

[b] Refers to number of distinct peptides matched to identified protein.

fibrinogen consumption, leading to the augmentation of consumptive coagulopathy [48]. This is supported by the potent procoagulant activity on human plasma in the present study.

Besides PLA_2 and KSPI, SVSP (the third major class of proteins, 6%) also plays a role in venom-induced hemostatic derangement. SVSP is a hemotoxic enzyme group commonly present in viper and pit viper venoms, acting as procoagulant, platelet aggregating or fibrinolytic agents [49]. The occurrence of Factor V activating enzyme (RVV-V) is a characteristic feature of Russell's viper venom. The enzyme selectively activates Factor V, thereby promoting the formation of the prothrombinase complex, a potent coagulant [50–52]. The presence of other procoagulant SVSP subtypes (fibrinogenases and thrombin-like enzymes) further supported that consumptive coagulopathy is a major hemotoxic complication of *D. russelii* envenomation [49,53,54]. Clinically, *D. russelii* envenomation warrants a longer course of meticulous monitoring of blood parameters and coagulation profile, as self-recovery is only possible when there is complete elimination of the toxin followed by restoration of the coagulant factors [55].

Viperid venoms are typically characterized by the dominating presence of snake venom metalloproteinases (SVMPs). Some SVMPs are also called hemorrhagins, as they can cause hemorrhage by damaging the vascular basal membranes, resulting in blood exudation from the vasculature [56,57]. However, unlike specimens from the other geographical regions, the SVMP content in the wild Pakistani *D. russelii* venom was exceptionally low, and this was corroborated by the lack of local hemorrhagic activity tested in mice in this study. In fact, the SVMP detected in the present study were virtually all matched to the Factor X activating enzyme (RVV-X), which is a form of P-III SVMP. Factor X activating enzyme is one of the main toxins responsible for the potent procoagulant activity of Russell's viper venom on human plasma [58–60]. It activates blood coagulant Factor X systemically by cleaving a specific peptide bond, triggering thrombin release from prothrombin that converts fibrinogen to fibrin, resulting in consumptive coagulopathy [56,61,62]. When tested *in vitro*, the coagulation of human plasma represented an overall or net effect of the venom activity. The *in vitro* coagulation did not exclude concurrent anti-coagulant activity as it could be masked by the *in vitro* procoagulant effect of the venom at the tested doses. Importantly, in clinical envenoming the *in vivo* venom effect is coagulopathic (loss of coagulability); this pathophysiology is likely a combined effect of the procoagulants (SVSP, SVMP, some KSPI, consuming the coagulant factors) and certain PLA_2 anticoagulants.

In addition, RVV-X is also known to interfere with platelet aggregation. RVV-X is a heterotrimeric complex of 79 kDa that consists of three covalently bonded peptide chains: a heavy chain with metalloproteinase, disintegrin-like and cysteine-rich domains and two C-type lectin-like light chains [59,63]. Both the heavy chains and the lectin-like light chains (see below) were also identified in the venom proteome of the wild Pakistani *D. russelii* in this study.

Snaclecs do not possess enzymatic activities but exhibit various pharmacological activities including anticoagulation, platelet aggregation induction or inhibition [64]. The low protein abundance of snaclecs in the wild Pakistani *D. russelii* venom (1.3%) is comparable to the west Indian specimen (1.8%) [24] but lower than that of the captive Pakistani snakes (6%) [20] and the Sri Lankan snakes (22.4%) [25]. Collectively, the snaclecs present in the venom may contribute to the overall hemotoxicity through anti-platelet (by dabocetin-like snaclecs) and anticoagulant activities (by snaclec Factor X light chain-1 and -2 which activate Factor IX, X and protein S) [60,65,66]. Nonetheless, the rather low abundance of these proteins in the venom implies that these bioactivities may not constitute the main pathophysiological mechanisms in envenoming by the Pakistani *D. russelli*.

Among the other venom proteins, VEGF has a relatively high abundance and may play a role in *D. russelii* envenoming. It was reported that snake venom VEGF could increase capillary permeability in the early course of envenoming, thereby facilitating venom dissemination in the bitten subjects [67–69]. The VEGF protein has also been shown to exhibit potent stimulatory effect on the proliferation of vascular endothelial cells [70]. The relatively high abundance of VEGF

Table 2
Overview of the venom proteome of Pakistani *Daboia russelii* by protein subtypes and relative abundances.

Protein family/protein subtype	Accession no.	Species	Designated protein ID (Fraction no.)	Relative abundance (%)
Phospholipase A_2(PLA_2)				**63.8%**
Basic phospholipase A_2 RVV-VD	P81458	*D. r. russelii*	DrP14 (7–15)	25.9%
Basic phospholipase A_2	B3RFI7	*D. r. limitis*	DrP15 (7–8), (10), (12 − 13), (15)	5.1%
Basic phospholipase A_2 Drk-b2	A8CG90	*D. r. russelii*	DrP9 (6), (11 − 12)	3.2%
Acidic phospholipase A_2 RV-7	P31100	*D. russelii*	DrP24 (12–15)	13.9%
Acidic phospholipase A_2 Drk-a1	A8CG86	*D. r. russelii*	DrP25 (12–13)	3.3%
Acidic phospholipase A_2 Drk-a2	A8CG87	*D. r. russelii*	DrP21 (10)	0.2%
Acidic phospholipase A_2 daboiatoxin B chain	Q7T3T5	*D. siamensis*	DrP17 (7)	~0.01%
Phospholipase A_2 (EC 3.1.1.4)	B2YHV1	*D. russelii*	DrP22 (11)	7.6%
Phospholipase A_2-IV	Q2ES51	*D. r. russelii*	DrP23 (12–13), (15)	3.6%
Ammodytin I1 (A) variant	Q6A3M8	*V. a. ruffoi*	DrP20 (10)	0.5%
Chain A, Crystal Structure Of Rv4RV7 complex	CL3663.Contig1_DRSL	*D. russelii*	DrP38 (13)	0.5%
Kunitz-type serine protease (KSPI)				**16.0%**
Kunitz-type serine protease inhibitor DrKIn-I	H6VC05	*D. r. russelii*	DrP1 (3–9), DrP42 (11–15)	6.9%
Kunitz-type serine protease inhibitor 2	P00990	*D. siamensis*	DrP4 (5–8)	3.7%
Kunitz-type serine protease inhibitor C6	A8Y7N9	*D. siamensis*	DrP2 (3–5)	2.0%
Kunitz-type serine protease inhibitor C8	P85039	*D. siamensis*	DrP6 (5)	1.5%
Protease inhibitor 4	Unigene17386_DRSL	*D. r. russelii*	DrP7 (5–6)	0.7%
Kunitz-type serine protease inhibitor DrKIn-II	H6VC06	*D. r. russelii*	DrP5 (5), (8)	0.5%
Trypsin inhibitor-4 precursor	A8Y7N7	*D. siamensis*	DrP3 (5)	0.5%
Kunitz-type serine protease inhibitor B6	A8Y7P6	*D. siamensis*	DrP19 (8)	0.1%
Kunitz-type serine protease inhibitor 2	Q2ES49	*D. r. russelii*	DrP18 (7)	0.1%
Kunitz-type protease inhibitor	H9BFA3	*D. russelii*	DrP16 (7–8)	~0.1%
Snake venom serine protease (SVSP)				**5.5%**
Vipera russelli proteinase RVV-V homolog 2	P86531	*D. r. russelii*	DrP33 (12–13)	1.5%
Factor V activator RVV-V gamma	P18965	*D. siamensis*	DrP26 (12), (14–15)	1.2%
Serine protease VLSP-1	CL2958.Contig6_DRSL	*D. russelii*	DrP35 (13), (15)	1.0%
Serine protease VLSP-3	E0Y420	*M. lebetina*	DrP28 (12)	0.3%
Beta-fibrinogenase-like	E5L0E4	*D. siamensis*	DrP27 (12)	0.4%
Venom serine proteinase-like protein 2	Q9PT40	*M. lebetina*	DrP31 (12–13)	0.4%
Alpha-fibrinogenase-like	E5L0E3	*D. siamensis*	DrP36 (13)	0.2%
Factor V activator RVV-V alpha	P18964	*D. siamensis*	DrP34 (13)	0.3%
Ancrod-like protein	A1E236	*C. rhodostoma*	DrP29 (12)	0.2%
Vascular endothelial growth factor (VEGF)				**4.3%**
Snake venom vascular endothelial growth factor toxin VR-1	P67861	*D. r. russelii*	DrP11 (6), (8–10), (13)	2.8%
Snake venom vascular endothelial growth factor toxin	Unigene30051_DRSL	*D. russelii*	DrP10 (6), (8–9)	1.5%
Snake venom metalloproteinase (SVMP)				**2.5%**
Factor X activator heavy chain	K9JAW0	*D. r. russelii*	DrP8 (6), (13–15)	1.4%
RVV-X-heavy chain	Q7LZ61	*D. russelii*	DrP37 (14–15)	1.1%
Coagulation factor X activating enzyme heavy chain	Q7LZ61	*D. siamensis*	DrP37 (13)	0.1%
Phosphodiesterase (PDE)				**2.5%**
Phosphodiesterase 1	Unigene21602_TW	*T. wagleri*	DrP46 (15)	1.9%
Phosphodiesterase	U3TBJ5	*O. okinavensis*	DrP45 (15)	0.4%
Phosphodiesterase 1	CL3655.Contig2_DRSL	*D. russelii*	DrP44 (15)	0.2%
Cystein-rich secretory protein (CRISP)				**1.3%**
Cysteine-rich seceretory protein Da-CRPa	F2Q6G0	*D. acutus*	DrP30 (12–13)	0.6%
Cysteine-rich seceretory protein Dr-CRPK	CL3658.Contig2_DRSL	*D. russelii*	DrP32 (12–13), (15)	0.6%
Cysteine-rich seceretory protein Dr-CRPB	F2Q6F3	*D. russelii*	DrP52 (15)	> 0.1%
Snaclec (C-type lectin and C-type lectin-like protein)				**1.3%**
P68 alpha subunit	K9JDF2	*D. r. limitis*	DrP50 (15)	0.5%
Snaclec 3	Q4PRD0	*D. siamensis*	DrP51 (15)	0.4%
Coagulation factor X activating enzyme light chain	Q4PRD1	*D. siamensis*	DrP40 (14–15)	0.2%
Factor X activator light chain 2	CL1101.Contig1_DRSL	*D. siamensis*	DrP43 (14)	0.1%
Snaclec agglucetin subunit alpha-1	Q8JIV9	*D. acutus*	DrP53 (15)	0.1%
Dabocetin alpha subunit	K9JBU0	*D. r. russelii*	DrP41 (14)	0.1%
Dabocetin beta subunit	Unigene920_DRSL	*D. r. russelii*	DrP42 (14)	0.1%
Agglucetin-alpha 2 subunit precursor	Q8AYA5	*D. acutus*	DrP54 (15)	0.1%
Venom nerve growth factor (VNGF)				**1.1%**
Venom nerve growth factor	V9I168	*V. ursinii*	DrP13 (7)	0.7%
Venom nerve growth factor	P30894	*D. r. russelii*	DrP12 (7–8)	0.5%
L-amino acid oxidase (LAAO)				**0.8%**
L-amino-acid oxidase	G8XQX1	*D. r. russelii*	DrP39 (13), (15)	0.4%
L-amino-acid oxidase	Q4F867	*D. siamensis*	DrP47 (15)	0.3%
L-amino acid oxidase Bs29	A0A024BTN9	*B. schlegelii*	DrP48 (15)	0.1%
Nucleotidase (5′-NUC)				**0.1%**
Snake venom 5′-nucleotidase	CL3322.Contig1_DRSL	*D. russelii*	DrP49 (15)	0.1%

(*continued on next page*)

Table 2 (*continued*)

Protein family/protein subtype	Accession no.	Species	Designated protein ID (Fraction no.)	Relative abundance (%)
Unidentified protein (UP)				**0.7%**
	–	–	– (1)	0.7%

D. r. russelii: Daboia russelii russelii; D. siamensis: Daboia siamensis; D. r. limitis: Daboia russelii limitis; V. ursinii: Vipera ursinii; V. a. ruffoi: Vipera ammodytes ruffoi; M. lebetina: Macrovipera lebetina C. rhodostoma: Calleoselasma rhodostoma; D. acutus: Deinagkistrodon acutus; O. okinavensis: Ovophis okinavensis; T. wagleri: Tropidolaemus wagleri; B. schlegelii: Bothriechis schlegelii.

in the wild Pakistani *D. russelii* venom is probably a source of snake venom-derived VEGF for further pharmacological characterization in the future. Besides, the venom contains two other non-enzymatic toxins *i.e.* cysteine-rich secretory proteins (CRiSP) and nerve growth factors (NGF) at lower abundances. CRiSP exhibits calcium channel inhibitory effect and is able to reduce potassium-induced smooth muscle contraction [71], but its pathogenic role in envenomation remains to be elucidated. NGF may potentiate venom spread from local envenoming site, but the mechanism is unclear [72]. Similarly, PDE and 5′-NUC present in the venom may play an ancillary role in promoting venom dissemination through the elevation of extracellular purines/purine derivatives that induce vasodilation [73,74]. It is noteworthy that a PDE isolated from Indian Russell's viper venom has been shown to exhibit potent inhibitory activity against ADP-induced platelet aggregation in human platelet-rich plasma [75]. This suggests that the relatively high abundance of PDE detected in the present study can contribute to the hemotoxic complication of Pakistani *D. russelii* envenoming. Typically, PDE constitutes < 1% of total venom proteins in snake venoms [74]. The present study also showed that the LAAO content in the Pakistani *D. russelii* venom is low, comparable to that reported for the captive Pakistani specimen and the west Indian Russell's viper (< 1%) [20,24]. Although viperid venom LAAO has diverse pharmacological activities *e.g.* antimicrobial, cytotoxic and anticoagulant effects [76,77], the very low content of LAAO in the Pakistani *D. russelii* venom indicates a minor role in the pathophysiology of envenomation.

The present venom proteome was quantitated based on the area under curve of the HPLC profile (215 nm) coupled to a label-free method by measuring the mean spectral intensity. The label-free method relies on the frequency of peptides matched to the database, and this may give rise to problem when the species dealt with is a non-sequenced organism. In this study, this limitation was partly overcome with the use of a custom database that integrated publicly available data and an in-house venom-gland transcriptomic dataset specific to *D. russelii*. Hence, in the absence of the full genome of this species, the integrated database provided an in-depth collection of homologous sequences and specific venom-gland transcripts, improving the significance of hits and frequency of peptides matched to the database. Nevertheless, a few faint bands present in fraction P11 might be minor proteins undetected by LCMS/MS due to the paucity of existing database. The amount, however, could also be very low and represent only a trace protein content. This ambiguity can be further elucidated using an updated database when the data of complete sequences specific to the Pakistani *D. russelii* is available.

Table 3
Procoagulant activity of wild Pakistani *Daboia russelii* venom and neutralization by Indian VINS Polyvalent Antivenom (VPAV).

Venom	Minimum coagulation dose (MCD) (μg/ml)	Challenge dose (2 MCD) (μg/ml)	Effective dose, ED (μg/μl)
Daboia russelii (Pakistan)	0.0145 ± 0.001	0.029	0.64 (0.58–0.78)

Procoagulant activity is defined as the dose of venom that causes plasma clotting in 3 min.
Effective Dose (ED): The ratio of μg venom/μl of antivenom that prolongs the clotting time of the citrated human plasma three times that of the control (2 MCD of venom, without antivenom).

Earlier, a study evaluated the ELISA immunoreactivity and *in vitro* neutralization of the procoagulant/anticoagulant effect of captive Pakistani *D. russelii* venom and its gel-filtration chromatographic fractions against the Indian polyvalent and monovalent antivenoms (tested at a fixed dose of antivenom) [20]. The immunological reactivity and *in*

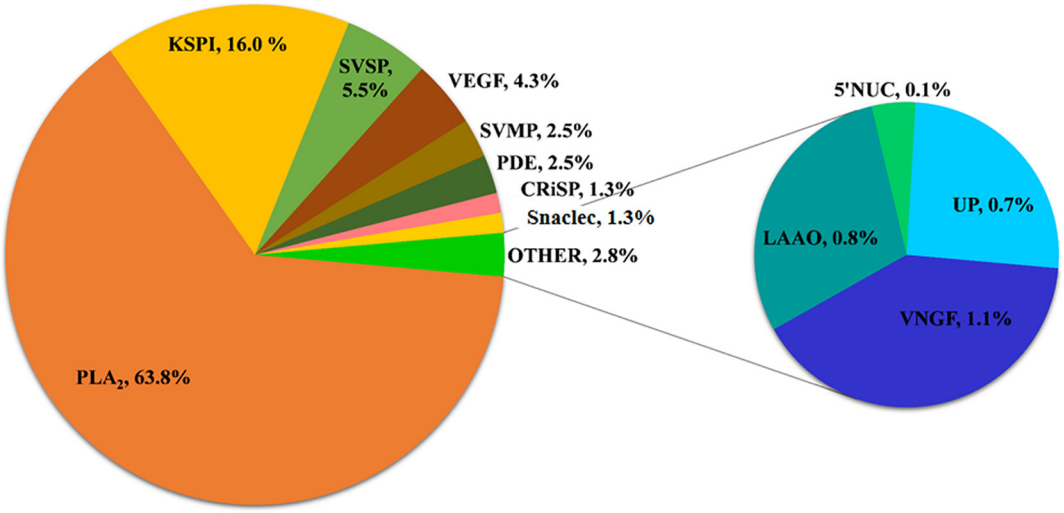

Fig. 2. Venom proteome of the wild Pakistani *Daboia russelii* venom.
Abbreviations: PLA_2, phospholipase A_2, SVMP, snake venom metalloproteinase; SVSP, snake venom serine protease; KSPI, Kunitz-type serine protease inhibitor; CRiSP: cysteine-rich secretory protein, NGF: nerve growth factor; VEGF: vascular endothelial growth factor; Snaclec: snake venom C-type lectin/lectin-like proteins; PDE, phosphodiesterase; LAAO: L-amino acid oxidase; 5′-NUC, 5′-nucleotidase; UP: unidentified protein.

Table 4
Lethality of wild Pakistani *Daboia russelii* venom and neutralization by Indian Polyvalent Antivenom (VPAV).

Venom	*i.v.* LD_{50} (μg/g)	Challenge dose (μg/g)	ED_{50} (μl of antivenom)	ER_{50} (mg/ml)	P (mg/ml)	Normalized P. (mg/g)
Daboia russelii (Pakistan)	0.19 (0.17–0.25)	5 LD_{50}	78.29 (63.98–95.8)	0.29 (0.26–0.31)	0.23	2.7

i.v.LD_{50}: Intravenous (*i.v.*) median lethal dose (LD_{50}).
Median Effective Dose, ED_{50}: Amount of antivenom in μl at which 50% of mice survived.
Median Effective Ratio, ER_{50}: The ratio of the amount of venom (mg) to the volume dose of antivenom (ml) at which 50% of mice survived.
Potency: The amount of venom (mg) completely neutralized per unit volume (ml) of antivenom.
Normalized potency: The amount of venom (mg) completely neutralized per unit gram of antivenom proteins.

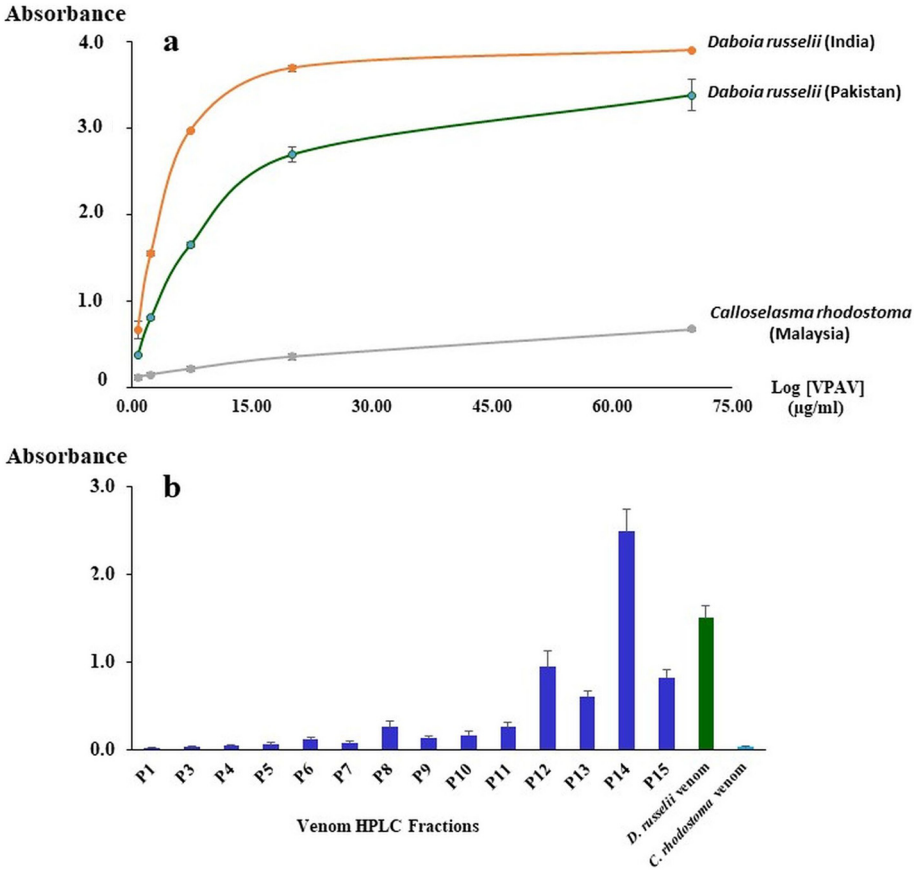

Fig. 3. Immunological binding activity of the Indian VINS Polyvalent Antivenom (VPAV) with (a) crude venoms of *Daboia russelii* from Pakistan and India; (b) protein fractions of the venom (Pakistan) eluted by reverse-phase HPLC. Absorbance values were obtained by indirect ELISA and expressed as mean ± S.E.M. from three experiments.

vitro neutralization of the procoagulant/anticoagulant activity of the venom and its fractions by the monovalent antivenom surpassed that of the polyvalent antivenom [20]. Similarly, the monovalent antivenom was found to be better than polyvalent antivenom in ELISA immunoreactivity and *in vitro* neutralization of selected pharmacological or enzymatic activities of the west Indian *D. russelii* venom [24]. Our present study further elucidated the immunological reactivity of the Pakistani *D. russelii* (wild caught) venom proteins against the Indian polyvalent antivenom of VINS Bioproduct which is used in the affected area in Pakistan. The concentration-dependent increase in the immunological binding activity of VPAV toward the venoms (both Indian and Pakistani origins) implied that the interaction is specific; however, the overall lower immunoreactivity exhibited by the Pakistani *D. russelii* venom (compared with the Indian sample) implies that the protein antigenicity varied to some extents between the two geographical venom samples. This finding is consistent with the recent reports of variable venom immunoreactivity of the Indian krait (*Bungarus caeruleus*) and Indian cobra (*Naja naja*) from different locales when tested against the Indian polyvalent antivenom (VINS Bioproduct) [17,78]. The finding is probably reflective of the compositional variation of the Pakistani *D. russelii* venom, in particular its high PLA_2 content and the variety of proteoforms which alter the overall protein antigenicity. Nonetheless, there was a substantial amount of conserved sequences and epitopes in the venom proteins between the two geographical specimens, thereby allowing the Indian antivenom to bind to the Pakistani venom, albeit at a lower binding efficacy.

Using an ELISA-based antivenomic approach, the individual venom fractions showed varying immunological binding activity with VPAV. The low molecular weight proteins in the fractions P1-P11 exhibited weaker venom-antivenom binding activity compared with the moderate and high molecular weight proteins (P12-P15). In general, antivenom antibodies have stronger binding to larger venom proteins, as larger proteins are usually more immunogenic. A previous study reacting a polyvalent antivenom against size-selected crotalid venom antigens revealed that the binding of antivenom antibodies to large (> 30,000 kDa), medium (13,000–30,000 kDa) and small (< 14,000 kDa) proteins coincided with high (> 0.7 absorbance units), moderate (0.3–0.7 absorbance units) and low (< 0.3 absorbance units) ELISA levels, respectively [79]. In the present study, fractions P1-P8 contained mainly KSPIs and/or VEGF whose molecular weights are small (< 10 kDa), and these proteins may have limited epitopes for antivenom binding. PLA_2-dominating fractions (P9-P12), on the other

hand, have medium molecular weights but their immunorecognition by VPAV was strong only in P12, which contained PLA_2 in addition to a small amount of high molecular weight proteins *e.g.* CRiSP, SVSP and SVMP. The highest immunoreactivity was shown in fraction P14, which contained predominantly SVMP (heavy chains), SVSPs and snaclecs; these are mainly large proteins (> 35 kDa) that usually possess higher immunogenicity. Fractions P12, P13 and P15 too showed strong antivenom immunorecognition probably because of the dominating presence of large proteins such as LAAO, PDE, 5′-NUC and SVMP. The better immunorecognition and stronger binding of protein fractions containing SVMP, SVSP and snaclec imply that the antivenom could neutralize the toxic effects of these toxins better, thereby preventing or reducing the coagulopathic complication and lethality caused by the Pakistani *D. russelii* venom. The weak binding to PLA_2 proteins, however, may be a limiting factor to the efficacy of the Indian antivenom in neutralizing the venom. Further research should aim at improving the immunogenicity of the toxin proteins for the production of a more effective pan-regional antivenom.

5. Concluding remarks

It is clear from the present study and other reports that there is much venom variation among the Russell's vipers from different geographical locales in the Indian subcontinent. It appears that all Russell's viper venoms consist of PLA_2 as the major toxin group, and a variety of toxins that can adversely affect hemostasis. The venom studied was sourced from wild snakes and hence may provide useful data for comparison with captive specimens reported earlier. VPAV could immunologically bind to the Pakistani venom proteins in a concentration-dependent manner, although the immunorecognition of small proteins and PLA_2-dominating fractions was weak to moderate. Further studies should be undertaken to examine the principal toxins and the epitopes for the *D. russelii* venoms from a wider geographical scale. The future production of an efficacious, pan-regional antivenom should incorporate the knowledge of intra-specific venom variability complicated by factors such as geographical origins and captive *vis-à-vis* wild specimens.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jprot.2018.05.003.

References

[1] E. Alirol, S.K. Sharma, H.S. Bawaskar, U. Kuch, F. Chappuis, Snake bite in South Asia: a review, PLoS Negl. Trop. Dis. 4 (1) (2010) e603.
[2] J. Chippaux, Guidelines for the production, control and regulation of snake antivenom immunoglobulins, Biologie aujourd'hui 204 (1) (2010) 87–91.
[3] M.S. Khan, The snakebite problem in Pakistan, Bull. Chicago Herp. Soc. 49 (2014) 165–167.
[4] C. Antonypillai, J. Wass, D. Warrell, H. Rajaratnam, Hypopituitarism following envenoming by Russell's vipers (*Daboia siamensis* and *D. russelii*) resembling Sheehan's syndrome: first case report from Sri Lanka, a review of the literature and recommendations for endocrine management, QJM 104 (2) (2010) 97–108.
[5] C. Ariaratnam, W. Meyer, G. Perera, M. Eddleston, S. Kuleratne, W. Attapattu, R. Sheriff, A. Richards, R. Theakston, D. Warrell, A new monospecific ovine Fab fragment antivenom for treatment of envenoming by the Sri Lankan Russell's viper (*Daboia Russelii Russelii*): a preliminary dose-finding and pharmacokinetic study, Am. J. Trop. Med. Hygiene 61 (2) (1999) 259–265.
[6] V. Jeevagan, P. Katulanda, C.A. Gnanathasan, D.A. Warrell, Acute pituitary insufficiency and hypokalaemia following envenoming by Russell's viper (*Daboia russelii*) in Sri Lanka: exploring the pathophysiological mechanisms, Toxicon 63 (2013) 78–82.
[7] R. Phillips, D. Warrell, Bites by Russell's viper (*Vipera russelli siamensis*) in Burma: haemostatic, vascular, and renal disturbances and response to treatment, Lancet 326 (8467) (1985) 1259–1264.
[8] R.E. Phillips, R.D.G. Theakston, D.A. Warrell, Y. Galigedara, D. Abeysekera, P. Dissanayaka, R.A. Hutton, D.J. Aloysius, Paralysis, rhabdomyolysis and haemolysis caused by bites of Russell's viper (*Vipera russelli pulchella*) in Sri Lanka: failure of Indian (Haffkine) antivenom, QJM 68 (3–4) (1988) 691–715.
[9] D.A. Warrell, Snake venoms in science and clinical medicine 1. Russell's viper: biology, venom and treatment of bites, Trans. R. Soc. Trop. Med. Hyg. 83 (6) (1989) 732–740.
[10] S. Kularatne, N. Senanayake, Venomous snake bites, scorpions, and spiders, Handb. Clin. Neurol. 120 (2014) 987–1001.
[11] T. Pe, R. Phillips, D. Warrell, R. Moore, T.N. Swe, Acute and chronic pituitary failure resembling Sheenan's syndrome following bites by Russel's viper in Burma, Lancet (Oct, 1987) 1987.
[12] R.S. Thorpe, C.E. Pook, A. Malhotra, Phylogeography of the Russell's viper (*Daboia russelii*) complex in relation to variation in the colour pattern and symptoms of envenoming, Herpetol. J. 17 (4) (2007) 209–218.
[13] G. Jayanthi, T.V. Gowda, Geographical variation in India in the composition and lethal potency of Russell's viper (*Vipera russelli*) venom, Toxicon 26 (3) (1988) 257–264.
[14] N.B. Prasad, B. Uma, S.K. Bhatt, V.T. Gowda, Comparative characterisation of Russell's viper (*Daboia/Vipera russelli*) venoms from different regions of the Indian peninsula, Biochimica et Biophysica Acta (BBA)-General Subjects 1428 (2) (1999) 121–136.
[15] M. Sharma, N. Gogoi, B. Dhananjaya, J.C. Menon, R. Doley, Geographical variation of Indian Russell's viper venom and neutralization of its coagulopathy by polyvalent antivenom, Toxin Rev. 33 (1–2) (2014) 7–15.
[16] M. Suzuki, T. Itoh, S.B. Athauda, A. Moriyama, Molecular diversity in venom proteins of the Russell's viper (*Daboia russellii russellii*) and the Indian cobra (*Naja naja*) in Sri Lanka, Biomed. Res. 31 (1) (2010) 71–81.
[17] A.M.F. Oh, C.H. Tan, G.C. Ariaranee, N. Quraishi, N.H. Tan, Venomics of *Bungarus caeruleus* (Indian krait): comparable venom profiles, variable immunoreactivities among specimens from Sri Lanka, India and Pakistan, J. Proteomics 164 (2017) 1–18.
[18] K.Y. Wong, C.H. Tan, N.H. Tan, Venom and purified toxins of the spectacled cobra *(Naja naja*) from Pakistan: insights into toxicity and antivenom neutralization, Am. J. Trop. Med. Hyg. 94 (6) (2016) 1392–1399.
[19] N.A. Quraishi, H.I. Qureshi, I.D. Simpson, A contextual approach to managing snake bite in Pakistan: snake bite treatment with particular reference to neurotoxieity and the ideal hospital snake bite kit, JPMA 58 (6) (2008) 325–331.
[20] A.K. Mukherjee, B. Kalita, S.P. Mackessy, A proteomic analysis of Pakistan Daboia russelii russelii venom and assessment of potency of Indian polyvalent and monovalent antivenom, J. Proteome 144 (2016) 73–86.
[21] C.M. Modahl, R. Doley, R.M. Kini, Venom analysis of long-term captive Pakistan cobra (Naja naja) populations, Toxicon 55 (2–3) (2010) 612–618.
[22] E. Saad, L.C. Barros, N. Biscola, D.C. Pimenta, S.R. Barraviera, B. Barraviera, R.S. Ferreira Jr., Intraspecific variation of biological activities in venoms from wild and captive Bothrops jararaca, J. Toxic. Environ. Health A 75 (16–17) (2012) 1081–1090.
[23] L. Freitas-de-Sousa, D. Amazonas, L. Sousa, S. Sant'Anna, M. Nishiyama, S. Serrano, I. Junqueira-de-Azevedo, H. Chalkidis, A. Moura-da-Silva, R. Mourao, Comparison of venoms from wild and long-term captive Bothrops atrox snakes and characterization of Batroxrhagin, the predominant class PIII metalloproteinase from the venom of this species, Biochimie 118 (2015) 60–70.
[24] B. Kalita, A. Patra, A.K. Mukherjee, Unraveling the proteome composition and Immuno-profiling of western India Russell's viper venom for in-depth understanding of its pharmacological properties, clinical manifestations, and effective antivenom treatment, J. Proteome Res. 16 (2) (2016) 583–598.
[25] N.H. Tan, S.Y. Fung, K.Y. Tan, M.K.K. Yap, C.A. Gnanathasan, C.H. Tan, Functional venomics of the Sri Lankan Russell's viper (Daboia russelii) and its toxinological correlations, J. Proteome 128 (2015) 403–423.
[26] N. Howard-Jones, A CIOMS ethical code for animal experimentation, WHO Chron. 39 (2) (1985) 51–56.
[27] C.H. Tan, K.Y. Tan, M.K. Yap, N.H. Tan, Venomics of *Tropidolaemus wagleri*, the sexually dimorphic temple pit viper: unveiling a deeply conserved atypical toxin arsenal, Sci. Rep. 7 (2017) 43237.
[28] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (5259) (1970) 680–685.
[29] C.H. Tan, K.Y. Wong, K.Y. Tan, N.H. Tan, Venom proteome of the yellow-lipped sea krait, Laticauda colubrina from Bali: insights into subvenomic diversity, venom antigenicity and cross-neutralization by antivenom, J. Proteome 166 (2017) 48–58.
[30] M.A. O'Leary, G.K. Isbister, A turbidimetric assay for the measurement of clotting times of procoagulant venoms in plasma, J. Pharmacol. Toxicol. Methods 61 (1) (2010) 27–31.
[31] D.J. Finney, F. Tattersfield, Probit Analysis, Cambridge University Press, Cambridge, 1952.
[32] C.H. Tan, N.H. Tan, K.Y. Tan, K.O. Kwong, Antivenom cross-neutralization of the venoms of Hydrophis schistosus and Hydrophis curtus, two common sea snakes in Malaysian waters, Toxins (Basel) 7 (2) (2015) 572–581.
[33] C.H. Tan, K.Y. Tan, S.E. Lim, N.H. Tan, Venomics of the beaked sea snake, Hydrophis schistosus: a minimalist toxin arsenal and its cross-neutralization by heterologous antivenoms, J. Proteome 126 (2015) 121–130.
[34] V. Morais, S. Ifran, P. Berasain, H. Massaldi, Antivenoms: potency or median effective dose, which to use? J. Venomous Animals Toxins Trop. Dis. 16 (2) (2010) 191–193.
[35] C.H. Tan, N.H. Tan, Toxinology of Snake Venoms: The Malaysian Context, Springer,

Netherlands, 2015.
[36] A. Mukherjee, S. Ghosal, C. Maity, Some biochemical properties of Russell's viper (Daboia russelli) venom from eastern India: correlation with clinico-pathological manifestation in Russell's viper bite, Toxicon 38 (2) (2000) 163–175.
[37] S. Kularatne, Epidemiology and clinical picture of the Russell's viper (*Daboia russelii russelii*) bite in Anuradhapura, Sri Lanka: A prospective study of 336 patients, (2003).
[38] R. Doley, X. Zhou, R.M. Kini, Snake venom phospholipase A2 enzymes, Handbook of Venoms and Toxins of Reptiles, vol. 1, 2010, pp. 173–205.
[39] I.-H. Tsai, P.-J. Lu, J.-C. Su, Two types of Russell's viper revealed by variation in phospholipases A2 from venom of the subspecies, Toxicon 34 (1) (1996) 99–109.
[40] E. Carredano, B. Westerlund, B. Persson, M. Saarinen, S. Ramaswamy, D. Eaker, H. Eklund, The three-dimensional structures of two toxins from snake venom throw light on the anticoagulant and neurotoxic sites of phospholipase A 2, Toxicon 36 (1) (1998) 75–92.
[41] A.K. Mukherjee, A major phospholipase A 2 from Daboia russelii russelii venom shows potent anticoagulant action via thrombin inhibition and binding with plasma phospholipids, Biochimie 99 (2014) 153–161.
[42] D. Saikia, S. Majumdar, A.K. Mukherjee, Mechanism of in vivo anticoagulant and haemolytic activity by a neutral phospholipase A 2 purified from Daboia russelii russelii venom: correlation with clinical manifestations in Russell's Viper envenomed patients, Toxicon 76 (2013) 291–300.
[43] I.-H. Tsai, H.-Y. Tsai, Y.-M. Wang, D.A. Warrell, Venom phospholipases of Russell's vipers from Myanmar and eastern India—cloning, characterization and phylogeographic analysis, Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics 1774 (8) (2007) 1020–1028.
[44] D.-Z. Hung, M.-L. Wu, J.-F. Deng, S.-Y. Lin-Shiau, Russell's viper snakebite in Taiwan: differences from other Asian countries, Toxicon 40 (9) (2002) 1291–1298.
[45] Y.M. WANG, P.J. LU, C.L. HO, I.H. TSAI, Characterization and molecular cloning of neurotoxic phospholipases A2 from Taiwan viper (Vipera russelli formosensis), FEBS J. 209 (2) (1992) 635–641.
[46] H. Inagaki, Snake Venom Protease Inhibitors: Enhanced Identification, Expanding Biological Function, and Promising Future, in: H. Inagaki, C.-W. Vogel, A.K. Mukherjee, T.R. Rahmy (Eds.), Snake Venoms, Springer, Netherlands, Dordrecht, 2017, pp. 161–186.
[47] A.K. Mukherjee, S.P. Mackessy, S. Dutta, Characterization of a Kunitz-type protease inhibitor peptide (Rusvikunin) purified from Daboia russelii russelii venom, Int. J. Biol. Macromol. 67 (2014) 154–162.
[48] A.-C. Cheng, H.-L. Wu, G.-Y. Shi, I.-H. Tsai, A novel heparin-dependent inhibitor of activated protein C that potentiates consumptive coagulopathy in Russell's viper envenomation, J. Biol. Chem. 287 (19) (2012) 15739–15748.
[49] R.M. Kini, K.J. Clemetson, F.S. Markland, M.A. McLane, T. Morita, Toxins and Hemostasis: From Bench to Bedside, Springer Science & Business Media, 2010.
[50] F. Tokunaga, K. Nagasawa, S. Tamura, T. Miyata, S. Iwanaga, W. Kisiel, The factor V-activating enzyme (RVV-V) from Russell's viper venom. Identification of isoproteins RVV-V alpha,-V beta, and-V gamma and their complete amino acid sequences, J. Biol. Chem. 263 (33) (1988) 17471–17481.
[51] A.K. Mukherjee, The pro-coagulant fibrinogenolytic serine protease isoenzymes purified from Daboia russelii russelii venom coagulate the blood through Factor V activation: role of glycosylation on enzymatic activity, PLoS One 9 (2) (2014) e86823.
[52] D. Nakayama, Y. Ben Ammar, T. Miyata, S. Takeda, Structural basis of coagulation factor V recognition for cleavage by RVV-V, FEBS Lett. 585 (19) (2011) 3020–3025.
[53] P. Sukkapan, Y. Jia, I. Nuchprayoon, J.C. Pérez, Phylogenetic analysis of serine proteases from Russell's viper (*Daboia russelli siamensis*) and Agkistrodon piscivorus leucostoma venom, Toxicon 58 (2) (2011) 168–178.
[54] G.K. Isbister, Snakebite doesn't cause disseminated intravascular coagulation: coagulopathy and thrombotic microangiopathy in snake envenoming, Semin. Thromb. Hemost. 36 (4) (2010) 444–451.
[55] WHO, Guidelines for the Management of Snake-bites, World Health Organization: Regional Office for South-East Asia, New Delhi, (2016).
[56] F.S. Markland, S. Swenson, Snake venom metalloproteinases, Toxicon 62 (2013) 3–18.
[57] J.M. Gutiérrez, A. Rucavado, T. Escalante, Snake venom metalloproteinases: Biological roles and participation in the pathophysiology of envenomation, in: S.P. Mackessy (Ed.), Handbook of Venoms and Toxins of Reptiles, CRC Press, Boca Raton, FL, 2010, pp. 115–138.
[58] W. Kisiel, M.A. Hermodson, E.W. Davie, Factor X activating enzyme from Russell's viper venom: isolation and characterization, Biochemistry 15 (22) (1976) 4901–4906.
[59] S. Takeda, T. Igarashi, H. Mori, Crystal structure of RVV-X: an example of evolutionary gain of specificity by ADAM proteinases, FEBS Lett. 581 (30) (2007) 5859–5864.
[60] H. Takeya, S. Nishida, T. Miyata, S. Kawada, Y. Saisaka, T. Morita, S. Iwanaga, Coagulation factor X activating enzyme from Russell's viper venom (RVV-X). A novel metalloproteinase with disintegrin (platelet aggregation inhibitor)-like and C-type lectin-like domains, J. Biol. Chem. 267 (20) (1992) 14109–14117.
[61] T. Morita, Proteases Which Activate Factor X, Enzymes From Snake Venoms, Fort Collins: Alaken, 1998, pp. 179–209.
[62] T. Matsui, Y. Fujimura, K. Titani, Snake venom proteases affecting hemostasis and thrombosis, Biochimica et Biophysica Acta (BBA)-Protein Structure and Molecular Enzymology 1477 (1) (2000) 146–156.
[63] J.W. Fox, S.M.T. Serrano, Snake venom metalloproteinases, in: S.P. Mackessy (Ed.), Handbook of Venoms and Toxins of Reptiles, CRC Press, Boca Raton, FL, 2010, pp. 95–113.
[64] K.J. Clemetson, Q. Lu, J.M. Clemetson, Snake venom proteins affecting platelets and their applications to anti-thrombotic research, Curr. Pharm. Des. 13 (28) (2007) 2887–2892.
[65] D. Gowda, C.M. Jackson, P. Hensley, E.A. Davidson, Factor X-activating glycoprotein of Russell's viper venom. Polypeptide composition and characterization of the carbohydrate moieties, J. Biol. Chem. 269 (14) (1994) 10644–10650.
[66] H.S. Chen, J.M. Chen, C.W. Lin, K.H. Khoo, I.H. Tsai, New insights into the functions and N-glycan structures of factor X activator from Russell's viper venom, FEBS J. 275 (15) (2008) 3944–3958.
[67] I. de LM Junqueira-de-Azevedo, M.B. da Silva, A.M. Chudzinski-Tavassi, P.L. Ho, Identification and cloning of snake venom vascular endothelial growth factor (svVEGF) from *Bothrops erythromelas* pitviper, Toxicon 44 (5) (2004) 571–575.
[68] Y. Tokunaga, Y. Yamazaki, T. Morita, Specific distribution of VEGF-F in Viperinae snake venoms: isolation and characterization of a VEGF-F from the venom of *Daboia russelli siamensis*, Arch. Biochem. Biophys. 439 (2) (2005) 241–247.
[69] Y. Yamazaki, Y. Matsunaga, Y. Tokunaga, S. Obayashi, M. Saito, T. Morita, Snake venom vascular endothelial growth factors (VEGF-Fs) exclusively vary their structures and functions among species, J. Biol. Chem. 284 (15) (2009) 9885–9891.
[70] Y. Yamazaki, K. Takani, H. Atoda, T. Morita, Snake venom vascular endothelial growth factors (VEGFs) exhibit potent activity through their specific recognition of KDR (VEGF receptor 2), J. Biol. Chem. 278 (52) (2003) 51985–51988.
[71] Y. Yamazaki, T. Morita, Structure and function of snake venom cysteine-rich secretory proteins, Toxicon 44 (3) (2004) 227–231.
[72] M.F. Lavin, S. Earl, G. Birrell, L.St. Pierre, L.W. Guddat, J.d. Jersey, P. Masci, Snake Venom Nerve Growth Factors, in: S.P. Mackessy (Ed.), Handbook of Venoms and Toxins of Reptiles, CRC Press, Boca Raton, FL, 2010, pp. 377–391.
[73] S.D. Aird, Ophidian envenomation strategies and the role of purines, Toxicon 40 (4) (2002) 335–393.
[74] B. Dhananjaya, C.J. D'souza, An overview on nucleases (DNase, RNase, and phosphodiesterase) in snake venoms, Biochem. Mosc. 75 (1) (2010) 1–6.
[75] J. Mitra, D. Bhattacharyya, Phosphodiesterase from *Daboia russelli russelli* venom: purification, partial characterization and inhibition of platelet aggregation, Toxicon 88 (2014) 1–10.
[76] F. Lazo, D.E. Vivas-Ruiz, G.A. Sandoval, E.F. Rodriguez, E.E.G. Kozlova, F. Costal-Oliveira, C. Chavez-Olortegui, R. Severino, A. Yarleque, E.F. Sanchez, Biochemical, biological and molecular characterization of an L-amino acid oxidase (LAAO) purified from *Bothrops pictus* Peruvian snake venom, Toxicon 139 (2017) 74–86.
[77] N.H. Tan, C.H. Tan, Cytotoxicity of snake venoms and toxins: mechanisms and applications, in: Y.N. Utkin, A.V. Krivoshein (Eds.), Snake Venoms and Envenomation. Modern Trends and Future Prospects, Nova Science Publishers, New York, USA, 2016, pp. 217–253.
[78] K.Y. Wong, C.H. Tan, K.Y. Tan, N.H. Quraishi, N.H. Tan, Elucidating the biogeographical variation of the venom of *Naja naja* (spectacled cobra) from Pakistan through a venom-decomplexing proteomic study, J. Proteome 175 (2018) 156–173.
[79] R.C. Schaeffer Jr., H. Randall, J. Resk, R.W. Carlson, Enzyme-linked immunosorbant assay (ELISA) of size-selected crotalid venom antigens by Wyeth's polyvalent antivenom, Toxicon 26 (1) (1988) 67–76.