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Phylovenomics of *Daboia russelii* across the Indian subcontinent. Bioactivities and comparative *in vivo* neutralization and *in vitro* third-generation antivenomics of antivenoms against venoms from India, Bangladesh and Sri Lanka

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ABSTRACT

Russell's viper (*Daboia russelii*) is, together with *Naja naja*, *Bungarus caeruleus* and *Echis carinatus*, a member of the medically important 'Big Four' species responsible for causing a large number of morbidity and mortality cases across the Indian subcontinent. Despite the wide distribution of Russell's viper and the well-documented ubiquity of the phenomenon of geographic variability of intraspecific snake venom composition, Indian polyvalent antivenoms against the "Big Four" venoms are raised against venoms sourced mainly from Chennai in the southeastern Indian state of Tamil Nadu. Biochemical and venomics investigations have consistently revealed notable compositional, functional, and immunological differences among geographic variants of Russell's viper venoms across the Indian subcontinent. However, these studies, carried out by different laboratories using different protocols and involving venoms from a single geographical region, make the comparison of the different venoms difficult. To bridge this gap, we have conducted bioactivities and proteomic analyses of *D. russelii* venoms from the three corners of the Indian subcontinent, Pakistan, Bangladesh, and Tamil Nandu (India) and Sri Lanka, along with comparative *in vivo* neutralization and *in vitro* third-generation antivenomics of antivenoms used in India, Bangladesh and Sri Lanka. These analyses let us to propose two alternative routes of radiation for Russell's viper in the Indian subcontinent. Both radiations, towards the northeast of India and Bangladesh and towards south India and Sri Lanka, have a common origin in Pakistan, and provide a phylovenomics ground for rationalizing the geographic variability in venom composition and their distinct immunoreactivity against available antivenoms.

Biological significance: Russell's viper (*Daboia russelii*), the Indian cobra (*Naja naja*), the common krait (*Bungarus caeruleus*), and the saw-scaled viper (*Echis carinatus*) constitute the 'Big Four' snake species responsible for most snakebite envenomings and deaths in the Indian subcontinent. Despite the medical relevance of *Daboia russelii*, and the well documented variations in the clinical manifestations of envenomings by this wide distributed species, which are doubtless functionally related to differences in venom composition of its geographic variants, antivenoms for the clinical treatment of envenomings by *D. russelii* across the Indian subcontinent are invariably raised using venom sourced mainly from the southeastern Indian state of Tamil Nadu. We have applied a phylovenomics approach to compare the venom proteomes of Russell's vipers from the three corners of the Indian subcontinent, Pakistan, Bangladesh, and South India/Sri Lanka, and have assessed the *in vitro* (third-generation antivenomics) and *in vivo* preclinical efficacy of a panel of homologous antivenoms. The identification of two dispersal routes of ancestral *D. russelii* into the Indian subcontinent provides the ground for rationalizing the variability in composition and immunoreactivity of the venoms of extant geographic variants of

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Russell's viper. Such knowledge is relevant for envisioning strategies to improve the clinical coverage of anti-*D. russelii* antivenoms.

1. Introduction

Named in honor of Scottish herpetologist Patrick Russell (1726–1805), author of "Indian Serpents" [1,2], Russell's viper (Shaw and Nodder 1797) [3] is a usually nocturnal, highly venomous snake found throughout the Indian subcontinent, including Sri Lanka, much of Southeast Asia, southern China and Taiwan [4]. According to molecular phylogeographic studies [5] (Thorpe et al., 2007), there are currently two full species, *Daboia russelii* (Indian Russell's viper, South Asia, west of the bay of Bengal) and *Daboia siamensis* (Eastern Russell's viper, east of the Bay of Bengal, Southeast Asia and China). Previous subspecies defined by morphology [6,7], *Vipera russelii nordicus* (north India) (Deraniyagala 1945) [8], *D. r. russelli* (Welch 1994) (remainder of India, Bangladesh, Pakistan) [9], and *Daboia russelii pulchella* (Gray 1842) (Sri Lanka) [10], have now been incorporated into *D. russelii*. Java-Indonesian *V. r. limitis* (Mertens 1927), *V.r. formosensis* (Maki 1931) (Taiwan), and *D. r. siamensis* (Myanmar, Thailand, South China, Cambodia) are considered as synonymous species with *D. siamensis* [10,11]. Russell's viper, together with the Indian cobra (*Naja naja*), the common krait, (*Bungarus caeruleus*), and the saw-scaled viper (*Echis carinatus*), constitute 'The Big Four', the four main species responsible for causing most snakebite incidents and deaths in the Indian subcontinent. The medical relevance of Russell's viper is due to many factors, such as its wide distribution, fast speed, well deserved reputation for aggressiveness and striking without warning, and its frequent occurrence in open, grassy or bushy areas in highly urbanized areas and settlements in the countryside, where rodent preys are abundant [12,13]. The quantity of venom produced by this heavy-bodied ambush rodent hunter is considerable, with reported venom yields for adult specimens ranging from 130 to 250 mg. Its Median Lethal Dose (LD_{50}) in mice is 0.13 mg/kg intravenous, 0.40 mg/kg intraperitoneal, and 0.75 mg/kg subcutaneous [14] (http://snakedatabase.org/pages/ld50.php). However, geographic variation in lethal potency of Russell's viper venom has been reported across India [15,16] and Myanmar [17].

While usually not lethal if properly treated with antivenom, envenomings by Russell's vipers cause marked local effects, e.g. pain at the site of the bite immediately followed by swelling of the affected extremity, which may progress to tissue necrosis. Systemic manifestations include coagulopathy and hemorrhage, with bleeding from the gums and, in severe cases, disseminated intravascular coagulation leading to spontaneous bleeding from vital organs; acute kidney injury; hypotension and cardiovascular shock. These are common clinical manifestations in envenomings throughout the geographical range of Russell's vipers [18–30]. Other clinical presentations are known to vary between geographical regions. In some parts of India, such as Kerala, there is generally increased capillary permeability which may lead to systemic capillary leakage syndrome. Sri Lankan and South Indian populations of *D. russelii* cause clinically significant neurotoxicity in humans [19,23,24,31] not commonly reported in other geographical regions where Russell's viper is found (Chauhan and Thakur, 2016). Neurotoxicity following Sri Lankan Russell's viper envenoming is primarily due to the pre-synaptic neurotoxin U1-viperitoxin-Dr1a, a major PLA_2 molecule [P86368] (13672.82 Da) that constitutes 19.2% of the crude venom [32]. Although damage to the central nervous system, particularly ischemic complications, after *D. russelii* bite are rare, the risk of anterior pituitary infarction resulting in fatal acute pituitary adrenal failure or chronic panhypopituitarism has been reported in southern India [20,29,30,33,34]. Moreover, rare cases of ischemic stroke following Russell's viper snake bites in India have been documented [35–37]. However, clinical studies involving Russell's viper bites from different parts of India, Bangladesh and Pakistan are missing. Thus, a complex spectrum of clinical pictures characterizes envenomings by this snake in different regions of its distribution range.

Variations in the clinical manifestations are doubtless functionally related to differences in venom composition of Russell's vipers inhabiting different geographical areas. Past studies have demonstrated geographical variation in the venom effects of Russell's vipers from different geographical areas in the Indian Peninsula, Sri Lanka, Myanmar, and Thailand [5,15,20,38,39]. Thus far, proteomics studies have been conducted on venoms from *D. siamensis* from Myanmar [40], and *D. russelii* from Sri Lanka [41], specimens of Pakistan origin kept in the Kentucky Reptile Zoo [42], South India [43], and Western India [44]. Here, we have applied a venomics approach to compare the venom proteomes of *D. russelii* from Bangladesh, Pakistan, South India and Sri Lanka. This study was also designed to assess the preclinical efficacy of a panel of antivenoms manufactured in India, Bangladesh, and Costa Rica to neutralize key effects of *D. russelii* venoms, i.e. lethal, hemorrhagic, coagulant, and phospholipase A_2 activities, by the combination of *in vivo* and *in vitro* neutralization assays and *in vitro* third-generation antivenomics analysis to assess the toxin recognition landscape of the antivenom and quantify the fraction of therapeutic antivenom molecules. These data contribute to enrich our knowledge on venom variations among Russell's vipers from different geographical areas, understand their associated toxicity, and envision strategies to improve the design of antivenoms used for the clinical treatment of envenomations by *D. russelii* across the Indian subcontinent.

2. Materials and methods

2.1. Venoms and antivenoms

Venom from *D. russelii* from Bangladesh was collected by Dr. Ulrich Kuch; venom from *D. russelii* from Pakistan was purchased from Latoxan SAS (26800 Portes lès Valence, France); another sample of Pakistani Russell's viper venom and homologous venom from the Irula tribal snake venom extraction cooperative (Tamil Nadu, South India) were kindly donated by Dr. Claire F. Komives (Dept. of Biomedical, Chemical and Materials Engineering, San Jose State University, San Jose, CA, USA, 95192–0082). Venom of *D. russelii* from Sri Lanka was pooled from 30 wild caught specimens following the granting of a collecting permit from the Department of Wildlife, Sri Lanka numbered WL/3/2/1/7, and maintained in captivity at a serpentarium in Sri Lanka run by the organization Animal Venom Research International (AVRI http://www.usavri.org).

Antivenoms used in this study were manufactured by Incepta Vaccine LtD. (Dhaka, Bangladesh) (410.6 mg/vial; expiry date: August 2017); Haffkine Bio-Pharmaceutical Corporation Ltd. (Mumbai, India) (1325.0 mg/vial; expiry date: August 1998, and 509.6 mg/vial; expiry date: March 2016); VINS Bioproducts Ltd. (Hyderabad, India) (555.3 mg/vial; expiry date: July 2016); Biological E. Ltd. (Hyderabad, India) (473.6 mg/vial; expiry date: July 2015); Premium Serums & Vaccines Pvt Ltd. (Narayangaon, Maharashtra, India) (628.1 mg/vial; expiry date: February 2021); ICP (San José, Costa Rica) (597 mg/vial; expiry date: November 2020). All Indian polyvalent antivenoms consist of equine $F(ab')_2$ fragments of IgGs raised against the venoms of *Naja naja*, *Bungarus caeruleus*, *Daboia russelii*, and *Echis carinatus* sourced mainly from Chennai area, the capital of the south Indian State of Tamil Nadu. These antivenoms follow a standard set out in the Indian Pharmacopoeia requiring products to neutralize 0.6 mg *Naja naja* venom/mL antivenom, 0.6 mg/mL *Daboia russelii* venom, 0.45 mg/mL *Bungarus caeruleus* venom and 0.45 mg/mL *Echis carinatus* venom (National Snakebite Management Protocol, India, 2009) [45]. A new

whole IgG, freeze-dried, polyspecific antivenom was prepared at Instituto Clodomiro Picado (San José, Costa Rica) from the plasma of horses immunized with the venoms of the snakes *Daboia russelii*, *Echis carinatus*, *Hypnale hypnale*, and *Naja naja* from Sri Lanka, as reported [46]. Its median effective dose (ED_{50}) against Sri Lankan *D. russelii* venom was 2.6 (95% confidence limits: 2.0–3.3) mg venom/mL antivenom [46].

2.2. Toxic and enzymatic venom activities and their neutralization by antivenoms

2.2.1. Lethal activity

For the estimation of the Median Lethal Dose (LD_{50}), groups of five CD-1 mice (18–20 g) received various doses of venom, by the intravenous (i.v.) route, dissolved in a volume of 0.2 mL 0.12 M NaCl, 0.04 M phosphate, pH 7.2 (PBS). Deaths occurring within 24 h were recorded and the LD_{50} was estimated by probits [47]. For the neutralization of lethality, mixtures containing a fixed dose of venom and various dilutions of antivenom were prepared, and incubated at 37 °C for 30 min. Aliquots of 0.2 mL of each mixture, containing a dose of venom corresponding to 3 LD_{50}s, were then injected intravenously (i.v.) into groups of five mice (18–20 g). Mixtures corresponded to various ratios of mg venom/mL antivenom A control group was injected with 3 LD_{50}s of venom incubated with PBS instead of antivenom. Deaths occurring during 24 h were recorded, and the neutralizing ability of antivenom was expressed as the Median Effective Dose (ED_{50}), i.e. the venom/antivenom ratio at which half of the population of injected mice is protected, estimated by probits. The protocols involving the use of mice were approved by the Institutional Committee for the Care and Use of Laboratory Animals (CICUA) of the University of Costa Rica (permit CICUA 27–13).

2.2.2. Hemorrhagic activity

To assess the hemorrhagic activity of venoms, groups of four CD-1 mice (18–20 g) received an intradermal (i.d.) injection, in the ventral abdominal region, of 0.1 mL of PBS containing various venom amounts. Mice were sacrificed by CO_2 inhalation 2 h after injection, the skin was removed, and the area of the hemorrhagic lesion in the inner side of the skin was measured. The Minimum Hemorrhagic Dose (MHD) corresponds to the dose of venom that induces a hemorrhagic area of 10 mm diameter [48]. For the assessment of the neutralizing capacity of antivenoms, mixtures containing a fixed dose of venom and various dilutions of antivenom were prepared, and incubated at 37 °C for 30 min [48]. Then, aliquots of 0.1 mL of each mixture, containing a dose of venom corresponding to 5 Minimum Hemorrhagic Doses (MHDs), were injected i.d. into groups of five mice, as described. Mixtures corresponded to various ratios of mg venom/mL antivenom. A control group of mice was injected with the same dose of venom incubated with PBS instead of antivenom. Mice were sacrificed as described 2 h after injection, and the area of the hemorrhagic lesion was measured. Neutralizing ability was expressed as the Median Effective Dose (ED_{50}), corresponding to the ratio venom/antivenom at which the diameter of the hemorrhagic spot is reduced by 50% when compared to the diameter of the hemorrhagic lesion in mice injected with venom incubated with no antivenom [48].

2.2.3. Coagulant activity

Coagulant activity of venom was determined based on the turbidimetric assay described by O'Leary and Isbister [49] with some modifications. Different amounts of venom, dissolved in 25 μL TBS (25 mM Tris-HCl, 137 mM NaCl, 3.4 mM KCl, pH 7.4) were added to wells in a 96-well plate and incubated for 5 min at 37 °C in a microplate reader (Cytation 3 Imaging Reader, BioTek). Then, 0.04 μL of 0.4 M $CaCl_2$ was added to 100 μL of human citrated plasma previously incubated at 37 °C, and this mixture was added immediately to each venom-containing well using a multichannel pipette. Controls consisted of plasma incubated with TBS alone. Samples were mixed 5 s in a shaking step, and the absorbance at 340 nm was monitored every 30 s over 15 min. The increase in absorbance reflects the formation of a clot. Coagulant activity was expressed as the Minimum Coagulant Dose (MCD), corresponding to the minimum dose of venom that induces a change in absorbance of 0.1 units within 1 min. For the study of neutralization, mixtures containing a fixed dose of venom and various dilutions of antivenom were prepared, and incubated at 37 °C for 30 min [50]. Then, aliquots of 25 μL of each mixture, containing a venom dose corresponding to 2 Minimum Coagulant Doses (MCDs), were tested for coagulant activity as described above. Mixtures corresponded to various ratios of mg venom/mL antivenom. A control group was included, corresponding to plasma incubated with venom that was previously incubated with PBS instead of antivenom. Changes in absorbance were recorded and neutralization was expressed as Effective Dose (ED), corresponding to the ratio of venom/antivenom in which the change in absorbance is prolonged three times as compared to plasma incubated with venom alone.

2.2.4. Phospholipase A_2 (PLA_2) activity

The titrimetric method described by Gutiérrez et al. (1986) [51] was followed, using egg yolk phospholipids as substrate. Activity was expressed as μEq fatty acid released per mg protein per min. For neutralization, mixtures were prepared containing a fixed amount of venom and variable dilutions of antivenom. Mixtures corresponded to various ratios of mg venom/mL antivenom. Controls included venom incubated with PBS instead of antivenom. After an incubation of 30 min at 37 °C, the PLA_2 activity of the mixtures was assessed as described. Neutralization was expressed as Median Effective Dose (ED_{50}), corresponding to the ratio of venom/antivenom in which PLA_2 activity was reduced by 50% as compared to the activity of venom alone.

2.3. Isolation and initial characterization of D. russelii venom proteins

Two milligrams of crude lyophilized venom were dissolved in 300 μL of 0.05% trifluoroacetic acid (TFA) and 5% acetonitrile (ACN). Insoluble material was removed by centrifugation in an Eppendorff centrifuge at 13000 × *g* for 10 min at room temperature, and the proteins contained in 15 μL were separated by RP-HPLC using a Agilent LC 1100 High Pressure Gradient System equipped with DAD detector and a Discovery® BIO Wide Pore C18 (15 cm × 2.1 mm, 3 μm particle size, 300 Å pore size) column and a DAD detector. The column was developed at a flow rate of 0.4 mL/min with a linear gradient of 0.1% TFA in MilliQ® water (solution A) and 0.1% TFA in acetonitrile (solution B), isocratic (5% B) for 1 min, followed by 5–25% B for 5 min, 25–45% B for 35 min, and 45–70% B for 5 min. Protein detection was carried out at 215 nm with a reference wavelength of 400 nm. Fractions were collected manually across the entire elution range, dried in a vacuum centrifuge (Savant), and redissolved in MilliQ® water. Molecular masses of the purified proteins were estimated by non-reduced and reduced SDS-PAGE (on 12 or 15% polyacrylamide gels), or determined by electrospray ionization (ESI) mass spectrometry. For SDS-PAGE analysis sample aliquots were mixed with ¼ volume of 4x sample buffer (0.25M Tris-HCl pH 6.8, 8% SDS, 30% glycerol, 0.02% bromophenol blue, with or without 10% 2-mercaptoethanol) and heated at 85 °C for 15 min, run under non-reducing and reducing conditions, and the gels were stained with Coomassie Brilliant Blue G-250. For ESI-MS, the proteins eluted in the different RP-HPLC fractions were separated by nano-Acquity UltraPerformance LC® (UPLC®) using BEH130 C18 (100μm × 100mm, 1.7 μm particle size) column in-line with a Waters SYNAPT G2 High Definition Mass Spectrometry System. The flow rate was set to 0.6 μL/min and the column was develop with a linear gradient of 0.1% formic acid in water (solution A) and 0.1% formic acid in ACN (solution B), isocratically 1% B for 1 min, followed by 1–12% B for 1 min, 12–40% B for 15 min, 40–85% B for 2 min. Monoisotopic and isotope-averaged molecular masses were calculated by manually deconvolution of the

isotope-resolved multiply-charged MS1 mass spectra.

2.4. Proteomics caracterization and quantification of the proteomes of D. russelii venoms

Protein bands of interest were excised from Coomassie Brilliant Blue-stained SDS-PAGE gels and subjected to in-gel reduction (10 mM dithiothreitol, 30 min at 65 °C) and alkylation (50 mM iodacetamide, 2 h in the dark at room temperature), followed by overnight sequencing-grade trypsin digestion (66 ng/μL in 25 mM ammonium bicarbonate, 10% ACN; 0.25 μg/sample) in an automated processor (Genomics Solution ProGest Protein Digestion Workstation) following the manufacturer's instructions. Tryptic digests were dried in a vacuum centrifuge (SPD SpeedVac®, ThermoSavant), redissolved in 15 μL of 5% ACN containing 0.1% formic acid, and submitted to LC-MS/MS. To this end, tryptic peptides were separated by nano-Acquity UltraPerformance LC® (UPLC®) as above. Doubly and triply charged ions were selected for CID-MS/MS. Fragmentation spectra were interpreted i) manually (*de novo* sequencing), ii) using the on-line form of the MASCOT Server (version 2.6) at http://www.matrixscience.com against the last update (release 230, February 15, 2019) of NCBI non-redundant database, and iii) processed in Waters Corporation's ProteinLynx Global SERVER 2013 version 2.5.2. (with Expression version 2.0). The following search parameters were used: Taxonomy: all entries; Enzyme: trypsin (1 missed cleavage allowed); MS/MS mass tolerance was set to ± 0.6 Da; carbamidomethyl cysteine and oxidation of methionine were selected as fixed and variable modifications, respectively. All matched MS/MS data were manually checked. Peptide sequences assigned by *de novo* MS/MS were matched to homologous proteins available in the NCBI non-redundant protein sequences database using the online BLASTP program [52] at https://blast.ncbi.nlm.nih.gov/Blast.cgi.

The relative abundances of the chromatographic peaks obtained by reverse-phase HPLC fractionation of the whole venom were calculated as "% of total peptide bond concentration in the peak" by dividing the peak area by the total area of the chromatogram [53–55]. For chromatographic peaks containing single components (as judged by SDS-PAGE and/or MS), this figure is a good estimate of the % by weight (g/100 g) of the pure venom component [56]. When more than one venom protein was present in a reverse-phase fraction, their proportions (% of total protein bands area) were estimated by densitometry of Coomassie-stained SDS-polyacrylamide gels using MetaMorph® Image Analysis Software (Molecular Devices). Conversely, the relative abundances of different proteins contained in the same SDS-PAGE band were estimated based on the relative ion intensities of the three most abundant peptide ions associated with each protein by MS/MS analysis. The relative abundances of the protein families present in the venom were calculated as the ratio of the sum of the percentages of the individual proteins from the same family to the total area of venom protein peaks in the reverse-phase chromatogram.

2.5. Two-dimensional (IEF/SDS-PAGE) gel electrophoresis (2-DE)

Two-dimensional gel electrophoresis (2-DE) was performed essentially according to the manufacturer's (GE Healthcare Amersham Biosciences) instructions unless otherwise indicated. For the first dimension, isoelectric focusing (IEF), ~150 μg of venom were dissolved in 7 M urea, 2 M thiourea, 4% CHAPS, and 0.5% IPG Buffer pH 3–10 and applied onto 7-cm pH 3–10 linear immobilized pH gradient (IPG) strips. IEF was carried out with an Ettan-IPGphor isoelectric focusing unit at 20°C applying the following conditions: 300 V (0.5 h), ramping to 1000 V (0.5 h), ramping to 5000 (1.3 h) and 5000 V (0.5 h). After IEF, the IPG strips were kept at −70°C until use. For the second dimension, SDS-polyacrylamide gel electrophoresis (SDS-PAGE), the IPGs were equilibrated for 15 min with gentle shaking and at room temperature in equilibration buffer (6 M urea, 2% [w/v] SDS, 30% [v/v] glycerol, 75 mM Tris–HCl, pH 8.8), with or without 40 mM DTT. IPG strips were then placed on top of an SDS-15% polyacrylamide gels and run in a Protean II (Bio-Rad) electrophoresis unit at room temperature. Protein spots were visualized by Coomassie Brilliant Blue G250 staining.

2.6. Molecular phylogenetic analysis by Maximum Likelihood method

Multiple sequence alignment of full-length PLA_2 sequences identified in *D. russelii* and *D. siamensis* venom proteomes was completed using MEGA version 7.0.26 [57]. A cladogram was inferred by using the Maximum Likelihood method based on the JTT matrix-based model [58].

2.7. Third-generation antivenomics

Third-generation antivenomics [59,60] was applied to assess the immunoreactivity of the Bangladeshian (Incepta) and Indian (Haffkine and BE Ltd.) antivenoms against *D. russelii* from Bangladesh; VINS against *D. russelii* venom from Bangladesh, Sri Lanka, Pakistan, and Tamil Nadu; Premium Serums & Vaccines against Sri Lanka, Pakistan, and Tamil Nadu; and Costa Rican (ICP) against the venom of *D. russelii* from Sri Lanka, Bangladesh, and Pakistan (Supplementary Tables S11–S24). To this end, one vial of each antivenom was dissolved in 10 mL of the supplied diluent, dialyzed against MilliQ® water, lyophilized, and reconstituted in 10 mL of 0.2 M $NaHCO_3$, 0.5 M NaCl, pH 8.3 (coupling buffer). The concentrations of the antivenom stock solutions were determined spectrophotometrically using an extinction coefficient for a 1 mg/mL concentration ($\varepsilon^{0.1\%}$) at 280 nm of 1.36 $(mg/mL)^{-1}$ cm^{-1} [61]. Antivenom affinity columns were prepared in batch. 3 mL of CNBr-activated Sepharose™ 4B matrix (Ge Healthcare, Buckinghamshire, UK) packed in a ABT column (Agarose Bead Technologies, Torrejón de Ardoz, Madrid) and washed with 10x matrix volumes of cold 1 mM HCl, followed by two matrix volumes of coupling buffer to adjust the pH of the column to 7.0–8.0. CNBr-activated instead of N-hydroxysuccinimide (NHS)-activated matrix was employed because NHS released during the coupling procedure absorbs strongly at 280 nm, thus interfering with the measurement of the concentration of antibodies remaining in the supernatant of the coupling solution. 40 mg of each antivenom was dissolved in the 2x matrix volume of coupling buffer and incubated with the 3 mL NHS-activated matrix for 4 h at room temperature. Antivenom coupling yield, estimated measuring A_{280nm} before and after incubation with the matrix, were 29 mg (Incepta Vaccine Ltd), 30.54 mg and 27.2 mg (Haffkine Bio-Pharmaceutical Corporation Ltd., expiry dates August 1998 and March 2016, respectively), 25.3 mg (VINS Bioproducts Ltd) and 26.35 mg (Biological E. Ltd.) antivenoms. After the coupling, remaining active matrix groups were blocked with 12 mL of 0.1 M Tris-HCl, pH 8.5 at room temperature for 4 h. Five affinity columns each containing 300 μL of immobilized (7 mg) antivenom were alternately washed with three matrix volumes of 0.1 M acetate containing 0.5 M NaCl, pH 4.0–5.0, and three matrix volumes of 0.1 M Tris-HCl, pH 8.5. This procedure was repeated 6 times. The columns were then equilibrated with 5 volumes of working buffer (PBS, 20 mM phosphate buffer, 135 mM NaCl, pH 7.4) and incubated with increasing amounts (100–1000 μg of total venom proteins) of *D. russelii* from Bangladesh dissolved in ½ matrix volume of PBS, and the mixtures incubated for 1 h at 25 °C in an orbital shaker. As specificity controls, 300 μL CNBr-activated Sepharose™ 4B matrix, without (mock) or with 8 mg of immobilized control (naïve) horse IgGs, were incubated with venom and developed in parallel to the immunoaffinity columns. The non-retained eluates of columns incubated with 100–500 μg and 750–1000 μg venom were recovered with 5x and 10x matrix volume of PBS, respectively, and the immunocaptured proteins were eluted, respectively, with 5x and 10x matrix volume of 0.1 M glycine-HCl, pH 2.7 buffer and brought to neutral pH with 1 M Tris-HCl, pH 9.0. The entire fractions eluted with 5x and ½ of the fractions recovered in 10x matrix volume were concentrated in a Savant

SpeedVac™ vacuum centrifuge (ThermoFisher Scientific, Waltham, MA USA) to 40 µL, which were then fractionated by reverse-phase HPLC using an Agilent LC 1100 High Pressure Gradient System (Santa Clara, CA, USA) equipped with a Discovery® BIO Wide Pore C18 (15 cm × 2.1 mm, 3 µm particle size, 300 Å pore size) column and a DAD detector as above. Eluate was monitored at 215 nm with a reference wavelength of 400 nm. The fraction of non-immunocaptured molecules was estimated as the relative ratio of the chromatographic areas of the toxin recovered in the non-retained (NR) and retained (R) affinity chromatography fractions using the equation %NRi = 100-[(Ri/(Ri + NRi)) × 100], where Ri corresponds to the area of the same protein "i" in the chromatogram of the fraction retained and eluted from the affinity column. However, for some toxins that were poorly recovered in the column-retained fraction owing to their high binding affinity to the immobilized antivenom likely preventing their elution from the column [62], the percentage of non-immunocaptured toxin"i" (% NRtoxin"i") was calculated as the ratio between the chromatographic areas of the same peak recovered in the non-retained fraction (NRtoxin"i") and in a reference venom (Vtoxin"i") containing the same amount of total protein that the parent venom sample and run under identical chromatographic conditions, using the equation %NRtoxin"i" = (NRtoxin"i"/Vtoxin"i") × 100.

2.8. Calculation of the venom toxin-specific and venom neutralizing antibody content of Russell's viper antivenoms

The percentage of antivenom anti-toxin $F(ab')_2$ or IgG molecules was calculated by dividing [(1/2 maximal amount (in µmoles) of total venom proteins bound per antivenom vial) × molecular mass (in kDa) of antibody (IgG, 160 kDa or $F(ab')_2$, 100 kDa) molecule] by the [total amount of antibody (IgG or $F(ab')_2$) (in mg) per antivenom vial [63,64].

The percentage of toxin neutralizing anti-toxin molecules was calculated by dividing the antivenom's potency (P) by the maximal amount of total venom proteins bound by mL of antivenom. The potency (P) is the amount of venom (mg) completely neutralized per mL of antivenom. P was calculated using the formula $P = [(n-1)/ED_{50}] \times LD_{50}$, where "n" is the number of median lethal doses (LD_{50}s) used as challenge dose to determine the antivenom's median effective dose, ED_{50}. For the calculation of P, LD_{50} and ED_{50} are expressed, respectively, as (mg venom/mouse) and (mL of antivenom that protect 50% of the mice population inoculated with $n \times LD_{50}$). In the calculation of P, $(n-1) \times LD_{50}$ is used instead of the total amount of venom, $n \times LD_{50}$, because at the endpoint of the neutralization assay, one LD_{50} remains unneutralized and causes the death of 50% of mice [65–67].

2.9. Statistical analyses

The significance of the differences between the mean values of pairs of samples was assessed by the Mann–Whitney U test. When more than two groups were compared, the method of Kruskal–Wallis was used, followed by Dunn's test to compare pairs of means. A value of $p < 0.05$ was considered significant. In lethality, values are considered significantly different if there is no overlap of the 95% confidence limits.

3. Results and discussion

Variation in the venom effects of Russell's vipers from different geographic origin has been repeatedly documented [5,15,20,38,39,68]. Haemostatic abnormalities have been described in all *D. russelii* venoms investigated. Other clinical signs of *D. russelii* envenomings show geographical variations, i.e., neurotoxicity and intravascular haemolysis are apparently confined to Sri Lankan and South Indian Russell's viper populations (Introduction, but consult also [28]). Likewise, systemic capillary leakage syndrome has been described only in envenomings occurring in some geographical settings [69]. Variable clinical manifestations of envenoming in different areas indicate geographical variations in venom composition. However, only venom obtained from a single source, the Irula tribal snake venom extraction cooperative situated in the south Indian state of Tamil Nadu, is being used for manufacturing Indian antivenoms to treat any Russell's viper bite in India [70]. On the basis of the wide geographical variation in the venom composition of this species, it seems thus highly unlikely that the currently used antivenoms are adequate to fully neutralize all the clinically relevant components of *D. russelii* venoms from different geographic origin and to prevent all the complications across the different regions of the Indian subcontinent. The aims of this work were i) to compare the biological effects of venoms from the northern (Pakistan) and southern (Sri Lanka) regions of *D. russelii* range; ii) to carry out a comparative venomics study to correlate conserved and geographic-specific clinical manifestations of envenomings by Russell's vipers from different geographic regions of the Indian subcontinent; and iii) to apply third generation antivenomics and *in vivo* neutralization analyses to assess the immunoreactivity and preclinical profiles of a panel of anti-*D. russelii* antivenoms produced in India and Costa Rica towards venoms from geographic variants of Russell's viper.

3.1. Toxic and enzymatic activities of Daboia russelii venoms from Pakistan and Sri Lanka and their neutralization by antivenoms

Table 1 displays the results of the quantification of the *in vivo* Median Lethal Dose (LD_{50}) and hemorrhagic effect, and the *in vitro* coagulant and PLA_2 activities, of the venoms from Sri Lankan and Pakistani Russell's viper, and the neutralization of Sri Lankan, Pakistani, and Bangladeshi Russell's viper venoms by two Indian (VINS and Premium Serums) and a Costa Rican (ICP) polyvalent antivenoms raised against *D. russelii* venom from Tamil Nadu (India) and Sri Lanka, respectively. Both Sri Lankan and Pakistani venoms presented similar hemorrhagic, *in vitro* coagulant, and PLA_2 activities but differed in their lethality, with the Pakistani venom being more potent than the venom from Sri Lanka. The value of LD_{50} of venom from Bangladesh was very similar to the one from Pakistan, and therefore more potent than Sri Lankan venom (Table 1). Toxic activities other than lethality of the venom of Russell's viper from Bangladesh were not addressed due to scarcity of venom.

Regarding neutralization, the two Indian antivenoms neutralized the lethality of heterologous Sri Lankan and Pakistani *D. russelii* venoms with 2–3-fold higher neutralization potency (P) than their nominal specification included in their prospects of "not less than 0.6 mg of Russell's viper (*D. russelii*) venom/mL antivenom". No significant difference was observed between the Indian and Costa Rican antivenoms in the neutralization of lethal activity of the Sri Lankan and Pakistani *D. russelii* venoms, as the 95% confidence limits overlapped (Table 1). In agreement, when neutralization of lethality was expressed as 'potency', the three antivenoms showed similar values against these two venoms. On the other hand, VINS and Premium Serums antivenoms were not effective in neutralizing the lethal activity of venom from Bangladesh (ED_{50} < 1.5 mg V/mL AV; Table 1), wheras the ICP antivenom prevented lethality with an ED_{50} of 2.64 (Potency = 1.76) mg V/mL AV (Table 1). Incepta antivenom, which is produced by Premium Serums but manufactured in Bangladesh, was ineffective in the neutralization of lethality induced by i.v. injection of 3 LD_{50}s of venom from Bangladesh specimens (ED_{50} < 1.5 mg V/mL AV; data not shown).

When antivenoms were compared for their ability to neutralize hemorrhagic activity caused by Pakistani and Sri Lankan venoms, there was a significant difference ($p < 0.05$) only between ICP and VINS antivenoms (Table 1). In the case of the neutralization of coagulant activity, significant differences ($p < 0.05$) were found only when comparing Premium Serums and ICP antivenoms (against Pakistan venom) and ICP and VINS antivenoms (for Sri Lanka venom) (Table 1). On the other hand, neutralization of PLA_2 activity revealed significant differences ($p < 0.05$) between ICP and VINS antivenoms (against venom of Pakistan), and between ICP and Premium Serums (against

Table 1
Biological activities of *Daboia russelii* venoms from Sri Lanka, Pakistan and Bangladesh, and their neutralization by antivenoms. LD50, Median Lethal Dose; MHD, Minimum Hemorrhagic Dose; MCD, Minimum Coagulant Dose; PLA2, phospholipase A2 activity.

	***Daboia russelii* venom**								
	Sri Lanka				**Pakistan**				**Bangladesh**
	LD_{50}(i.v.)	**MHD**	**MCD**	**PLA_2**	**LD_{50}(i.v)**	**MHD**	**MCD**	**PLA_2**	**LD_{50}(i.v.)**
	µg/18–20 g mouse		µg/100 µL plasma	µEq/mg/min	µg/18–20 g mouse		µg/100 µL plasma	µEq/mg/min	µg/18–20 g mouse
	7.89 (7.16–10.9)*	4.27 ± 1.18	0.52 ± 0.03	78.03 ± 6.34	3.67 (3.01–4.37)*	4.71 ± 0.77	0.13 ± 0.01	59.45 ± 8.18	3.69 (2.00–5.86)
Neutralization by antivenom (ED50)**									
Antivenom ***	mg V/mL AV	mg V/mL AV	mg V/mL AV	mg V/mL AV	mg V/mL AV	mg V/mL AV	mg V/mL AV	mg V/mL AV	mg V/mL AV
VINS	1.89 (1.49–2.84)	8.71 ± 0.64	3.90 ± 0.14	0.22 ± 0.01	1.86 (1.14–3.66)	5.04 ± 0.53	1.96 ± 0.00	0.68 ± 0.02	< 1.5
ICP	2.60 (2.00–3.30)	16.15 ± 1.29	7.28 ± 0.20	0.47 ± 0.02	2.30 (1.22–4.42)	12.76 ± 2.08	2.27 ± 0.06	1.01 ± 0.02	2.64 (2.01–3.47)
Premium Serums	2.33 (1.62–5.12)	13.06 ± 1.04	6.26 ± 0.08	0.18 ± 0.01	2.66 (1.73–5.48)	10.51 ± 0.80	4.63 ± 0.42	0.95 ± 0.08	< 1.5
Potency of antivenom (P)									
	mg V/mL AV	mg V/g AV			mg V/mL AV	mg V/g AV			mg V/mL AV	mg V/g AV
VINS	1.26	22.68			1.24	22.32			< 1.0	
ICP	1.73	28.98			1.53	25.62			1.76	29.48
Premium Serums	1.55	24.68			1.78	28.34			< 1.0	

* Significant difference between venoms.
** Neutralization is expressed as ED_{50} for lethal, hemorrhagic and PLA_2 activities, and as ED for coagulant activity (see Section 2 for details).
*** See results of statistical analyses of the comparisons of the three antivenoms in Section 3.

venom of Sri Lanka) (Table 1). In general, regardless of these quantitative variations observed in the neutralization of some effects, the three antivenoms tested had a similar neutralizing profile against venoms from Pakistan and Sri Lanka. This is in line with the similarities observed between these two venoms in the protemics analysis (see below). However, ICP antivenom had a higher neutralizing potency than the other antivenoms when tested against venom from Bangladesh.

The outcome of the neutralization assays are in line with a previous study showing that Indian mono- and polyvalent antivenoms produced against South India *D. russelli* venom, manufactured by VINS and Bharat Serum and Vaccines Limited, partially neutralized the pro- and anticoagulant activities of crude venom pooled from Pakistani Russell's viper maintained in the Kentucky Reptile Zoo, and of fractions enriched in procoagulant SVMPs and SVSPs and anticoagulant PLA_2s and/or Kunitz-like inhibitor type proteins [42]. These authors did not quantify the neutralization capability of the Indian antivenoms tested. More recently, Faisal and colleagues [71] reported that the VINS polyvalent antivenom moderately neutralized the *in vitro* procoagulant and *in vivo* lethal effect of venom from wild caught *D. russelii* (Indus Delta, Pakistan), with a neutralization potency against lethality of 0.23 mg venom neutralized per mL antivenom. However, despite the fact that the LD_{50} of the venom employed by these authors (i.v. LD_{50} = 0.19 (0.17–0.25) µg/g mouse) was similar to that used in our work (Table 1), the VINS antivenom potency reported by Faisal et al. [71] was one order of magnitude lower than the one determined in the present work for the same antivenom (Table 1). Such difference might be due to geographic or ontogenetic venom variability or to the fact that different 'challenge doses' of venom were used in these studies for assessing the neutralization of lethality. To assess the landscape of para-specificity of available commercial antivenoms towards the venoms of geographical variants of *D. russelii* across the Indian subcontinent, we have compared the venom proteomes of *D. russelii* from Bangladesh, Pakistan, South India and Sri Lanka, and analyzed the antivenomics profiles of homologous antivenoms produced in India and Costa Rica.

3.2. The venom proteomes of Russell's vipers across the Indian subcontinent

Samples (1–2 mg) of *D. russelii* venoms from Bangladesh, Pakistan, Sri Lanka, and South India (Tamil Nadu) were fractionated by reverse-phase (RP) HPLC (Fig. 1, panels A–D), and the RP fractions analyzed by SDS-PAGE (insets in Fig. 1A–D). The venoms of *D. russelii* from Bangladesh, Pakistan, Sri Lanka, and South India (Tamil Nadu) were also fractionated by two-dimensional electrophoresis (2DE), with isoelectric focusing of the venom proteins solubilized in denaturing but non-reducing buffer as first dimension, and orthogonal SDS-PAGE separation of the isoelectric focused proteins under both non-reduced and reduced conditions (Fig. 2, panels A–D). The 1D and 2D electrophoretically-resolved protein bands were identified applying a bottom-up tryptic peptide-centric MS/MS approach and database matching through the MASCOT search engine or BLAST analysis of *de novo* gathered peptide ion sequences. Supplementary Tables S1, S3, S5 and S7 display the assignments of RP-HPLC/SDS-PAGE decomplexed venom proteomes of *D. russelii* from Bangladesh, Pakistan, Sri Lanka, and South India (Tamil Nadu), respectively, and Supplementary Tables S2, S4, S6 and S8 show the MS/MS-based identifications of the 2DE-separated proteins from the same *D. russelii* venoms. Panels A–D in Fig. 3 display, respectively, the number of distinct proteins and the relative abundances (in percentage of the total venom proteins) of the 15, 14, 12, and 14 peptide and protein classes identified, respectively, in the venoms of Russell's vipers from Bangladesh (B), Sri Lanka (SL), Pakistan (P), and South India (SI) origin (Supplementary Table S9). These toxin classes were unevenly distributed in the four venom proteomes, with PLA_2s representing the most abundant family, ranging from 47.5% of the total venom proteins in the venom from Bangladesh to 70.6% in that of the Tamil Nadu (South India) (Fig. 3). A set of proteins with more moderate

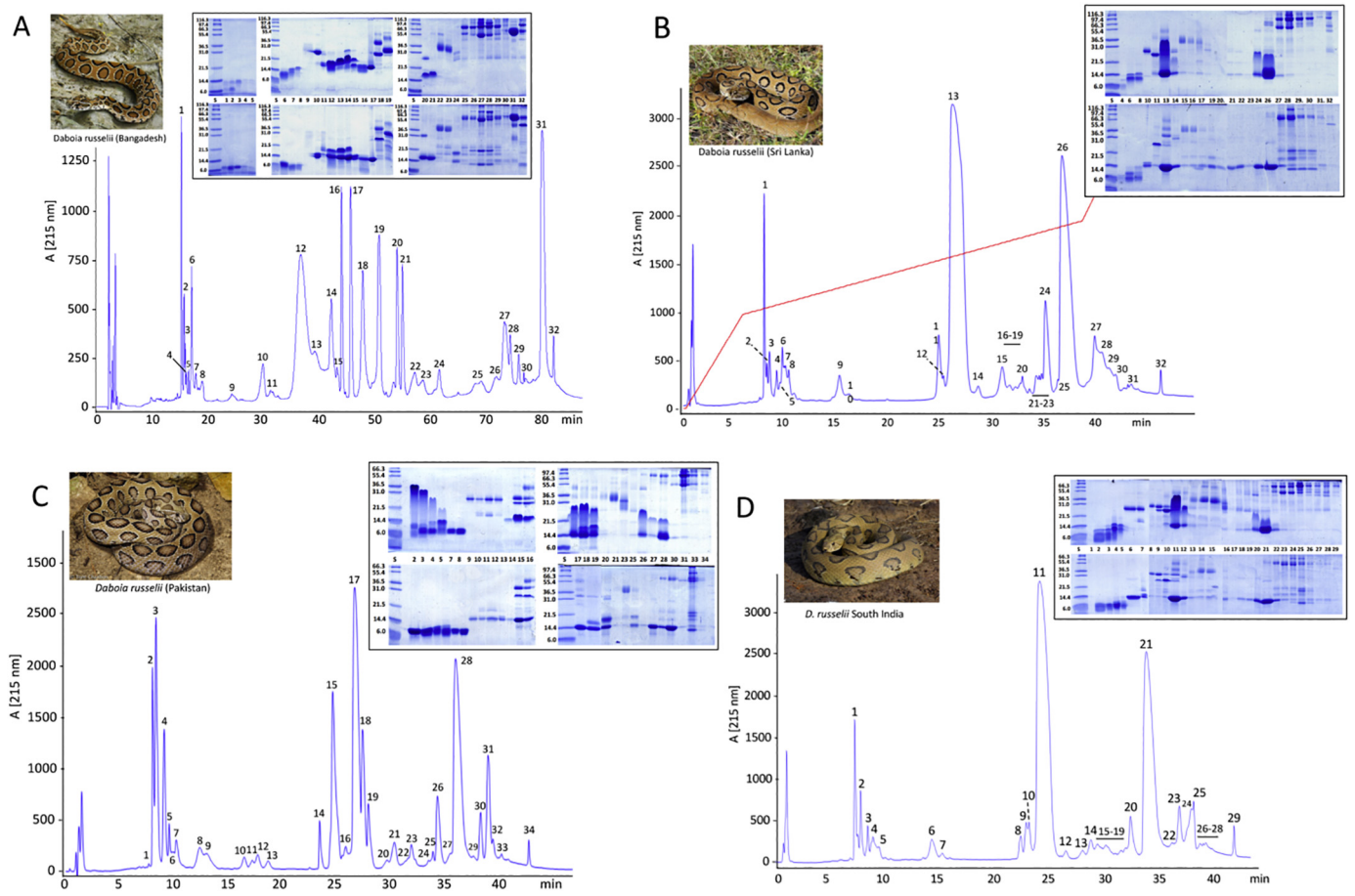

Fig. 1. Reverse-phase chromatographic separations of the venom proteins of *D. russelii* from Bangladesh (panel A), Sri Lanka (panel B), Pakistan (panel C), and South India (panel D). Chromatographic fractions were collected manually and analyzed by SDS-PAGE (inset) under non-reduced (upper panels) and reduced (lower panels) conditions. Protein bands were excised, in-gel digested with trypsin, and the resulting tryptic peptides sequenced by LC-nESI-MS/MS and identified by database searching or *de novo* sequencing (Supplementary Tables S1, S3, S5, and S5).

expression comprised snake venom serine proteinases (SVSP, 8.5–14%), snake venom metalloproteinases (SVMP, 3.4–12.6%), Kunitz-type inhibitor-like molecules (KUN, 2.4–17.8%), C-type lectin-like proteins (CTL, 3.9–6.8%), L-amino acid oxidases (LAO, 2.5–3.9%), cysteine-rich secretory proteins (CRISP, 2.1–3.3%), vascular endothelial growth factors (svVEGF, 1.8–2.7%), and the SVMP tripeptide inhibitor ZNW (3–3.5%) (Fig. 3). The relative abundances of the remaining protein families, which included 5′-nucleotidase (5'NT), phosphodiesterase (PDE), aminopeptidase (AP), glutaminyl cyclotrasferase (GC), disintegrin (DISI), disintegrin-like/cysteine-rich (DC), and snake venom nerve growth factor (vNGF), showed wide variation among the four *D. russelii* venoms sampled, representing 0.01–0.9% of their respective venom proteome (Fig. 3). Traces of hyaluronidase (< 0.01%) were only detected in the venom proteome from South India (Fig. 3D).

The combined LC-MS/MS analyses of proteins separated by the RP-HPLC/SDS-PAGE and 2DE approaches provided compelling evidence for the existence, in the venom proteomes of Russell's vipers from different geographic origin, of 57 (B), 43 (SI), 48 (SL), and 49 (P) peptide/proteins (Supplementary Table S9). The majority of these proteins behave as monomeric molecules upon 2DE separation under nonreducing conditions in both dimensions (Fig. 3A–D, upper panels). The occurrence and subunit composition of covalent protein complexes were uncovered through 2DE separation under nonreducing (IEF) and reducing (SDS-PAGE) conditions (Fig. 3A–D, lower panels). This approach showed the existence in all *D. russelii* venoms of Russell's viper venom Factor X activator (RVV-X), a PIIId SVMP that consists of three covalently bonded peptide chains: a PIII-SVMP heavy chain and two disulphide-linked C-type lectin-like (CTL) light chains (LC) (Takeda et al., 2007). Our analysis revealed, in the four *D. russelii* venoms investigated, at least 4 PIIId-SVMP heavy chains (PIV RVV-X HC) [ADJ67475, AAB22478, Q7LZ61, AUF41652], promiscuously associated with a number of light chains, particularly RVV-X LC-1 [AAB22478, Q4PRD1, AUF41654], RVV-X LC-2 [ADJ67473 and Q4PRD2], CTL P68 α-subunit [ADK22827] and CTL-3 [Q4PRD0]. On the other hand, P31 α and β-subunits [ADK22829, ADK22834]; Daboceti α and β-subunits [ADK22821, ADK22822]; CTL-7 [Q4PRC6]; and CTL-3 and CTL-5 [Q4PRC8] are associated into canonical disulphide-bonded αβ dimers (Fig. 2, Supplementary Tables S2, S4, S6 and S8).

3.3. Conserved toxins among geographic variants of Russell's viper contribute to relevant clinical features (kidney injury, bleeding and coagulopathy) that are widespread across the D. russelii complex range

Supplementary Table S9 displays the number and distribution of the proteins from the different toxin families identified among the venom proteomes of the Russell's vipers from Bangladesh, South India, Sri Lanka, and Pakistan (this work), along with literature data on biochemical and venomics studies of *D. russelii* from Pakistan [42,71], western India [44], eastern India [72,73], southern India [43,74], and Sri Lanka [41]. Fig. 4 highlights the number of geographic variant-specific and conserved (identical or highly similar) proteins between two or more conspecific venoms. Twenty nine proteins out of 77 distinct proteins (38%) were shared between the four *D. russelii* venoms

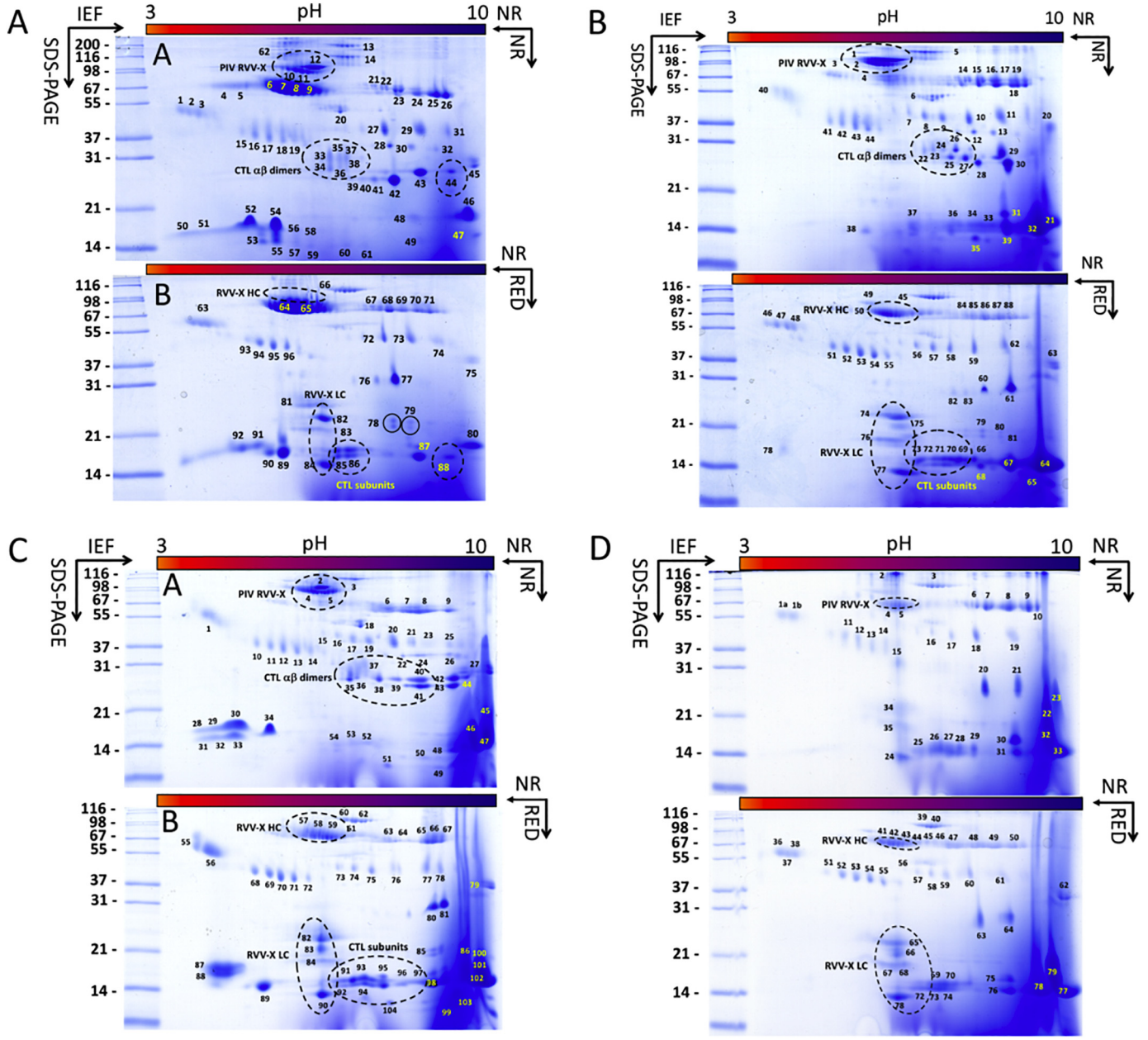

Fig. 2. Two-dimensional electrophoretic separations (IEF/SDS-PAGE) of the venom proteins of *D. russelii* from Bangladesh (panel A), Sri Lanka (panel B), Pakistan (panel C), and South India (panel D). In the first (IEF) dimension, the venom proteins were focused to their isoelectric points under non-reducing (NR) conditions. For the second (SDS-PAGE) dimension, the IPGs were equilibrated at room temperature in equilibration buffer without (NR) and with (RED) 40 mM DTT. Protein spots were excised, in-gel digested with trypsin, and the resulting tryptic peptides submited to LC-nESI-MS/MS sequencing and matched by database searching or *de novo* sequencing (Supplementary Tables S2, S4, S6, and S8).

sampled (Fig. 4). Another 35 proteins were present in two or more conspecific venoms, whereas 4, 2, 6 and 1 proteins were uniquely expressed in the venom from Bangladesh, Sri Lanka, Pakistan, and South India, respectively (Fig. 4 and Supplementary Table S9). With the notable exception of the PLA_2s and KUN molecules, the conserved set of venom proteins among the geographic variants of Russell's viper comprised toxins from all other major, and some minor, venom protein families (Fig. 4), and together account for 24% (SI), 30% (P), 32% (B) and 33% (SL) of the venom proteome.

SVSPs represent 8.5%, 9.1%, 12.8%, and 14.2% of the venom proteomes of South India, Pakistan, Sri Lanka, and Bangladesh, respectively (Fig. 3). Iso- or proteoforms of Factor V (RVV-V) activating serine proteinases [P18965, Q9PT40] [75–77], and thrombin-like α- and β-fibrinogenases [ES5L03, E5L0E4 (RVAF) and [E0Y418, E0Y419 and E0Y420 (RVBF)] [78] represent the major serine proteinases conserved among the venoms of the geographic variants of Russell's viper (Figs. 3 and 4, and Supplementary Table S9). Procoagulant thrombin-like and Factor V-activating serine proteases have been purified, and their pharmacological properties characterized, from venom of Russell's viper from Pakistan [79,80] and the close phylogenetically related *Macrovipera lebetina* subspecies (reviewed by Siigur et al. [77]). The occurrence of Factor V activating enzyme is a characteristic feature of the venom proteomes of Russell's vipers (both *D. russelii* and *D. siamensis*) from different geographic origin, including Sri Lanka [41], Pakistan [43], South India [42], Western India [44], and Myanmar [40]. RVV-V converts coagulation factor V to Va by selectively cleaving the Arg1545-Ser1546 bond [81], thereby promoting the formation of the prothrombinase complex, a potent procoagulant [75,80]. The

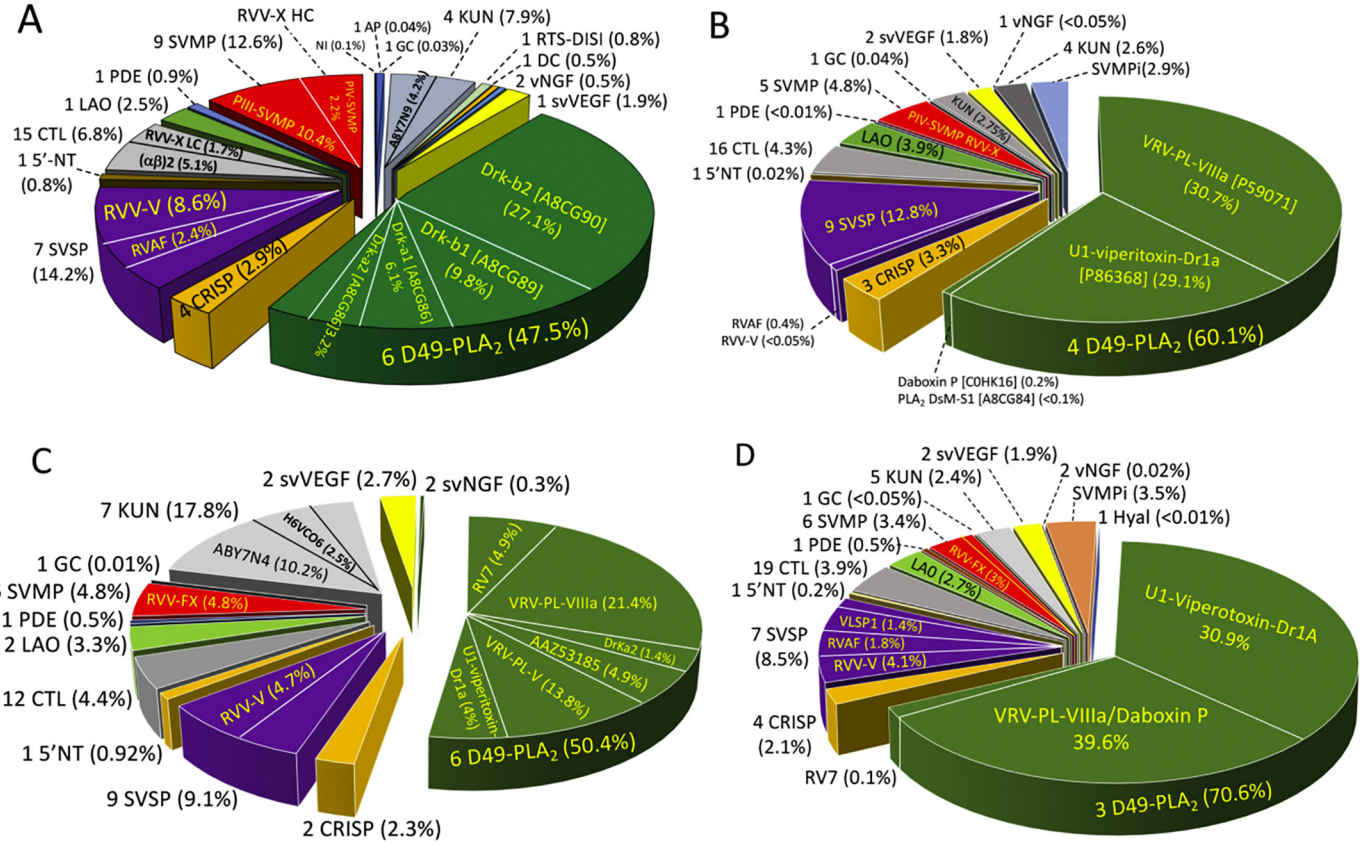

Fig. 3. Pie chart displaying the relative occurrence (in percentage of total venom proteins) of toxins from different protein families in the venom proteome of *D. russelii* from Bangladesh (panel A), Sri Lanka (panel B), Pakistan (panel C), and South India (panel D). Acronyms, PIII-SVMP, snake venom metalloproteinase of class PIII; AP, aminopeptidase; GC, glutaminyl cyclase; KUN, Kunitz-type serine proteinase inhibitor-like protein; RTS-DIS, RTS-disintegrin; DC, disintegrin-like/cysteine-rich of PIII-SVMP; vNGF, venom nerve growth factor; svVEGF, snake venom vascular endothelial growth factor; D49-PLA_2, D49 phospholipase A_2; CRISP, cysteine-rich secretory protein; SVSP, snake venom serine protease; 5'NT, 5′-nucleotidase; CTL, C-type lectin-like; LAO, L-amino acid oxidase; PDE, phosphodiesterase; SVMPi, tripeptide inhibitors of SVMP; Hyal, hyaluronidase; NI, not identified.

conserved occurrence of RVV-V and procoagulant SVSP subtypes (thrombin-like α- and β-fibrinogenases), along with α-fibrinogenolytic PIII-SVMP Daborhagin-K [B8K1W0] found in venoms from Bangladesh (10.4% of the venom proteome, Fig. 1A) and East India [82], and minor *M. lebetina* VLAIP-A [Q4VM08] and *D. siamensis* DSAIP [AUF41660] homologs [77,83] (Supplementary Table S9), may underly the molecular basis of consumption coagulopathy and defibrinogenation [84], which are major hemotoxic complications of Russell's viper envenomings.

Another characteristic component of *D. russelii* venom which plays a key role in the coagulopathy characteristic of these envenomings is the factor X activator. PIIId-SVMPs found in the venoms of the four geographic variants of Russell's viper sampled (Figs. 3 and 4, and Supplementary Table S9) were matched to Factor X activating isoenzymes (RVV-X) from *D. russelii*, *D. siamensis* and *M. lebetina*, and account for 3–5% of their respective venom proteome (Fig. 3). RVV-X is a non-hemorrhagic PIIId-SVMP that consists of three covalently bonded peptide chains, a PIII-SVMP heavy chain and two C-type lectin-like light chains [85]. Factor X activating enzyme is one of the main toxins responsible for the potent procoagulant activity of Russell's viper venom on human plasma [86,87]. RVV-X activates Factor X systemically by cleaving a specific peptide bond. Activated factor X forms, in combination with activated factor V, calcium and phospholipids, the prothrombinase complex, triggering thrombin release from prothrombin that converts fibrinogen to fibrin, resulting in consumption coagulopathy and defibrinogenation [88]. On the other hand, hemorrhage, a common finding in envenomings by Russell's viper, results from the degradation of basement membrane proteins by SVMPs, with the consequent damage of blood vessels [89,90]. Thus, the combined actions of hemorrhagic SVMPs, which disrupt the integrity of the microvasculature, and procoagulant enzymes causing consumption coagulopathy and defibrinogenation are responsible for the local and systemic hemorrhage characteristic of *D. russelii* envenomings.

C-type lectin-like proteins comprise another major toxin family that contributes with 11 members to the set of conserved venom proteins among the geographic variants of Russell's viper *D. russelii* (Fig. 4). Some of these CTLs represent light chains of the PIIId-SVMP RVV-X described above. Others, such as the P31 α and β-subunits; Dabocetin α and β-subunits; CTL-7, CTL-3 and CTL-5 associate into canonical disulphide-bonded αβ dimers. This type of CTLs exhibits various pharmacological activities including anticoagulation and platelet aggregation induction or inhibition [91]. However, the biological activities of Russell's viper CTLs remain poorly studied. The exception is Dabocetin, a heterodimeric GPIb-binding CTL purified from *D. siamensis* venom [92], identical to *D. russelii* (Kolkata, India) [ADK22821/ADK22822], which inhibits ristocetin-induced platelet agglutination in platelet-rich plasma in a dose-dependent manner with an IC50 value of 0.35 μM [92] r. These data point out CTLs as possible contributors to the hemostatic perturbances caused by *D. russelii* venoms through anti-platelet (Dabocetin) and defibrinogenating (RVV-X light chain-1 and -2) activities.

Besides the role that the major toxin families play in venom-induced imbalance of the hemostatic system, conserved low-abundance proteins could play an ancillary role, maximizing the overall venom effectiveness. Thus, NGF may contribute to venom spread from local envenoming site [93]. PDE and 5'-NT may also promote venom dissemination by inducing vasodilation through elevation of the

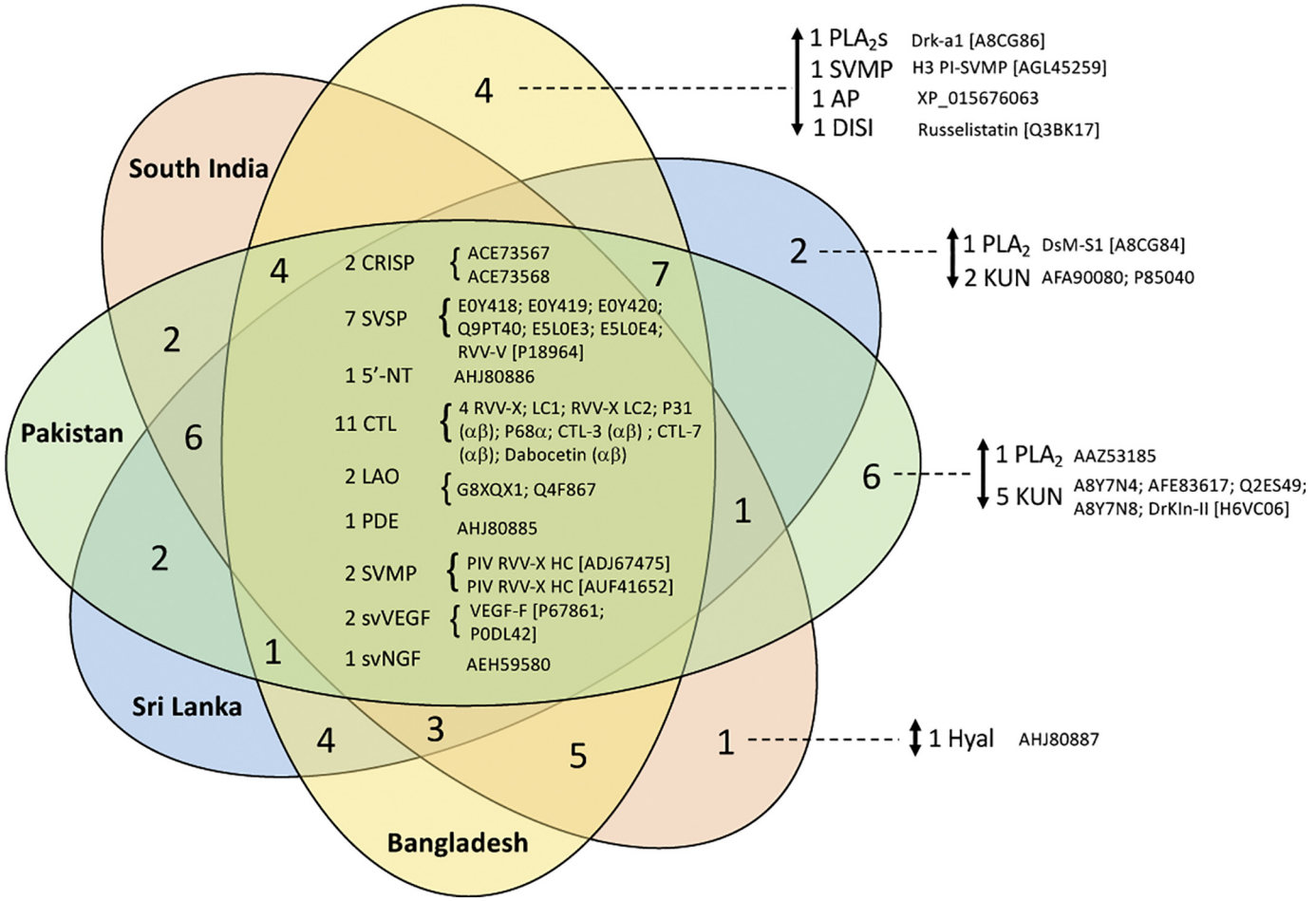

Fig. 4. Venn diagram showing the occurrence of unique and shared venom proteins among the venom proteome of *D. russelii* from Bangladesh, Sri Lanka, Pakistan, and South India.

concentration of extracellular purine derivatives [94,95]. In addition of hypotension, svVEGF may cause increased capillary permeability, thereby promoting hemoconcentration and venom spread in envenomed subjects [96,97]. Moreover, in severe cases svVEGF is also responsible of systemic capillary leakage syndrome (CLS) [98], characterized by parotid gland swelling, chemosis, periorbital edema, hypotension, albuminuria, hypoalbuminemia, and hemoconcentration, and a very high fatality rate (ranging from 43% to 67% in the absence of treatment), mainly due to acute kidney injury [99,100]. On the other hand, although a variety of pharmacological effects have been attributed to LAOs and CRISPs [101–103], their pathogenic role in envenoming remains to be elucidated.

3.4. Non-conserved toxins among the venom proteomes of geographic variants of Russell's viper may be responsible for the differential pathological manifestations of their envenomings

3.4.1. Kunitz-type serine proteinase inhibitor-like proteins

Venoms of Russell's vipers across the Indian subcontinent contain highly diversified sets of KUN proteins (Supplementary Table S9). This toxin family represents, respectively, 7.9%, 2.6%, 17.8%, and 2.4% of the total venom proteomes of *D. russelii* from Bangladesh, Sri Lanka, Pakistan, and South India (Fig. 3). KUN homologs of *D. siamensis* A8Y7N9, A8Y7P1, and *D. russelii* Q2ES46/47, were found in Bangladesh, Pakistan, south India (this work) and western India [42,44] venoms; *D. russelii* AFD04724 in Bangladesh and south India; *D. russelii* Q2ES48 in south India, Sri Lanka, and Pakistan; *D. siamensis* A8Y7P5 in south India and Sri Lanka; and *D. siamensis* A8Y7N7 in western India and Sri Lanka (Supplementary Table S9). Venoms of Russell's viper from western India, Sri Lanlka, and Pakistan each express a number of unique KUN molecules (Supplementary Table S9). The physiological significance of these venom molecules remains obscure. However, another KUN, DrKln-I [H6VC05], potently inhibited (with IC50 of 3.5 nM and 88.9 nM in the presence or absence of heparin, respectively) activated protein C, an important serine protease that negatively regulates blood coagulation by inactivating FVa [104]. DrKln-I also showed a synergistic action with the SVMP RVV-X [Q7LZ61] [104]. Activated factors X (FXa) and V (FVa), in the presence of calcium and phospholipids, form the prothrombinase complex that catalyzes the conversion of prothrombin to α-thrombin [105]. Thus, KUN molecules may play a role in the pathogenesis of Russell's viper envenomation by exacerbating fibrinogen consumption, thereby leading to the augmentation of consumption coagulopathy and bleeding. Enhanced pharmacological effects have been described by the association of PLA_2 [ABD24038] and the non-enzymatic KUN DNTx-II [H6VC06] into the hemorrhagic and neurotoxic complex DR–HC-I (LD_{50} dose of 0.7 mg/kg mouse body weight), isolated from venom of *D. russelii* (Kolkata, eastern India) [106]. In line with this view, other studies have shown that Rusvikunin, a 6.9 kDa KUN isolated from the venom of *D. russelii* of Pakistan origin, forms a basic complex (Rusvikunin complex) with a 7.1 kDa peptide, Rusvikunin-II, from the same venom, and this interaction resulted in enhancement of the anticoagulant activity of the individual components of the complex [107,108].

3.4.2. Phospholipases A_2

Variable clinical manifestations of envenoming by the same species

in different areas of its range indicate the occurrence of geographical variability in the composition of its venom [109–113]. A major distinguishing feature among the four regional *D. russelii* venoms investigated is their distinct complement of PLA_2s and KUN proteins (Supplementary Table S9; Fig. 4). Despite similarities in their structures and common catalytic properties, PLA_2s exhibit a wide spectrum of pharmacological activities including neurotoxic, hemolytic, myotoxic, anticoagulant, and antiplatelet activities [114,115]. In addition to structural-functional variability, the expression of shared PLA_2 genes is also subject to quantitative differences between *D. russelii* populations. Qualitative and quantitative changes may account for variable envenoming manifestations observed across Russell's viper range. The physiological significance of KUN molecules in the venom remains largely elusive, however.

PLA_2s represent the dominant toxin family in the venom proteomes of *D. russelii* from Bangladesh, Sri Lanka, Pakistan, and South India (Fig. 3). The major venom PLA_2 molecule of Russell's viper from Bangladesh is basic Drk-b2 [A8CG90] (Fig. 3A). This PLA_2 molecule is also present in conspecific venoms from western India [44], eastern India (Kolkata) [72,73], and Pakistan [42] (Supplementary Table S10), albeit its expression varies geographically. Thus, Drk-b2 represents 27.1% of the total venom proteome (57% of the PLA_2 subproteome) in Bangladesh (Fig. 3A) but it constitutes only 0.2% of Russell's viper venom from eastern India [72,73]. Although its toxic effects have not been unveiled, the closest homolog (84% identity) of PLA_2 Drk-b2, is RVV-VD [P81458], a basic PLA_2 of low catalytic activity, being weakly lethal (LD_{50} > 10 μg/g mouse) but strongly anticoagulant [116]. It has been proposed that the anticoagulant site resides within a Lys-rich region between residues 53 and 76 [117], a region where both Drk-b2 and RVV-VD have several lysine residues. Other major PLA_2 molecules of *D. russelii* venom from Bangladesh are the basic PLA_2 Drk-b1 [A8CG89] and the acidic PLA_2 Drk-a1 [A8CG86], which constitute, respectively, 9.8% and 6.1% of the venom proteome (Fig. 3A, Supplementary Table S10). Drk-a1 [A8CG86] represents the major (17% of the venom proteome), and the most neurotoxic and lethal (0.06 < i.p. LD_{50} < 0.10 μg/g mouse), among the PLA_2s of the venom of *D. russelii* caught near Kolkata (eastern India) [72]. In contrast, the intraperitoneal LD_{50} of the basic PLA_2 DrK-b1 [A8CG89] was > 3.0 μg/g mouse [72]. Daboiatoxin-B [DbTx-B, Q7T3T5] [2H4C] [118], a minor component of *D. russelii* (0.4% of the venom proteome) from Bangladesh, has been identified as the major lethal factor (LD_{50} i.p. 0.05 μg/g mouse body weight) of the Burmese Russell's viper venom, *D. siamensis* (Myanmar) [119]. Mice injected intraperitoneally with DbTx-B showed neurotoxic symptoms, such as hind-limb paralysis, convulsions and respiratory distress). DbTx-B also exhibited edema-inducing and myotoxic activities. The strong anticoagulant activity of major Drk-b2 and the low abundance of neurotoxic Drk-a1 and DbTx-B are in line with the most common clinical symptoms of Russell's viper envenoming, hemostatic disturbances and extensive hemorrhage leading to spontaneous bleeding from vital organs. Anticoagulant PLA_2s are likely to act in concert with proteinases causing defibrinogenation in the pathogenesis of coagulopathy and bleeding. In contrast, neurotoxic features do not commonly develop in human victims of *D. russelii* envenomings in the north and west of the Indian subcontinent [120]. On the other hand, VRV-PL-VIIIa [P59071] and Daboxin P [C0HK16]/U1-viperitoxin-Dr1a [P86368] represent the major PLA_2 molecules of Russell's viper venom from Sri Lanka and South India (Fig. 3, panels B and D; Supplementary Table S10). VRV-PL-VIIIa [121,122] is a basic myotoxic and neurotoxic PLA_2 [123,124] and damages vital organs such as lung, liver and kidney at doses close to the LD_{50} (i.p., 5.3 μg/g mouse body weight) [123]. VRV-PL-VIIIa is found in venom samples of southern and northern regions of the Indian peninsula [16], comprising 21.4%, 30.7%, and 39.6% of the venom proteome of Pakistani, Sri Lankan, and South Indian Russell's viper, respectively (Fig. 3, panels B, C, and D; Supplementary Table S10). Daboxin P is a presynaptic neurotoxin [125] and also displays anticoagulant activity [121] by disrupting the function of the coagulation cascade through interaction with FXa [126]. Neurotoxicity in Sri Lankan Russell's viper envenomings is primarily

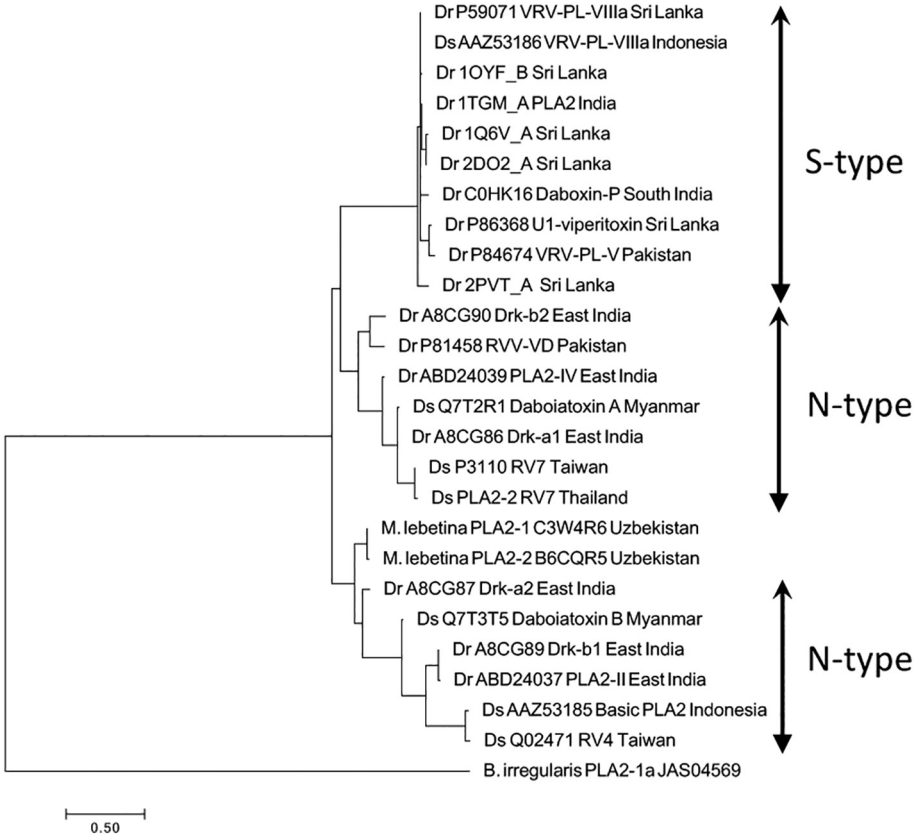

Fig. 5. Maximum Likelihood inferred phylogeny from the multiple sequence alignment of Russell's viper venom PLA_2s listed in Supplementary Table S11. Dr, *Daboia russelii*; Ds, *D. siamensis*. The two PLA_2 sequences from *Macrovipera lebetina* (Uzbekistan) venom available in the NCBI database were included in the alignment as outgroup for the Daboia sequences. *Boiga irregularis* PLA_2-1a represents the outgroup for the combined Russell's vipers and *M. lebetina* PLA_2 sequence alignment.

due to the pre-synaptic neurotoxic basic PLA_2 U1-viperitoxin-Dr1a [32].

The distinct venomics patterns of *D. russelii* venoms from northern and southern regions of the Indian subcontinent set the ground for rationalizing the documented North-South divide in Russell's viper envenoming in India, characterized by a clear distinction in the presentation all over north India into either hemotoxic or neurotoxic envenoming, and a more blurred clinical picture as one moves towards south India, where Russell's viper can cause a wide spectrum of clinical manifestations in the same patient [20,120].

3.5. Tracing Russell's viper dispersal across the Indian subcontinent

Intraspecific geographic variation in venom composition is of considerable importance in relationship to snakebite envenoming and therapy. Venom samples from northern and western India possess high acidic phospholipases, while acidic fractions with phospholipase activity were absent in the samples from southern India, which in contrast showed large basic fractions with phospholipase activity [15]. A correlation between higher LD_{50} values of the Russell's viper venoms from northern and western India than that from conspecific venoms of southern India, and between the high lethal potency and the high PLA_2 activity of the venoms, have been known since more than 30 years [15]. The data discussed in this manuscript strongly suggest that the geographic variability of PLA_2s underlies these functional correlations. Russell's viper venom PLA_2s (Supplementary Table S11) have been classified, based on their primary sequences, into two types, N-type and S-type [116]. Phylogenetic analysis segregates the two types of PLA_2 in different branches of the phylogram [122] (Fig. 5). Both PLA_2 types were found in *D. russelii* (Pakistan), whereas N-type PLA_2s predominate in the venoms of *D. russelii* from east India and Bangladesh, as well as in the venom of *D. siamensis* from Myanmar. Venom PLA_2s of *D. russelii* from Sri Lanka are primarily of the S-type. Within the western clade, Pakistani *D. russelli* is basal, with the Sri Lankan and southern Indian Russell's viper variants as sister group [5]. On the other hand, venom similarities between *D. russelii* from eastern India and *D. siamensis* from Myanmar indicate their close kinship within the Russell's viper complex [72].

For reconstructing dispersal routes of Russell's viper that contributed to the current biogeography of this highly adaptable and widely distributed snake species, we mapped the intraspecific variability landscape of *D.russelii* venom PLA_2s onto a physical map of its distribution range (Fig. 6). Connecting populations that share venom toxins provides a rough graphical view of the dispersal routes of the species under study [127]. The biogeographical pattern that emerged applying this approach to *D. russelii* is consistent with a two-major-radiations model, with origin in the northwest of the Indian subcontinent (Pakistan) and dispersal routes towards the northeast of India and

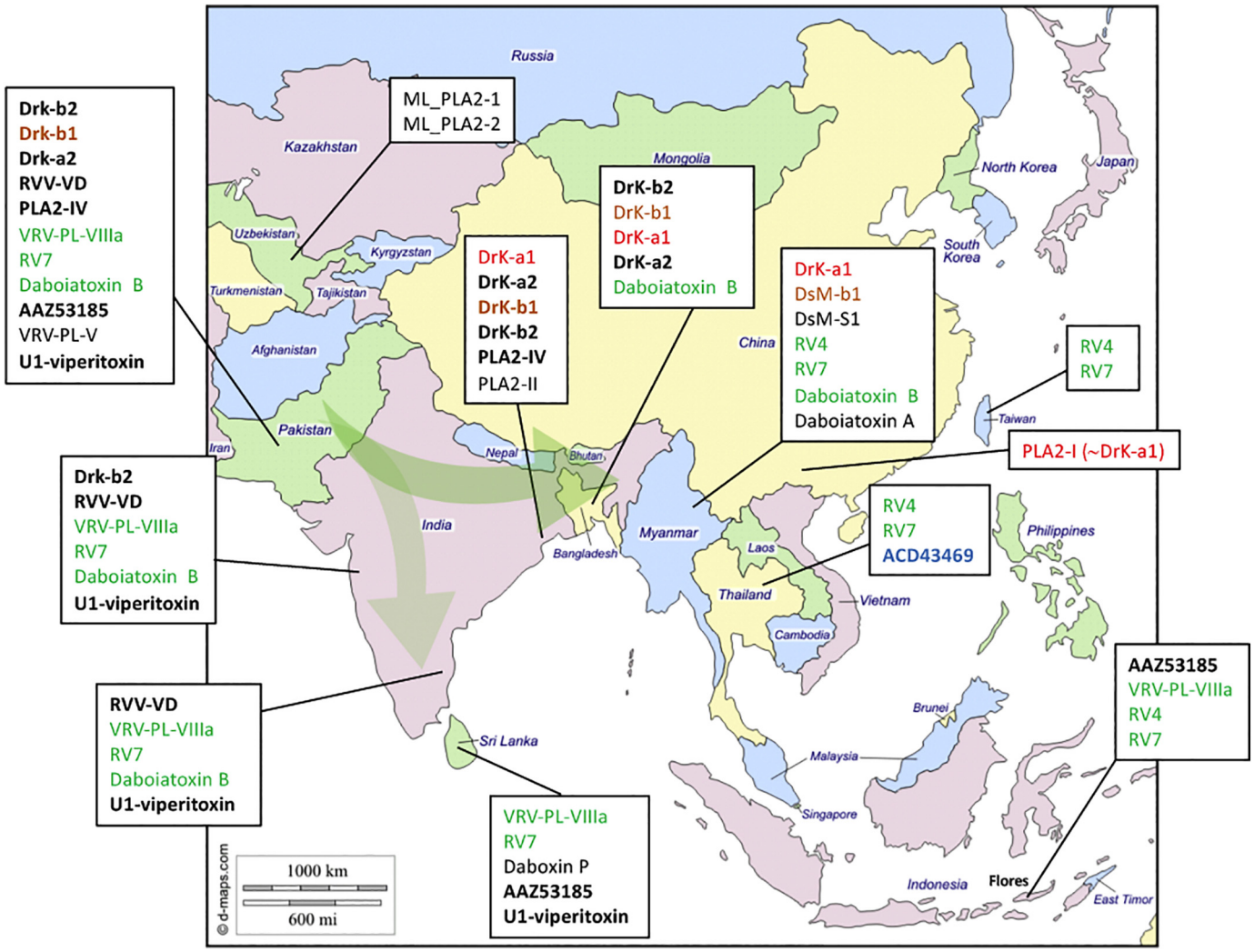

Fig. 6. Distribution of PLA_2 molecules across Russell's viper range. The pale green arrows highlight the proposed Northwest-South and Northwest-East dispersal routes that emerge by connecting geographic variations of Russell's viper venoms sharing PLA_2 molecules. Shared PLA_2s are color-coded (for primary data and bibliography, please consult Supplementary Table S9).

Bangladesh and towards southern India and Sri Lanka (Fig. 6). This scenario provides molecular support for the existence of a North-South divide in Russell's viper envenoming in India. In addition, the dispersal route towards the south explains the paraspecific neutralization activity of antivenoms raised against *D. russelii* venom from Tamil Nadu (VINS, Premium Serums) or Sri Lanka (ICP) towards the toxic effects, including lethality, of *D. russelii* venom from Pakistan (Table 1). To investigate the immunological consequences of the evolutionary diversification of the venom toxins of Russell's viper dispersed through the northern route, we carried out comparative third generation antivenomics analyses [57,128] on a panel of polyspecific (including Russell's viper) antivenoms manufactured in India, Bangladesh, and Costa Rica to assess their toxin recognition landscape and to quantify the fraction of therapeutic antibody molecules.

3.6. Third generation antivenomics: clues towards the formulation of a pan-Indian Russell's viper antivenom

The outcome of antivenomics analyses of antivenoms commercialized by Haffkine Bio-Pharmaceutical Corporation Ltd., VINS Bioproducts Ltd., Biological E. Ltd., and Premium Serums & Vaccines Pvt Ltd. (India); Incepta Vaccine Ltd. (produced by Premium Serums; manufactured in Dhaka, Bangladesh); and the Costa Rican ICP (for Sri Lanka) [46,129], against venoms of *D. russelii* from Bangladesh, south India, Sri Lanka, and Pakistan are displayed in Figs S1–S10 and Supplementary Tables S12–S25 and summarized in Table 2. It is clear that for all the antivenoms the percentage of antibodies immunorecognizing toxins from the venom of Bangladesh is significantly lower (average of 5.4%) than the percentage of antibodies against toxins from the venoms of south India (Tamil Nadu), Sri Lanka and Pakistan (average of 9.2%). All these antivenoms efficiently immunorecognize the toxins conserved among the venoms of the geographic variants of *D. russelii* from Bangladesh, south India, Sri Lanka, and Pakistan (Table 3). The restricted affinity of the Indian antivenoms for *D. russelii* (Bangladesh) venom toxins could be adscribed to their poor paraspecific recognition of PLA_2s Drk-b1 and Drk-a1 (but also Drk-b2, although the relative importance of this PLA_2 in venom lethality remains to be established) and PIII-SVMP (Daborrhagin K) (Table 3), which are major toxins in the venom proteome of the Russell's viper (Bangladesh) but are essentially absent from the venom proteomes of *D. russelii* populations along the Pakistan-western India-South India-Sri Lanka dispersal route (Fig. 6; Supplementary Table S10). The exception is the Costa Rican antivenom, which exhibited higher immunorecognition towards PLA_2s Drk-b1 and Drk-a1 and Daborrhagin K (Table 3). On the other hand, antivenoms raised against the highly similar *D. russelii* venoms from south India (VINS, Premium Serums) and Sri Lanka (ICP) exhibit high binding capacity towards major toxic PLA_2 molecules (VRV-PL-VIIIa/Daboxin-P, LD_{50} 5.5 μg/g mouse body weight, and U1-viperotoxin Dr1A, LD_{50} > 2 μg/g mouse body weight) [116], shared by south and west Indian, Sri Lankan and Pakistani venoms, and towards PLA_2 VRV-PL-V, a major (13.8%) component of the Pakistani *D. russelii* venom proteome which, although absent from the venom of south Indian and Sri Lankan taxa, exhibits 96% and 93% amino acid sequence identity with U1-viperotoxin Dr1A and VRV-PL-VIIIa/Daboxin-P, respectively.

These PLA_2s belong to the same clade of S-type PLA_2s, which is phylogenetically distant from the N-type clades that include the East India and Bangladesh major PLA_2 molecules Drk-b1, Drk-b2, Drk-a1, and Drk-a2 (Fig. 5). The amino acid sequence identity between U1-viperotoxin Dr1A and VRV-PL-VIIIa/Daboxin-P and the east India/Bangladesh N-type PLA_2s are, respectively, in the range of 51–58% and 53–60%. This moderate sequence similarity correlates well with the limited immunoreactivity of the Indian and Costa Rican antivenoms against the PLA_2 molecules of the Bangladesh/east India Russell's viper venoms (Table 3). On the other hand, the fact that the Indian and Costa Rican antivenoms exhibit paraspecificity towards PLA_2s Drk-b1 and Drk-a1 strongly suggests that the distinct immunization protocols used to generate Indian and Costa Rican antivenoms may have conferred the latter antivenom a higher neutralizing activity against the lethal effect of Russell's viper (Bangladesh) venom compared to the Indian antivenoms. Although the impact of the other venoms of the immunization mixtures used for the production of the Indian and Costa Rican antivenoms, i.e. {*N. naja*, *B. caeruleus*, and *E. carinatus*} and {*N. naja, H. hypnale*, and *E. carinatus*}, respectively, deserves future detail studies, extensive cross-reactivity of anti-*Hypnale hypnale* antiserum with *D. russelii* (Sri Lanka) and *D. siamensis* (Myanmar) venoms [130] supports this hypothesis. In addition, the Thai Red Cross hemato polyvalent antivenom raised produced in horses hyperimmunized by venoms from the three common viper and pit vipers in Thailand, Green pit viper (*C. albolabris*), eastern Russell's viper (*D. siamensis*), and Malayan pit viper (*C. rhodostoma*), neutralized the lethal effect of *H. hypnale* (Sri Lanka) and *Daboia russelii* (Sri Lanka) venoms with ED_{50}s of 1.52 and 2.50 mg venom/mL antivenom, respectively [131]. Such paraspecific venom neutralization evidence the presence of similar antigenic determinants between the venoms of *C. rhodostoma* and its sister group, the genus *Hypnale* [132,133], and between the PLA_2-dominated venom proteomes of Sri Lankan *Hypnale hypnale* [134,135] and *D. siamensis* venom. It is worth noting that *D. siamensis* (Myanmar) and *D. russelii* (Bangladesh) venoms share identical or highly similar PLA_2 molecules (e.g. Drk-a1, RV7, Daboiatoxin B) [40,72]. Taken together, these data suggest the existence of a network of antigenic determinants among the venoms of

Table 2
Summary of third generation antivenomics analyses of polyclonal Russell's viper antivenoms against venoms from *D. russelii* in the Indian subcontinent.

Antivenom	mg/vial	*D. russelii* venom	Maximal venom binding		Anti-toxin antibodies	Lethality neutralizing antibodies	
			(mgV/mL AV)	(mgV/g AV)	%	% of anti-toxin Abs	% of vial Abs
Incepta	411	Bangladesh	0.86	21.6	5.9		
BE	474	Bangladesh	0.75	15.9	4.4		
Haffkine (2016)	510	Bangladesh	0.91	17.8	4.9		
Haffkine (1998)	1325	Bangladesh	1.78	13.5	3.3		
VINS	555	Bangladesh	1.01	18.4	5.0		
ICP	580	Bangladesh	1.12	21.9	8.8 (Average, 5.4%)	100	8.8
Premium Serums	357	Tamil Nandu (I)	1.21	33.9	9.3		
		Sri Lanka	0.93	26.1	7.2		
		Pakistan	1.39	39.1	10.7	100	10.7
VINS	555	Tamil Nandu (I)	1.41	25.5	7.0	100	7.0
		Sri Lanka	1.89	34.2	9.4	66	6.2
		Pakistan	1.51	27.2	7.5	82	6.2
ICP	580	Sri Lanka	2.31	39.8	14.8	75	11.2
		Pakistan	1.72	29.3	8.0 (Average, 9.2%)	96	7.7

Table 3
Percentages of immunodepletion by third generation antivenomics of major toxins of venoms from geographic variants of D. russelii.

Antivenom	PLA2s (Bangladesh)			PLA2s (Sri Lanka and South India)		PLA2 (Pakistan)	PIII-SVMP (Bangladesh)	Conserved toxins	
	Drk-b2	Drk-b1	Drk-a1	VRV-PL-VIIIa	U1-viperotoxin Dr1A	VRV-PL-V	Daborrhagin K	PIV-SVMP RVV-FX	SVSP RVV-V
Incepta	7.0	7.8	7.1				24	69	68
BE	8.6	5.5	8.9				15	56	60
Haffkine (2016)	7.9	7.6	7.7				26	58	85
ICP	1.5	10.3	10.8	46	39	74	61	94	59
VINS	8.0	4.9	3.5	32	38	86	24	59	58
Premium Serums				46	42	55			

H. hypnale (Sri Lanka), *D. siamensis* (Myanmar) and *D. russelii* (Bangladesh) that potentially explain why the ICP antivenom outperformed Indian antivenoms in the neutralization of the lethal effect of Russell's viper (Bangladesh) venom.

3.7. Preclinical calculations from antivenomics data

Phospholipase Drk-a1 (6.1% and 17% of Bangladesh and eastern India *D. russelii* venom, respectively) exhibits an i.p. LD_{50} of 0.06–0.13 μg/g mouse (1.2–2.6 μg/20g mouse) [72], and represents the most lethal PLA_2 of *D. russelii* venom from eastern India (i.p. LD_{50} 1.0–1.33 μg/g mouse) [72]. Allometric conversion [136,137] suggests an LD_{50} dose for humans of 6.35–8.45 mg crude venom/70 kg body weight (0.38–051 mg (Bangladesh) and 1.07–1.43 mg (eastern India) Drk-a1/70 kg body weight). The amount of PLA_2 Drk-a1 injected into a victim of an adult Russell's viper bite, whose reported average venom yield is 63 ± 7 mg [138], can be 3.9 mg (Bangladesh) and 10.7 mg (eastern India) (7.5–11 crude venom LD_{50}s; 0.8–1.1 and 2.2–3 Drk-a1 LD_{50}s for Bangladesh and eastern India venoms, respectively). Reversing the symptoms of an envenoming depends on shifting the balance between the amount of circulating and target-bound venom toxins, with toxin–antibody complexes contributing to the latter. Incepta, VINS, and ICP antivenoms bind, respectively, 8.6, 10.1, and 11.2 mg of total *D. russelii* (Bangladesh) venom proteins per vial of antivenom (Table 2), and immunodeplete 7.1%, 3.5%, and 10.8% of PLA_2 Drk-a1, respectively (Table 3). From these values, and without taking into account the effect of the pharmacokinetics of the antivenom antibodies in the *in vivo* neutralization, we calculated that clearing 3.9 mg of Drk-a1 would theoretically requires at least 103 (Incepta), 178 (VINS), and 52 (ICP) antivenom vials. For comparison, Premium Serums, VINS, and ICP antivenoms bind, respectively, 15.5 mg, 12.6 mg, and 17.3 mg south Indian *D. russelii* venom proteins per vial (Table 1), and immunodeplete, respectively, 46%/63%, 32%/38%, and 57%/37% of PLA_2s VRV-PL-VIIIa/U1-viperotoxin Dr1A from the same venom. Neutralizing the amounts of these two major toxins present in 63 ± 7 mg Russell's viper (South India) (25.7 mg VRV-PL-VIIIa and 20 mg U1-viperotoxin Dr1A) would theoretically need 7–11 (Premium Serums), 14–16 (VINS), and 7–11 (ICP) antivenom vials.

4. Concluding remarks and perspectives

It has been repeatedly documented that the toxin composition of Russell's viper venom is subject to geographic variability across the Indian subcontinent. However, the different analytical approaches applied by various laboratories to match and quantify the venom toxins prevent a comprehensive comparison of the different venom proteomes. Thus, the venom proteome of Sri Lankan *D. russelii* was elucidated by 1D-SDS-PAGE followed by shotgun LC-MS/MS proteomics [41]. Pakistan and south Indian Russell's viper venom proteomes were analyzed by gel filtration fractionation and shotgun LC-MS/MS analysis of trypsin digested peptides of the chromatographic fractions [42,43,71]. Western India Russell's viper venom was fractionated by gel-filtration (GF) chromatography and the GF fractions subjected to a second separation step using an anion-exchange column before submitting each chromatographic fraction to trypsinolysis and shotgun LC-MS/MS analysis of the digests [44]. In all cases the relative toxin abundance in the chromatographic fractions was estimated by label-free spectral counting, where the relative number of spectra matched to peptides from a protein is used as a surrogate measure of protein abundance. However, this strategy was developed for label-free quantification of proteomes of model organisms for which comprehensive genomic or transcriptomic databases are available, and thus matching the MS/MS spectra does not represent a limiting factor [139–144]. When a comprehensive homolog database is not available, deriving reliable quantitative information from peptide-centric MS/MS data should be based on a procedure that does not depend on the sequence coverage of the database used for identifying a venom's toxin composition [128]. A simple and reliable option is reverse-phase HPLC fractionation monitoring the eluate at the absorbance wavelength of the peptide bond [54,55]. This procedure combines excellent separation of venom components and the relative quantification, as % by weight of the chromatographic fractions [56], in the same experiment. Here we have applied our standard bottom-up venomics protocol to qualitatively and quantitatively compare venom proteomes of Russell's viper from regions of the three corners of the Indian subcontinent. This knowledge is intended to serve as a reference for achieving an increasingly detailed overview of the venomic and antivenomics phylogeography of Russell's viper across the Indian subcontinent. The present study, despite dealing with a limited sample, illustrates and consolidates our belief that unveiling the spatial distribution of Russell's viper venom variation can shed light on the mutually enlightening relationship between evolutionary and clinical toxinology, an alliance of applied importance for optimizing the formulation of antivenoms with enhanced clinical efficacy. Future studies should expand venom sampling in both the geographic and the ontogenetic context. The latter aspect is particularly relevant because the clinical picture observed following bites by young and adult snakes varies [145].

Our present comparative venomics analysis allowed us to propose two alternative routes of radiation for Russell's viper in the Indian subcontinent. Both have their origin in Pakistan and are directed towards the northeast of India and Bangladesh and towards southern India and Sri Lanka. Antivenoms raised against the highly similar venoms of *D. russelii* venoms from South India (Tamil Nadu) and Sri Lanka efficiently recognized the major toxic component of *D. russelii* (Pakistan) venom proteome, but exhibited much lower paraspecificity towards the major venom toxins, PLA_2s (Drk-b2, Drk-b1, and Drk-a1) and PIII-SVMP (Daborrhagin K), of conspecific Russell's viper from Bangladesh. However, a percentage of patients suffering envenomings by Russell's viper bites develop severe nephrotoxicity despite early antivenom administration. Moreover, the neuroparalysis due to presynaptic toxins is generally unresponsive to antivenom, and it takes 2–4 days for the reversal of paralysis [146]. In these cases, hemodialysis and ventilatory support are required for survival. In good agreement with our antivenomics calculations, a retrospective study of 142 cases of snakebite (with haemostatic abnormalities attributable to viper bites in 52 (36.6%) of them) from the period January 1997 to December 2001,

carried out at a referral government hospital in north India for patients from the states of Punjab, Haryana, Uttar Pradesh, and Himachal Pradesh, reported a mean dose antivenom of 32 vials administered for viper (*E. carinatus* and *D. russelii*) bites [147]. In highly severe cases, some patients received as many as 130 vials for bites by Russell's vipers [147]. These data are in agreement with preclinical calculations from the antivenomics outcome for the Indian antivenoms investigated. Our *in vitro* and *in vivo* analyses showing the low potency of Indian antivenoms (raised against an immunization mixture of "Big Four" venoms, including venom of *D. russelii* from Tamil Nadu, south India) against venom from Bangladesh, underline the need to reformulate the immunization mixture to include venoms from the Pakistan-western India-South India-Sri Lanka and the northern India-east India-Bangladesh dispersal routes to achieve an effective pan-Indian subcontinent antivenom against Russell's viper envenomings.

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Conflict of interest statement

M.V., G.L. and JMG work at Instituto Clodomiro Picado, where one of the antivenoms used in this study was manufactured.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jprot.2019.103443.

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