Biogeographic venom variation in Russell’s viper (Daboia russelii) and the preclinical inefficacy of antivenom therapy in snakebite hotspots

R. R. Senji Laxme1* , Suyog Khochare1*, Saurabh Attarde1*, Vivek Suranse1, Ashwin Iyer1, Nicholas R. Casewell2, Romulus Whitaker3, Gerard Martin4, Kartik Sunagar1*

1 Evolutionary Venomics Lab. Centre for Ecological Sciences, Indian Institute of Science, Bangalore, Karnataka, India, 2 Centre for Snakebite Research & Interventions, Liverpool School of Tropical Medicine, Pembroke Place, Liverpool, United Kingdom, 3 Madras Crocodile Bank Trust/Centre for Herpetology, East Coast Road, Mamallapuram, Tamil Nadu, India, 4 The Liana Trust, Survey #1418/1419 Rathnapuri, Hunsur, Karnataka, India

* These authors contributed equally to this work.

ksunagar@iisc.ac.in

Abstract

Background
Snakebite in India results in over 58,000 fatalities and a vast number of morbidities annually. The majority of these clinically severe envenomings are attributed to Russell’s viper (Daboia russelii), which has a near pan-India distribution. Unfortunately, despite its medical significance, the influence of biogeography on the composition and potency of venom from disparate D. russelii populations, and the repercussions of venom variation on the neutralisation efficacy of marketed Indian antivenoms, remain elusive.

Methods
Here, we employ an integrative approach comprising proteomic characterisation, biochemical analyses, pharmacological assessment, and venom toxicity profiling to elucidate the influence of varying ecology and environment on the pan-Indian populations of D. russelii. We then conducted in vitro venom recognition experiments and in vivo neutralisation assays to evaluate the efficacy of the commercial Indian antivenoms against the geographically disparate D. russelii populations.

Findings
We reveal significant intraspecific variation in the composition, biochemical and pharmacological activities and potencies of D. russelii venoms sourced from five distinct biogeographic zones across India. Contrary to our understanding of the consequences of venom variation on the effectiveness of snakebite therapy, commercial antivenom exhibited surprisingly similar neutralisation potencies against the majority of the investigated populations,
with the exception of low preclinical efficacy against the semi-arid population from northern India. However, the ability of Indian antivenoms to counter the severe morbid effects of *Daboia* envenoming remains to be evaluated.

**Conclusion**

The concerning lack of antivenom efficacy against the north Indian population of *D. russelii*, as well as against two other ‘big four’ snake species in nearby locations, underscores the pressing need to develop pan-India effective antivenoms with improved efficacy in high snakebite burden locales.

**Author summary**

The Russell’s viper (*Daboia russelii*), with a near-countrywide distribution, is arguably the deadliest snake species in India. Despite being responsible for the majority of snakebite deaths in the country, the influence of biogeography on its venom composition and potency, and the impact of this variation on snakebite therapy, is yet to be understood. Evaluation of the composition, biochemical activities, pharmacological implications and potencies of *D. russelii* venoms from five distinct biogeographic zones in India (>5,800 km) revealed remarkable intraspecific differences in venom profiles. Surprisingly, these observed differences did not seem to affect the marketed effectiveness of the commercial Indian antivenoms in countering the lethal effects of *D. russelii* venoms, with the exception of the North Indian semi-arid population. A similar lack of antivenom potency has also been documented in two other ‘big four’ snake species in these regions that suffer the brunt of snakebite. These alarming findings underscore the pressing need to develop pan-India efficacious antivenoms, especially for the treatment of snakebites in regions that are worst affected by this neglected tropical disease.

**Introduction**

Globally, 5.4 million people suffer from snakebite, which results in over 137,000 annual deaths and nearly three times as many morbidities [1]. Asia accounts for over 70% of these cases, with India being its snakebite capital [1,2]. In India, the so-called ‘big four’ snakes, namely the spectacled cobra (*Naja naja*), common krait (*Bungarus caeruleus*), Russell’s viper (*Daboia russelii*) and saw-scaled viper (*Echis carinatus*), are considered to be the most medically important, with *D. russelii* seemingly being responsible for the majority of fatal envenomings and cases of long-term morbidity [2].

Variation in venom composition among and within snake species is seemingly driven by differing ecologies and environment and is a well-documented phenomenon [3–7]. These adaptive changes, particularly in a biodiverse region like the Indian subcontinent, can critically impact the clinical efficacy of antivenoms and, thus, poses an arduous challenge for countering geographical variations in snakebite pathologies. Considering its geological history and highly variable climatic and topological conditions, India can be divided into ten biogeographic zones: 1. Himalayas; 2. Trans-Himalayas; 3. Semi-arid regions; 4. Desert; 5. Western Ghats; 6. Deccan Plateau; 7. Gangetic Plain; 8. Coasts; 9. Northeast India; and 10. Islands [8]. Russell’s viper is arguably amongst the most widely distributed of the medically important Indian
snakes and can be found in all biogeographical zones, with the exception of Trans-Himalayas, most of Northeast India and the Islands. The near-countrywide distribution across diverse habitats and presence even at elevations of 2,000 m and above in the Himalayan region is suggestive of the remarkable adaptability of this snake species. Despite this wide distribution, the influence of varying ecology and environment on *D. russelii* venom has not been comprehensively investigated to date. While some studies have assessed variability in venom proteomes and the influence of this variation on antivenoms via *in vitro* binding experiments [9–13], neutralisation potencies of commercial antivenoms are yet to be determined. Consequently, the absence of preclinical data has impeded the identification of the most medically important populations that may require more targeted therapy.

Here, we aim to bridge this knowledge gap by performing proteomic, biochemical and pharmacological characterisation of *D. russelii* venoms from five distinct biogeographic zones in India. We evaluate the influence of varying ecology and environment on the toxicity profiles of the pan-Indian populations of this species. Furthermore, using the World Health Organization (WHO)-recommended murine efficacy assays, we investigate the capability of conventional antivenoms to neutralise the venoms of geographically disparate *D. russelii* populations.

**Methods**

**Ethics statement**

WHO-recommended preclinical experiments were performed in the mouse model (CD-1 male mice; 18–22 g), following guidelines issued by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India. All experiments were performed after obtaining the requisite approval from the Institutional Animal Ethics Committee (IAEC), Indian Institute of Science (IISc), Bangalore (CAF/Ethics/643/2018). A single best-binding antivenom was down-selected using *in vitro* assays (enzyme-linked immunosorbent assay) for testing the *in vivo* neutralisation potency of antivenom against *D. russelii* venoms. This down-selection strategy was crucial in greatly reducing the number of animals used in the *in vivo* experiments and minimising animal suffering. For investigating the coagulopathic effects of *D. russelii* venoms on human blood, ethical clearance was obtained from the Institute Human Ethical Committee (IHEC No: 5–24072019), and subsequently, blood was collected from healthy volunteers with informed consent.

**Sampling permits, snake venoms and antivenoms**

Snake venoms were sourced from 48 individuals across a range of over 5800 km from various biogeographic zones of India, with appropriate permissions from the respective State Forest Departments: North- (Punjab: #3615;11/10/12), South- (Tamil Nadu), Southeast- (Andhra Pradesh:#13526/2017/WL-3), East- (West Bengal: 386/WL/4R-6/2017), Southwest- (Maharashtra: Desk-22(8)/Research/CR-80(16–17) /943/2017-18), and Central- (Madhya Pradesh: #/TK-1/48-II/606) India. The venoms were sampled individually or by pooling, flash-frozen with liquid nitrogen, and stored at -80˚C after lyophilisation until further use. Sourced venom samples were subjected to preliminary reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as a quality control measure and, thereafter, representative samples were down-selected for assessment. The details of *D. russelii* venom and commercial Indian antivenom samples analysed in this study are provided in Table 1 and S1 Table, respectively.

Locations of *D. russelii* venoms sourced from various biogeographic zones in the country are listed in this table. State codes are indicated in parentheses, and the number of individual snakes from which these venoms were collected, and their protein concentrations have also
been provided. The asterisk indicates the venom sample sourced from the Irula Snake Catchers Industrial Cooperative Society.

**Protein concentration**

Lyophilised venom was reconstituted with molecular grade water, and the Bradford method was used for estimating protein concentration, with Bovine Serum Albumin (BSA) used as a standard \[[14]\]; Table 1]. The total IgG content of antivenom was estimated by reconstituting the lyophilised contents of antivenom vials following the manufacturer’s guidelines and using the Bovine Gamma Globulin (BGG) standard curve (S1 Table).

**Gel electrophoresis**

Reducing SDS-PAGE was performed to separate venom toxins. Venom samples, which were normalised for their protein content (12 μg), were run on a 12.5% gel in Tris-Glycine-SDS (TGS) buffer at 80 V \[[15]\], and the Precision Plus Protein Dual Color Standard (Bio-Rad) was used as a marker. The gel was stained with Coomassie Brilliant Blue R-250 (Sisco Research Laboratories Pvt. Ltd, India) and visualised in an iBright CL1000 gel documentation system (Thermo Fisher Scientific, USA).

**Reversed-phase high-performance liquid chromatography (RP-HPLC)**

A slightly modified version of a previously published protocol \[[16]\] was used to fractionate the reconstituted venom in a Shimadzu LC-20AD series HPLC system (Kyoto, Japan). A reverse phase C18 column with dimensions 4.6 x 250 mm, 5 μm particle size, and pore size of 300 Å (Shimadzu, Japan), was equilibrated with solution A [0.1% Trifluoroacetic acid (TFA) in water (v/v)] and loaded with 200 μg of each venom sample for fractionation. A constant flow rate of 1 ml/min was used for eluting fractions with the following concentration gradients of solution B [0.1% TFA in 100% acetonitrile (v/v)]: 5–15% for 10 mins, 15–45% over the next 60 mins, and finally 45–70% for 10 mins, and the absorbance was monitored at 215 nm.

**Liquid chromatography-tandem mass spectrometry (LC-MS/MS)**

HPLC fractions (40 μg each) were subjected to electrospray ionisation tandem mass spectrometry (ESI-MS/MS) for the characterisation of proteomic profiles. Samples were reduced with dithiothreitol (DTT; 10 mM), alkylated using iodoacetamide (IAA; 30 mM), and subsequently digested with trypsin (0.2 μg/μl) overnight and desalted. A Thermo EASY nLC 1200 series system (Thermo Fisher Scientific, MA, USA) with a C18 nano-LC column (dimension 50 cm x 75 μm, 3 μm particle size and 100 Å pore size) was used for liquid chromatography of these processed samples. A sample volume of 2 μl was injected into the column and run with buffer A (0.1% formic acid in HPLC grade water) and buffer B (0.1% formic acid in 80% acetonitrile).

| Region | Biogeographical Zone | No. of individuals | Protein content (mg/ml) |
|--------|----------------------|--------------------|-------------------------|
| North India Nawanshahr, Punjab (PB) | Semi-arid | 2 | 0.430 |
| South India Kancheepuram, Tamil Nadu (TN) | Coastal | *Multiple | 0.680 |
| Southeast India Visakhapatnam, Andhra Pradesh (AP) | Coastal | 1 | 0.449 |
| East India Kolkata, West Bengal (WB) | Gangetic Plain | 1 | 0.488 |
| West India Mahad, Maharashtra (MH) | Western Ghats | 5 | 0.95 |
| Central India Jabalpur, Madhya Pradesh (MP) | Deccan Plateau | 3 | 0.293 |

https://doi.org/10.1371/journal.pntd.0009247.t001
solutions at a constant flow rate of 300 nl/min for 120 mins. The gradient of buffer B (10–45%) was used for the elution over the first 98 mins, followed by 45–95% over the next 4 mins and finally 95% for the last 18 mins. A Thermo Orbitrap Fusion Mass Spectrometer (Thermo Fisher Scientific, MA, USA) was used for mass spectrometric analyses of the samples. MS scans were performed using the following parameters: scan range (m/z) of 375–1700 with a resolution of 120000 and maximum injection time of 50 ms. Fragment scans (MS/MS) were performed using an ion trap detector with high collision energy fragmentation (30%), scan range (m/z) of 100–2000, and maximum injection time of 35 ms.

For identification of various toxin families in the proteomic profiles of venom fractions, the raw MS/MS spectra were searched against the SwissProt database (www.uniprot.org) using PEAKS Studio X Plus (Bioinformatics Solutions Inc., ON, Canada) with the following parameters: parent and fragment mass error tolerance limits of 10 ppm and 0.6 Da, respectively; ‘monoisotopic’ precursor ion search type; and ‘semispecific’ trypsin digestion. Carbamidomethylation and oxidation were specified as fixed and variable modifications, respectively. Error in peptide identification was minimised by fixing the False Discovery Rate (FDR) for peptide-spectrum matching at 0.1%, and the corresponding -10lgP cutoff value was automatically determined by PEAKS Studio. Hits with at least one unique matching peptide were considered for downstream analyses. Mass spectrometry data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository [17], with data identifier: PXD021060. The relative abundance of each toxin hit in a fraction was determined by estimating its area under the curve (AUC) for spectral intensities, obtained from PEAKS analysis [18], relative to the total AUC for all toxins in that fraction. These values were further normalised across fractions using the percentage of peak areas for the respective RP-HPLC fractions [19]. Thus, the relative abundance of a toxin hit (X) was calculated as follows:

$$\text{Relative abundance of } X(\%) = \sum_{n=1}^{N} \frac{\text{AUC of } X \text{ in Fraction } F_n \times \text{AUC of the chromatographic fraction } F_n(\%)}{\text{Total AUC of all toxin hits in fraction } F_n(\%)}$$

Here, N indicates the number of fractions obtained from RP-HPLC.

Venom biochemistry

Venom samples were assayed for various biochemical activities following previously described protocols [20] and are thus detailed in brief below.

Phospholipase A₂ (PLA₂) assay

The PLA₂ activity of the venom samples was assessed via turbidimetric assay. The substrate for the reaction was freshly prepared with chicken egg yolk dissolved in 0.9% NaCl solution, and its absorbance was made up to one at 740 nm [20,21]. The time-dependent kinetic assay was performed in triplicate with different concentrations of each venom sample (0.01 μg, 0.1 μg, 0.5 μg, and 1 μg) prepared in a 20 mM Tris-Cl buffer. The resulting absorbance was measured at 1-minute intervals for 60 mins at 740 nm using an Epoch 2 microplate spectrophotometer (BioTek Instruments, Inc., USA). Unit activity was calculated as the amount of crude venom required to reduce the absorbance of the substrate by 0.01 OD unit per min at the given wavelength [22].
Snake venom protease assay

Proteolytic venom activity was assayed following a previously described protocol [23], wherein a pre-defined volume of azocasein substrate was incubated with a known concentration of crude venom at 37˚C for 90 mins. The reaction was stopped with trichloroacetic acid post-incubation, and the resulting mixture was further subjected to centrifugation at 1000 x g for 5 mins, mixed with an equal volume of 0.5 M NaOH and finally, absorbance was measured at 440 nm. The relative protease activity of crude venoms was calculated in comparison with the purified protease from the bovine pancreas (Sigma-Aldrich, USA).

L-amino acid oxidase (LAAO) assay

LAAO activity of snake venoms was evaluated with an endpoint assay that uses L-leucine as substrate, following a previously described protocol [20,24]. A mixture of crude venom and L-leucine (5 mM L-leucine, 50 mM Tris-HCl buffer, 5 IU/ml horseradish peroxidase, 2 mM o-phenylenediamine dihydrochloride) in 1:9 proportion was incubated at 37˚C for an hour. The reaction was terminated with 2 M H₂SO₄, and an Epoch 2 microplate spectrophotometer was used to record the absorbance at 492 nm.

DNase assay

The DNase activities of the venoms were assessed using a method described by Gercerker et al. [25] with slight modifications [20]. A predefined concentration of crude venom (0.05 μg/μl) was incubated with purified calf thymus DNA (Sigma-Aldrich, USA), reconstituted in phosphate buffer saline (PBS), followed by incubation at 37˚C for 60 mins. Reaction mixtures were then subjected to electrophoresis on a 0.8% agarose gel and visualised on an iBright CL1000 gel documentation system.

Fibrinogenolytic assay

The ability of Daboia venoms to degrade human fibrinogen was evaluated using an electrophoresis-based method, previously described by Ouyang and Teng [26]. Human fibrinogen (Sigma-Aldrich, USA) dissolved in PBS was incubated with a known concentration of venom (1.5 μg) at 37˚C for 60 mins. Following the addition of an equal volume of loading dye (1 M Tris-HCl pH 6.8, 50% Glycerol, 0.5% Bromophenol blue, 10% SDS, 20% β-mercaptoethanol), the mixture was heated at 70˚C for 10 mins. Samples were further subjected to 15% SDS-PAGE, and the gel was stained with Coomassie Brilliant Blue R-250 before visualisation in an iBright CL1000 (Thermo Fisher Scientific, USA) gel documentation system. The results were interpreted in comparison to a negative control consisting of human fibrinogen only, where all three bands (different chains of reduced fibrinogen) are observed intact.

Blood coagulation assays

To assess the capability of D. russelli venom to perturb the coagulation cascade, specifically the extrinsic and intrinsic pathways, we quantified venom-induced alterations to the prothrombin time (PT) and partial thromboplastin time (aPTT), respectively. Blood samples collected from healthy male volunteers were centrifuged at 3000 x g for 10 mins at 4˚C to separate platelet-poor plasma (PPP). Prewarmed calcium thromboplastin reagent (Uniplastin; Tulip diagnostics, Mumbai; 200 μl) and activated cephaloplastin reagent (Liquicelin-E; Tulip diagnostics, Mumbai; 100 μl) with 0.02 M Calcium chloride (CaCl₂; 100 μl) were mixed with 50 μl of PPP as per the manufacturer’s protocol, for measuring PT and aPTT, respectively. Following this, the mixture was treated with different concentrations of D. russelli venoms (0.5 or 5 μg in
50 μl), and the time taken for the appearance of the first fibrin clot was measured using a Hemostar XF 2.0 coagulometer. The results have been represented in the form of a heat-map generated using Graphpad Prism 8 (GraphPad Software, San Diego, California USA, www.graphpad.com).

**Turbidimetric coagulation assay**

Procoagulant activities of *D. russelli* venoms were assessed by mixing various concentrations of venoms (15.6 to 250 ng/ml) with equal volumes of freshly collected PPP and 0.2 M CaCl₂ (60 μl for 1 ml of PPP) at 37˚C, following a previously described protocol [27]. The increase in turbidity of the mixture was recorded by measuring the optical density (OD) at 340 nm for every 60 seconds, over a period of 60 mins, in an Epoch 2 microplate spectrophotometer. A graph showing time versus absorbance was plotted, and the clotting time was defined as the time point where the OD increases by 0.02 units over the average OD units measured at the first two time points [27]. Subsequently, the neutralisation of procoagulant activities by the Indian polyvalent antivenom (Premium Serums) was assessed by incubating various concentrations of the venom (15.6 to 250 ng/ml) with 0.25 μg/μl (1:4) and 0.0625 μg/μl (1:16) of the antivenom at 37˚C for 30 mins, followed by the addition of plasma and CaCl₂ [28]. The assays were performed in triplicates.

**Haemolytic assay**

The haemolytic activity of snake venom was determined by assessing the degradation of human red blood cells (RBC) using a previously described protocol [29]. RBCs isolated from whole blood were resuspended with 1X PBS and centrifuged at 3000 x g for 10 mins at 4˚C. After centrifugation, the supernatant was discarded, and the RBC pellet was resuspended again in 1X PBS. The above procedure was repeated five times to remove undesirable blood factors and cellular debris. Thereafter, a 1% RBC suspension was prepared and mixed with various concentrations of the venoms (5, 10, 20 and 40 μg) in a 10:1 ratio and the reaction mixture incubated at 37˚C for 24 hours. Following incubation, samples were centrifuged at 3000 x g for 10 mins, and the absorbance of the supernatant was measured at 540 nm using an Epoch 2 microplate spectrophotometer. The relative haemolytic activity of the venoms was calculated using 0.5% Triton X as the positive control and after accounting for background absorbance.

**Enzyme-linked immunosorbent assay (ELISA)**

We investigated the *in vitro* venom recognition (i.e., binding) of commercial antivenoms using a previously described ELISA protocol [20,30]. Venom samples (100 ng) were diluted in a carbonate buffer (pH 9.6) and coated onto 96-well plates. Following overnight incubation at 4˚C, the unbound venom was removed by washing with Tris-buffered saline with 1% Tween 20 (TBST). The venom-bound plate was then incubated with a blocking buffer (5% skimmed milk in TBST) for 3 hours at room temperature. The plate was then subjected to another round of TBST washing, which was followed by the addition of various dilutions of commercial equine Indian antivenoms (Premium Serums, VINS, Bharat, and Haffkine). Plate was then incubated overnight at 4˚C, and the unbound antibodies removed by a TBST wash the next day. Horseradish peroxidase (HRP)-conjugated rabbit anti-horse secondary antibody (Sigma-Aldrich, USA) diluted in PBS (1:1000) was added, and the plate was incubated at room temperature for 2 hours. Post incubation, ABTS (2,2’-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) substrate solution (Sigma-Aldrich, USA) was added and the absorbance measured at a wavelength of 405 nm for 40 mins in Epoch 2 microplate reader. IgG from a naive horse...
(Biorad) was used as the negative control to determine the cut-off for non-specific antibody binding, as described previously [20,30].

**Immunoblotting**

Venom and antivenom immunoblotting experiments were performed following a previously described protocol [20,30]. Following the electrophoretic separation of crude venoms using SDS-PAGE (12.5%), a nitrocellulose membrane was used to electrotransfer the proteins as per the manufacturer’s instructions (BioRad, USA). To verify transfer efficacy, Ponceau S reversible stain was used, and the membrane was then incubated with a blocking buffer at 4˚C overnight. After washing with TBST, the membrane was incubated with a known concentration of commercial antivenom at 4˚C. On the following day, an HRP-conjugated rabbit anti-horse secondary antibody was added at a dilution of 1:2000, followed by six TBST washes to remove the unbound antivenom. Finally, following the manufacturer’s instructions (Thermo Fisher Scientific, USA), an enhanced chemiluminescence substrate was used to visualise the binding efficacy of commercial antivenoms to venom, and the membrane was imaged in an iBright CL1000 (Thermo Fisher Scientific, USA).

**In vivo venom toxicity and antivenom efficacy assays**

Preclinical experiments were conducted using a murine model of envenoming to evaluate the toxicity of *D. russelii* venoms from various biogeographical zones and the efficacy of the marketed Indian antivenom in neutralising the lethal effects of these venoms.

**The intravenous median lethal dose (LD₅₀)**

The potencies of *Daboia* venoms from distinct biogeographic zones were determined by calculating the LD₅₀ values (or the amount of venom required to kill 50% of the test population) by following WHO-recommended murine assay protocols [31]. Five concentrations of each crude venom were prepared in physiological saline (0.9% NaCl) and injected intravenously into the caudal vein of male CD-1 mice (500 μl/mouse). Subsequently, the number of dead and surviving mice in each venom dose group (n = 5) was recorded 24 hours later, and the LD₅₀ and 95% confidence intervals were calculated using Probit statistics [32].

**The median effective dose (ED₅₀) of antivenom**

The preclinical efficacy of commercial antivenom in neutralising the venoms of pan-Indian populations of *D. russelii* was evaluated by calculating the ED₅₀ value or the minimum amount of antivenom required to protect 50% of the test population injected with lethal doses of venom [31]. We selected the antivenom manufactured by Premium Serums for use in ED₅₀ testing based on its superior in vitro venom recognition potential (determined by ELISA and western blotting experiments). This in vitro guided strategy significantly reduced the number of mice sacrificed in ED₅₀ experiments. Briefly, various volumes of antivenoms were mixed with the challenge dose of venom (equivalent to 5X LD₅₀ of each venom), followed by an incubation period of 30 mins at 37˚C. Post incubation, each venom-antivenom mixture (n = 4 per venom) was intravenously injected into a group of five male CD-1 mice (18–22 g). The number of surviving and dead animals were monitored over 24 hours, and the resulting ED₅₀ values and 95% confidence intervals were calculated using Probit statistics [32]. The volume of antivenom (μl) required to neutralise one milligram of venom was estimated following a recently proposed method [33]. The neutralisation potency of the commercial antivenom was calculated with the equation [20,34]. Here, n represents the number of LD₅₀ used as the
challenge dose.

\[ \text{Antivenom neutralisation potency (mg/ml)} = \frac{(n - 1) \times LD_{50} \text{ of venom (mg/mouse)}}{ED_{50} \text{ (ml)}} \]

Statistical analysis

Statistical comparisons of data generated in the various biochemical and ELISA assays were performed using One-way ANOVA and Two-way ANOVA with Tukey’s and Dunnett’s multiple comparison tests in GraphPad Prism (GraphPad Software 8.0, San Diego, California USA, www.graphpad.com).

Results

Venom proteomics

Venoms sourced from \( D. \) \( russelii \) populations from five distinct biogeographical zones across India were separated using SDS-PAGE under reducing conditions (Table 1 and Fig 1). Analyses of SDS-PAGE profiles revealed considerable differences in band intensities and patterns, highlighting the significant interpopulation venom variation in this species (Fig 1).

To further resolve intraspecific variation, \( D. \) \( russelii \) venom samples were subjected to RP-HPLC, which unravelled finer differences in the venom compositions across the

Fig 1. Map of India, prepared with QGIS 3.8.2 [35], depicts (A) sampling locations and (B) SDS-PAGE profiles of \( D. \) \( russelii \) venoms. M: Protein marker (units in kDa); PB: Punjab; TN: Tamil Nadu; AP: Andhra Pradesh; WB: West Bengal; MH: Maharashtra; MP: Madhya Pradesh.

https://doi.org/10.1371/journal.pntd.0009247.g001
biogeographical zones (Fig 2). RP-HPLC profiles revealed ten major fractions/peaks, with notable differences observed in peaks #1, #6, #7 and #8 between samples. Interestingly, peak #6 was completely absent in the Western Ghats (MH) and Gangetic Plain (WB) populations while dominating the venom of the Deccan Plateau (MP) population. Similarly, peak #8 enriched the venoms of the semi-arid (PB) and Deccan Plateau (MP) populations while being absent from one of the coastal (TN) populations.

Populations from the Gangetic Plain (WB), Western Ghats (MH) and Deccan Plateau (MP) were selected for mass spectrometry analyses based on their unique HPLC and toxicity profiles (Fig 2). RP-HPLC profiles revealed ten major fractions/peaks, with notable differences observed in peaks #1, #6, #7 and #8 between samples. Interestingly, peak #6 was completely absent in the Western Ghats (MH) and Gangetic Plain (WB) populations while dominating the venom of the Deccan Plateau (MP) population. Similarly, peak #8 enriched the venoms of the semi-arid (PB) and Deccan Plateau (MP) populations while being absent from one of the coastal (TN) populations.

Populations from the Gangetic Plain (WB), Western Ghats (MH) and Deccan Plateau (MP) were selected for mass spectrometry analyses based on their unique HPLC and toxicity profiles (see below). Tandem mass spectrometry of venom fractions (n = 10) from these samples identified between 49 and 66 proteins from 13 toxin families, including phospholipase A\(_2\) (PLA\(_2\)), Kunitz-type serine protease inhibitor (Kunitz), snake venom serine protease (SVSP), cysteine-rich secretory protein (CRIISP), snaclec, snake venom metalloproteinase (SVMP), three-finger toxin (3FTx; neurotoxic-3FTx (N-3FTx) and cytotoxic-3FTx (C-3FTx)), L-amino-acid oxidase...
(LAAO), nerve growth factor (NGF), disintegrin, vascular endothelial growth factor (VEGF), 5’ Nucleotidase (5’-NTD) and C-type lectin (CTL) (S2–S4 Tables, S1 Data). The identification of 3FTx in *Daboia* venoms is particularly noteworthy as these toxins are rarely reported in the venom proteomes of viperid snakes [36,37].

Tandem mass spectrometry revealed significant differences in the venom compositions of the investigated *D. russelii* populations (Fig 3). Kunitz-type serine protease inhibitors were found to be more abundant in the venom of the Gangetic Plain (WB) population (~39%), in line with previous reports [38], whereas the Deccan Plateau (MP) and the Western Ghats (MH) populations contained very limited amounts of this toxin: 7% and 4%, respectively (Fig 3 and S2–S4 Tables). Considerable differences in the proportions of PLA$_2$s were also observed. While 59% of the venom of the Western Ghats (MH) population consisted of PLA$_2$, the Gangetic Plain (WB) and the Deccan Plateau (MP) populations respectively contained 35% and 45% of this toxin in their venom. In contrast to the previous report [9], SVSPs were found to be comparatively more abundant in the Western Ghats (MH) population (27%) than the Deccan Plateau (MP, 13%) and Gangetic Plain (WB, 8%) populations. Interestingly, snaclcs constituted nearly ~20% of the venom proteome of the Deccan Plateau (MP) population, whereas the Gangetic Plain (WB) and the Western Ghats (MH) populations contained only 5% and 2% of this toxin, respectively. While VEGF, a family of toxins responsible for inducing vascular permeability and lower blood pressure [39,40], was only identified in trace amounts in the other populations of *D. russelii* (<1%), it was found to constitute ~8% of the venom of the Deccan Plateau (MP) population (S2–S4 Tables).
Venom biochemistry

The snake venom arsenal, which is composed of toxins with tremendous compositional and functional diversity, can inflict varied clinical manifestations in snakebite victims. To understand the differences in the biochemical and pharmacological effects of venoms from the pan-Indian populations of *D. russelii*, we subjected them to a battery of biochemical (PLA₂, protease, LAAO, DNase, fibrinogengenolytic) and pharmacological (haemolytic assay, plasma coagulation assay, prothrombin time and activated partial thromboplastin time) assessments.

PLA₂ assay

Snake venom PLA₂s are known to exert a wide range of pharmacological effects in bite victims, including cytotoxicity, neurotoxicity, myotoxicity and perturbation of haemostasis [41]. Given their medical relevance, we assessed the abilities of *D. russelii* venoms in hydrolysing phospholipid substrates. The enzymatic PLA₂ activity profiles of *D. russelii* differed between populations (p<0.05), with the Deccan Plateau (MP) population exhibiting the highest activity, while the coastal populations (TN and AP) showed intermediate activities that were indistinguishable from each other (S1A Fig). While *D. russelii* populations from the Western Ghats (MH) and Gangetic Plain (WB) were statistically similar to each other in their PLA₂ activities, they were significantly lower than all other *D. russelii* venom samples under study (p<0.05). The semi-arid (PB) population exhibited the lowest PLA₂ activity among all the tested populations. Low PLA₂ activity, despite relatively high abundance in the proteome, is probably suggestive of the presence of non-catalytic PLA₂s that enrich the venoms of many viperid snakes [42–44].

Snake venom protease assay

Venoms of many snake species may contain a variety of proteolytic enzymes that cause serious clinical pathologies in bite victims [45,46]. Viperid venoms, in particular, are known to be dominated by SVSPs and SVMPs that disrupt haemostasis by targeting various factors involved in the blood coagulation cascade (e.g., thrombin, fibrinogen, plasminogen and platelets), often synergistically [47–49]. Examination of *D. russelii* venoms from distinct biogeographic locations revealed highly variable degrees of proteolysis. While the Deccan Plateau (MP) population and one of the coastal populations (AP) exhibited the highest proteolytic activities, followed by the population from the Western Ghats (MH), all others showed negligible effects (p<0.05; S1B Fig). Interestingly, the Western Ghats (MH) population that was rich in the overall protease content (SVSP and SVMP) exhibited relatively reduced proteolytic activity in comparison to the SVMP-rich Deccan Plateau (MP) population (p<0.05). Similarly, the low proportion of proteases in the Gangetic Plain (WB) population could be reflective of the negligible protease activity observed.

LAAO assay

Snake venom LAAOs are theorised to contribute to the oxidative stress in bite victims as they catalyse the oxidative deamination of L-amino acids into α-keto acids, releasing H₂O₂ as a byproduct [50]. Consequently, despite being secreted in relatively minimal amounts in many snake venoms, LAAO may contribute to a diversity of toxic effects, including cytotoxicity, induction of cell death, haemorrhage and inhibition of platelet aggregation [51–53]. When the crude venoms of *D. russelii* were tested for their ability to catalyse the oxidative deamination of L-amino acids, they exhibited substantial activity (S1C Fig). Though statistically significant differences in LAAO activities were observed between populations (p<0.05), further experiments are required to assess the clinical importance of this difference.
DNase assay
Recently, it has been shown that the venoms of certain viperid snakes are capable of inducing a phenomenon called ETosis in bite victims, wherein cells extrude their nuclear DNA to form extracellular traps (ETs) [54,55]. As these traps restrict the diffusion of venom toxins, ETosis may significantly contribute to local tissue damage. However, the DNase activity of certain snake venoms can facilitate the diffusion of venom toxins by cleaving such extracellular traps [56,57]. Taking this into account, the DNase activities of the geographically distinct D. russelli venoms were assessed. With the exception of the semi-arid (PB) population, which showed atypically high activity, all other populations exhibited low to negligible DNase venom activities (S1D and S2 Figs). Additional experiments are necessary to understand the biological consequence of the unusually high DNase activity of the semi-arid (PB) D. russelli population.

Fibrinogenolytic assay
Snake venoms are capable of perturbing the haemostasis of bite victims by cleaving the fibrinogen glycoprotein complex [58], which is composed of Aα, Bβ and γ subunits. When the fibrinogenolytic potential of D. russelli venoms was assessed, barring the semi-arid (PB) and Gangetic Plain (WB) populations, all D. russelli venoms either completely or partially cleaved the Aα subunit, while none of the venoms degraded the Bβ- and γ-chains (S3 Fig).

Blood coagulation assays
Viperid venoms are known to perturb hemostasis by inducing a range of clinical symptoms, such as local and systemic haemorrhage, venom-induced consumption coagulopathy (VICC), and alteration of blood pressure and vascular permeability [59]. Amongst the plethora of toxins in viper venoms, PLA2, SVSP and SVMMP are known to exhibit potent procoagulant or anticoagulant effects by cleaving coagulation factors involved in the intrinsic or extrinsic coagulation cascade [60–62]. To evaluate the effects of D. russelli venoms on the intrinsic and extrinsic coagulation cascades, we estimated the prothrombin time (PT) and activated partial thromboplastin time (aPTT), respectively. The venoms of D. russelli from distinct biogeographies exhibited potent procoagulant activities, altering both the extrinsic and intrinsic pathways (Fig 4A and 4B). Notably, even at very low concentrations (5 μg), all Daboia venoms exhibited significant procoagulant effects within ~5 seconds (Fig 4A and 4B). In contrast, certain D. russelli populations have been preclinically [63] and clinically [64] documented to induce potent anticoagulant effects at higher venom concentrations. Anticoagulatory effects in bite victims are primarily caused by VICC, wherein the coagulation factors of the blood are depleted due to the activities of the procoagulant snake venom toxins, resulting in life-threatening haemorrhage [59]. As D. russelli can inject very large amounts of venom (an average of 200 mg of venom was extracted from 12 Russell’s vipers in this study), potent anticoagulant effects are likely seen in bite victims [65].

A turbidimetric plasma clotting time assay revealed similar procoagulatory effects of Daboia venoms. Consistent with the results of the PT and aPTT experiments, all populations were found to exhibit strong procoagulant effects (clotting time between 2 and 5 mins) (Fig 4C and S4 Fig). Moreover, when the ability of the best binding antivenom (Premium Serums; see the outcomes of binding experiments below) in neutralising these effects were tested under in vitro conditions, the clotting time of the plasma mixed with either the venom of the semi-arid (PB) population or the Deccan Plateau (MP) population was considerably prolonged (Fig 4D and S4 Fig). This is, perhaps, indicative of the neutralisation of procoagulant toxins, and not those responsible for anticoagulant effects. These experiments yielded identical results despite the repetition. In complete contrast, despite the treatment with the antivenom, considerable
procoagulant effects were still seen in the Gangetic Plain (WB) and one of the coastal (AP) populations (Fig 4D and S4 Fig). Interestingly, the addition of antivenom to the venom of *D. russelii* from Western Ghats (MH) and the other coastal (TN) region resulted in clotting times that were comparable to the blank, suggesting the presence of procoagulant toxin-neutralising antibodies in the antivenom (Fig 4D and S4 Fig).

**Haemolytic assay**

Intravascular haemolysis, which often results in adverse conditions, including renal failure, has been demonstrated as one of the major clinical pathologies of *D. russelii* envenomings [66]. The haemolytic effect of *D. russelii* venoms is predominantly attributed to secretory PLA$_2$s that hydrolyse the phospholipid bilayer on the cellular membrane [67,68]. Since a significant effect of the venom was observed on egg yolk phospholipids in PLA$_2$ assays, we assessed the direct haemolytic activity of *D. russelii* venoms on human erythrocytes. Here, the Gangetic Plain (WB) and coastal (AP and TN) populations of *D. russelii* exhibited negligible to no direct haemolytic activities (0 to 18%) at the highest venom concentration tested (40 μg),
whereas the semi-arid (PB), Western Ghats (MH) and Deccan Plateau (MP) populations exhibited moderately increased haemolytic effects [26 to 31%; (Fig 4E)]. It is interesting to note that the semi-arid (PB) population showed increased haemolysis despite low PLA$_2$ activity (S1A Fig), suggesting the potential pharmacological role of non-catalytic PLA$_2$s or other toxin constituents [42,43].

**In vitro venom recognition potential of commercial Indian antivenoms**

The compositional differences in snake venoms from distinct populations are known to significantly affect the immunological cross-reactivity of antivenoms [5,10,63,69], thereby reducing their potential clinical effectiveness. We, therefore, assessed the venom recognition potential of the major Indian commercial polyvalent antivenoms against the venoms of the pan-Indian populations of *D. russelii*. In indirect ELISA experiments, the Premium Serums antivenom consistently recognised the venoms of the pan-Indian populations of *D. russelii* to a greater extent (end-point titres between 1:2500 to 1:12500) than that of its comparators, followed by the VINS antivenom (1:2500) (Fig 5 and S5 Fig). On the contrary, antivenoms manufactured by Bharat Serums (1:500 to 1:2500) and Haffkine (1:500) exhibited considerably poorer venom recognition.

![Heatmaps showing venom recognition potential of Indian antivenoms](https://doi.org/10.1371/journal.pntd.0009247.g005)

**Fig 5. Immunological cross-reactivity of commercial Indian antivenoms against the pan-Indian populations of *D. russelii* venoms.** Heatmaps, shown here, quantify the binding of commercial Indian antivenoms to the venoms of pan-Indian populations of *D. russelii*. Multiple dilutions of antivenoms (1:500, 1:2500 and 1:12500) were tested in indirect ELISA experiments. Non-specific binding of naive horse IgGs (1:4 dilution) to *Daboia* venoms is also shown in the first plate for reference. A gradient colour scale has been shown indicating the degree of binding from low (black) to high (cream). PB: Punjab; TN: Tamil Nadu; AP: Andhra Pradesh; WB: West Bengal; and MH: Maharashtra; MP: Madhya Pradesh.
recognition capabilities. Surprisingly, despite sourcing venoms from the Maharashtra Daboia population as immunogens for antivenom production, the Haffkine antivenom failed to exhibit high binding titres against all populations, including this source population.

Furthermore, immunoblotting experiments, which were performed to identify venom toxins that are recognised by antivenoms, revealed that the Premium Serums antivenom recognised many low-, mid- and high-molecular-weight toxins found in the venom of the pan-Indian populations of D. russelii (S6A and S6B Fig). In contrast, the commercial products manufactured by VINS and Haffkine exhibited increased recognition only towards high-(> 50 kDa) and low-(< 15 kDa) molecular weight toxins, while poor immunorecognition was observed in the case of Bharat Serum antivenoms (S6A and S6B Fig).

**Venom potency by murine median lethal dose (LD$_{50}$) assay**

The proteomic composition and potency of venoms is significantly influenced by the ecology and environment of the snake species [4–7,70]. Evaluation of murine intravenous toxicity profiles revealed interesting differences between the pan-Indian populations of D. russelii venoms. While the Deccan Plateau (MP; 0.11 mg/kg) and the semi-arid (PB; 0.14 mg/kg) populations were highly potent, the Gangetic Plain (WB; 0.34 mg/kg) population was found to be considerably less potent, while the coastal (AP; 0.18 mg/kg) and Western Ghats (MH; 0.19 mg/kg) populations exhibited near equivalent, intermediary, potencies (Fig 6A and S5 Table).

**Antivenom efficacy via median effective dose (ED$_{50}$) assay**

Though the in vitro recognition and inhibition of biochemical/pharmacological activities by commercial antivenoms has been demonstrated against venoms of D. russelii from certain populations in India [9–13], their ability to neutralise the lethal effects in animal models is yet to be robustly evaluated. Considering its increased venom recognition capabilities, the Premium Serums antivenom was selected for use in the assessment of in vivo neutralisation against the pan-Indian venoms of D. russelii. Perhaps surprisingly, given the extent of venom recognition capabilities.
compositional and functional variation observed in this study, the venom neutralising potencies for all populations of *D. russelii* (0.84–0.99 mg/ml) met the manufacturer’s marketed claim (0.6 mg/ml), with the exception of observations of poor neutralisation of the semi-arid (PB) population (0.39 mg/ml) (Fig 6B and S6 Table). These findings contrast with those recently observed with venom sourced from Indian *Naja* populations, where venom variation resulted in a dramatic lack of preclinical antivenom efficacy [71].

**Discussions**

**Biogeographic variation in Russell’s viper venom**

Biotic and abiotic factors are well-known to dictate snake venom compositions and potencies [5]. Unfortunately, the influence of diverse biogeographic conditions on the composition and potency of the medically most important Indian snakes, and the consequence of this variation on snakebite treatment, remains elusive. Here, evaluation of proteomic profiles of the pan-Indian populations of *D. russelii* from distinct biogeographic zones revealed considerable compositional differences in venom toxins, including PLA₂, Kunitz, SVSP and snaclec. As variation in venom proteomes can significantly alter biochemical and pharmacological activities of snake venoms, we also subjected *D. russelii* venoms to a variety of biochemical assessments, including PLA₂, LAAO, DNase, fibrinogenolytic, haemolytic and blood coagulation assays. The outcomes of these experiments highlighted significant differences in the activities of *D. russelii* venoms from distinct biogeographical zones. Moreover, they are suggestive of the differential abilities of these populations in inflicting cytotoxic, haemotoxic and procoagulant effects in human snakebite victims [65,72,73]. Such stark differences in composition and activities may be underpinned by a variety of factors across distinct agro-climatic conditions, including temperature, humidity, altitude, phylogenetic divergence and prey and predator abundance. Differences in toxin composition were also found to correlate with altered potencies of *Daboia* venoms, where the protease-rich Deccan Plateau (MP) and Kunitz-rich Gangetic Plain (WB) populations of *D. russelii* were the most and the least toxic populations, respectively (Figs 3 and 6A and S2–S4 Tables).

**Inadequacy of antivenom therapy in North Indian snakebite hotspots**

Despite being the only curative therapeutic for snakebite in India, commercial polyvalent antivenoms that are marketed by several manufacturers across the country suffer from several critical limitations. Perhaps, their major potential limitation is a lack of effectiveness against geographically disparate snake populations, as antivenoms are customarily manufactured using venoms sourced from the ‘big four’ snakes in the southeastern part of the country [74]. Venom recognition experiments in this study revealed that the majority of marketed products lacked antibodies specific to several high-, mid- and low-molecular-weight toxins (S6A Fig), which was in line with previous findings [9,11,13,20]. Preclinical experiments conducted in a murine model of envenoming were undertaken to evaluate the neutralising potential of the Premium Serums antivenom, which exhibited superior *in vitro* venom recognition potential over its competitors. Though this antivenom met the marketed neutralising potency (0.60 mg/ml) for four out of the five *D. russelii* populations sampled across the various biogeographical zones in India (0.84 to 0.99 mg/ml), its potency against the semi-arid (PB) population was significantly lower (0.39 mg/ml). Similar concerning inefficacy of this antivenom has been previously reported against two of India’s other ‘big four’ snake species (*B. caeruleus* and *N. naja*) in this region [20,71], while a complete lack of neutralisation (challenge dose: 5X LD₅₀) has also been recently reported against the Desert (Rajasthan) population of *N. naja* [71]. Improved neutralisation potential of the other commercial antivenoms (i.e., VINS, Bharat Serums and
Haffkine) seems unlikely, as these products largely rely on the same venom source for immunisation and follow very similar immunisation protocols. This assertion is further supported by the outcomes of our in vitro binding experiments, which revealed comparably worse immunological binding of these antivenoms to venoms. Similarly, deficiencies in the neutralisation potency of Indian antivenoms (VINS) towards *D. russelii* from Pakistan has also been documented previously [75]. These disturbing findings, perhaps, explain the alarming rates of snakebite mortalities in the northern and (north)western regions of the country (Fig 7; [2]), further highlighting the pressing need to develop a pan-India effective antivenom therapy [20].

Fig 7. The preclinical inefficacy of Indian antivenom therapy in snakebite hotspots. This figure depicts the alarming preclinical ineffectiveness of commercial antivenoms in the major snakebite hotspots of India (highlighted in red circles). The relative differences in neutralisation potencies of antivenoms against the geographically distinct populations of *N. naja* (green vials) [71], *D. russelii* (yellow vials) and *B. caeruleus* (purple vial) [20] are shown in comparison to the venom source (for antivenom production) population in southern India. The red dotted lines on vials represent the marketed neutralising potency of the commercial products. Sampling locations have been indicated with uniquely coloured markers (top right box) on the biogeographical map of India that was prepared with QGIS 3.8.2 [35]. The intensity of purple clouds on the map is indicative of the estimated standardised snakebite death rates per million reported by Suraweera et al. 2020 [2], with the brighter regions representing the major hotspots.
Compositional variation in venoms cannot predict the underlying clinical outcomes

Intraspecific differences in venom composition, resulting from multiple biotic and abiotic factors, has been well-documented in snakes [63,70,76]. As a local adaptation to the changing ecology and environment, species that are characterised by large geographical distribution exhibit stark variations in the proteomic composition and toxicity. For instance, the venoms sourced from the pan-Indian populations of *N. naja*, yet another medically important Indian snake species with a near-countrywide distribution, has been shown to exhibit substantial differences in the abundances of lethal neurotoxins and cytotoxins [71,77–79]. These compositional differences led to reduced preclinical effectiveness of the marketed antivenoms in mitigating snakebite pathologies [71]. In complete contrast, despite the observed geographic variability in venom composition and toxicity profiles, we find that the lethal effects induced by venoms of the majority of investigated *D. russelii* populations are neutralised by the Premium Serums polyvalent antivenom, matching the marketed claim of effectiveness (S6 Table). Thus, the considerable variation observed in the venom proteomes of both *N. naja* and *D. russelii*, surprisingly, mostly translate into treatment challenges only for the former species. These contrasting findings highlight that intraspecific venom variation in itself cannot be a predictor for the (pre)clinical effectiveness of antivenoms. It should be noted, however, that the results presented here do not shed light on the effectiveness of antivenoms in mitigating the local, morbidity-causing effects associated with *D. russelii* envenomings, and this would be a valuable research line to pursue in the future.

In summary, comparative proteomics, biochemical and pharmacological assessments, and toxicity profiling experiments performed in this study reveal significant intraspecific variation in *D. russelii* venoms from five distinct biogeographic regions of India. The results of in vitro immunological assays identified the Premium Serums antivenom to be superior to its competitors (VINS, Bharat Serums and Haffkine) in terms of its venom recognition potential. Despite the considerable differences in venom proteomic profiles revealed, the Premium Serums antivenom exhibited surprisingly similar efficacy in countering the lethal effects of venom from four out of the five *D. russelii* populations in a mouse model of envenoming. However, this antivenom was found to be inefficacious in neutralising the lethal effects of the North Indian semi-arid (PB) population, a caveat previously also highlighted for two other ‘big four’ snakes [20,71]. These inadequacies of existing antivenom further highlight the compelling need to develop pan-India effective antivenoms to safeguard human lives in high snakebite burdened locales of India.

Limitations of the study

Russell’s viper is amongst the most medically important Indian snakes, as it accounts for over 40% of snakebite fatalities in the country [2]. Surprisingly, though the polyvalent antivenom neutralised the lethality of *D. russelii* venoms from four out of five biogeographic zones investigated in this study, these conclusions were derived with respect to the neutralisation potency advertised by the antivenom manufacturers. However, since Indian antivenoms have not been evaluated through robust human clinical trials, it is essential to validate the accuracy of this cut off for effective treatment of *D. russelii* envenomings. Moreover, the neutralisation experiments in the mouse model employed here do not inform us of the ability of this commercial product in countering the morbid symptoms that incapacitate hundreds of thousands of Indians annually. Therefore, future work is warranted to evaluate the abilities of antivenoms to neutralise local pathologies (e.g., necrosis and haemorrhage) that result in immutable morbidities. Moreover, certain populations of *D. russelii* from Sri Lanka have been shown to secrete a
form of basic PLA₂ (U1-viperitoxin-Dr1a) that makes their venoms highly neurotoxic [73]. While their counterparts in South India have also been proposed to exhibit such neurotoxic effects [64,80], robust clinical evidence has been lacking. In our in vivo toxicity experiments, we did not observe any neurotoxic symptoms (e.g., ptosis, paralysis of the limbs, etc.) in mice injected with the venoms of the pan-Indian populations of Daboia snakes. Nonetheless, it will be important in the future to conduct neurotoxicity assays in mammalian model systems to delineate the abilities of the pan-Indian populations of D. russelii in inflicting neurotoxic symptoms. Furthermore, systematic documentation of epidemiological data and clinical evaluation of antivenom’s effectiveness is indispensable for assessing the shortcomings of the conventional antivenom therapy, particularly in regions that suffer the brunt of snakebite. Finally, assessing the avidity of venom-antivenom interactions and characterisation of unrecognised medically important toxins could further provide valuable information for improving the efficacy of conventional antivenoms.

Supporting information

S1 Fig. Biochemical venom variation in the pan-Indian populations of D. russelii. (DOCX)
S2 Fig. DNase activities of D. russelii venoms demonstrated through agarose gel electrophoresis. (DOCX)
S3 Fig. Fibrinogenolytic activities of D. russelii venoms from distinct biogeographical locations across India. (DOCX)
S4 Fig. Plasma clotting times of D. russelii venoms and neutralisation by commercial antivenom. (DOCX)
S5 Fig. IgG reactivity of commercial Indian antivenoms against D. russelii venoms determined by indirect ELISA. (DOCX)
S6 Fig. Immunoblotting of commercial Indian antivenoms against D. russelii venoms. (DOCX)
S1 Table. Details of commercial Indian antivenoms. (DOCX)
S2 Table. The proteomic composition of the Gangetic Plain (WB) population. (DOCX)
S3 Table. The proteomic composition of the Western Ghats (MH) population. (DOCX)
S4 Table. The proteomic composition of the Deccan Plateau (MP) population. (DOCX)
S5 Table. Toxicity profiles of the pan-Indian D. russelii populations. (DOCX)
S6 Table. Median effective doses and neutralisation potencies of Premium Serums anti-venom against the pan-Indian *D. russelii* venoms.

(Document)

S1 Data. Archive containing the results of proteomics analyses.

(Zip)

Acknowledgments

The authors are thankful to Navneet Kaur, Aratrika Ray, Aditi Singh, and Bharat Ahuja for assistance with biochemical experiments. Authors are also thankful to the following State Forest Departments for the kind support and permits for venom collection: Punjab, Tamil Nadu, Andhra Pradesh, West Bengal, Maharashtra, and Madhya Pradesh. For the invaluable assistance in the collection of various samples, the authors are thankful to Ajay Kartik (MCBT), Sumanth Madhav (Humane Society International), Vivek Sharma, Allwin Jesudasan (MCBT) and Dr Aditya Malladi (IISc). For contributing snake photographs used in Fig 1 of the manuscript, the authors are thankful to Ajay Kartik (TN), Varinder Singh (PB), and Vivek Sharma (all others).

Author Contributions

Conceptualization: Kartik Sunagar.

Data curation: R. R. Senji Laxme, Suyog Khochare, Saurabh Attarde, Ashwin Iyer.

Formal analysis: R. R. Senji Laxme, Suyog Khochare, Saurabh Attarde, Vivek Suranse, Kartik Sunagar.

Funding acquisition: Kartik Sunagar.

Investigation: R. R. Senji Laxme, Suyog Khochare, Saurabh Attarde, Vivek Suranse, Ashwin Iyer, Kartik Sunagar.

Methodology: R. R. Senji Laxme, Suyog Khochare, Saurabh Attarde, Ashwin Iyer, Kartik Sunagar.

Project administration: Kartik Sunagar.

Resources: Romulus Whitaker, Gerard Martin, Kartik Sunagar.

Supervision: Kartik Sunagar.

Visualization: Kartik Sunagar.

Writing – original draft: R. R. Senji Laxme, Suyog Khochare, Saurabh Attarde, Vivek Suranse, Ashwin Iyer, Kartik Sunagar.

Writing – review & editing: R. R. Senji Laxme, Nicholas R. Casewell, Kartik Sunagar.

References

1. World Health Organization (WHO). Snakebite envenoming: WHO Press; 2019 [https://www.who.int/news-room/fact-sheets/detail/snakebite-envenoming].

2. Suraweera W, Warrell D, Whitaker R, Menon G, Rodrigues R, Fu SH, Begum R, Sati P, Piyasena K, Bhatia M, Brown P, Jha P. Trends in snakebite deaths in India from 2000 to 2019 in a nationally representative mortality study. *Elife*. 2020; 9.

3. Holding ML, Biardi JE, Gibbs HL. Coevolution of venom function and venom resistance in a rattlesnake predator and its squirrel prey. *Proc Biol Sci*. 2016; 283(1829).
4. Daltry JC, Wuster W, Thorpe RS. Diet and snake venom evolution. Nature. 1996; 379(665):537–40. https://doi.org/10.1038/379537a0 PMID: 8596631

5. Casewell NR, Jackson TNW, Laustsen AH, Sunagar K. Causes and Consequences of Snake Venom Variation. Trends in Pharmacological Sciences. 2020; 41(8):570–81. https://doi.org/10.1016/j.tips.2020.05.006 PMID: 32564899

6. Zancolli G, Calvete JJ, Cardwell MD, Greene HW, Hayes WK, Hegarty MJ, Herrmann HW, Holycross AT, Lannutti DJ, Mulley JF, Sanz L, Travis ZD, Whorley JR, Wuster CE, Wuster W. When one phenotype is not enough: divergent evolutionary trajectories govern venom variation in a widespread rattlesnake species. Proc Biol Sci. 2019; 286(1898):20182735. https://doi.org/10.1098/rspb.2018.2735 PMID: 30862287

7. Durban J, Sanz L, Trevisan-Silva D, Neri-Castro E, Alagon A, Calvete JJ. Integrated Venomics and Venom Gland Transcriptome Analysis of Juvenile and Adult Mexican Rattlesnakes Crotalus simus, C. tzaban, and C. culminatus Revealed miRNA-modulated Ontogenetic Shifts. J Proteome Res. 2017; 16(9):3370–90. https://doi.org/10.1021/acs.jproteome.7b00414 PMID: 28731347

8. Rodgers W, Panwar H. Planning a wildlife protected area network in India. 1988.

9. Kalita B, Patra A, Das A, Mukherjee AK. Proteomic Analysis and Immuno-Profiling of Eastern India Russell’s Viper Venom (Daboia russellii) Venom: Correlation between RVV Composition and Clinical Manifestations Post RV Bite. J Proteome Res. 2018; 17(8):2819–33. https://doi.org/10.1021/acs.jproteome.8b00291 PMID: 2938511

10. Pla D, Sanz L, Quesada-Bernat S, Villalta M, Bael J, Chowdhury MAW, Leon G, Gutierrez JM, Kuch U, Calvete JJ. Phylovenomics of Daboia russellii across the Indian subcontinent. Bioactivities and comparative in vivo neutralization and in vitro third-generation antivenom of antivenoms against venoms from India, Bangladesh and Sri Lanka. J Proteomics. 2019; 207:103443. https://doi.org/10.1016/j.jprot.2019.103443 PMID: 31326506

11. Kalita B, Patra A, Mukherjee AK. 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. 2017; 16(2):583–98. https://doi.org/10.1021/acs.jproteome.6b00693 PMID: 27936776

12. Sharma M, Gogoi N, Dhananjaya B, Menon JC, Doley R. Geographical variation of Indian Russell’s viper venom and neutralization of its coagulopathy by polyvalent antivenom. Toxin Reviews. 2014; 33(1–2):7–15.

13. Kalita B, Singh S, Patra A, Mukherjee AK. Quantitative proteomic analysis and antivenom study revealing that neurotoxic phospholipase A2 enzymes, the major toxin class of Russell’s viper venom from southern India, shows the least immuno-recognition and neutralization by commercial polyvalent antivenom. Int J Biol Macromol. 2018; 118(Pt A):375–85. https://doi.org/10.1016/j.ijbiomac.2018.06.083 PMID: 29924981

14. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976; 72:248–54. https://doi.org/10.1016/abio.1976.9999 PMID: 942051

15. Smith BJ. SDS Polyacrylamide Gel Electrophoresis of Proteins. Methods Mol Biol. 1984; 1:41–55. https://doi.org/10.3855/0-89603-062-8:41 PMID: 20512673

16. Lomonte B, Calvete JJ. Strategies in ‘snake venomics’ aiming at an integrative view of compositional, functional, and immunological characteristics of venoms. J Venom Anim Toxins Incl Trop Dis. 2017; 23:26. https://doi.org/10.1186/s40409-017-0117-8 PMID: 28465677

17. Perez-Riverol Y, Csrordas A, Bai J, Bernal-Linares M, Hewapathirana S, Kundu DJ, Inuganti A, Griss J, Mayer G, Eisenacher M, Perez E, Uszkoreit J, Pfeuffer J, Sachsenb erg T, Yilmaz S, Tiwary S, Cox J, Audain E, Walzer M, Brazma A, Vizcaino JA. The PRIDE database and related tools and resources in 2019: improving support for quantification data. Nucleic Acids Res. 2019; 47(D1):D442–D50. https://doi.org/10.1093/nar/gky1106 PMID: 30395289

18. PEAKS Studio Technical Notes. Peptide Feature Area. Bioinformatics Solution Inc.; [https://www.bioinf.com/tutorial-peptide-feature-area/].

19. Tan NH, Wong KY, Tan CH.Venomics of Naja sputatrix, the Javan spitting cobra: A short neurotoxin-driven venom needing improved antivenom neutralization. J Proteomics. 2017; 157:18–32. https://doi.org/10.1016/j.jprot.2017.01.018 PMID: 28159706

20. Senji Laxme RR, Khochare S, de Souza HF, Ahuja B, Suranse V, Martin G, Whitaker R, Sunagar K. Beyond the ‘big four’: Venom profiling of the medically important yet neglected Indian snakes reveals disturbing antivenom deficiencies. PLoS Negl Trop Dis. 2019; 13(12):e0007899. https://doi.org/10.1371/journal.pntd.0007899 PMID: 31805055

21. Marinetti GV. The action of phospholipase A on lipoproteins. Biochim Biophys Acta. 1965; 98(3):554–65. https://doi.org/10.1016/0005-2760(65)90152-9 PMID: 5891200
Maisano M, Trapani MR, Parrino V, Parisi MG, Cappello T, D’Agata A, Benenati G, Natalotto A, Mauceri
World Health Organization (WHO). World Health Organization guidelines for the production, control and
31.
30.
Casewell NR, Cook DA, Wagstaff SC, Nasidi A, Durfas N, Wuster W, Harrison RA. Pre-clinical assays
28.
27.
Tan CH, Liew JL, Tan NH, Ismail AK, Maharrani T, Khomvilaiv S, Sitprija V. Cross-reactivity and lethality
26.
25.
Joubert FJ, Taljaard N. Purification, some properties and amino-acid sequences of two phospholipases

22. Joubert FJ, Taljaard N. Purification, some properties and amino-acid sequences of two phospholipases
A (CM-II and CM-III) from Naja naja kaouthia venom. Eur J Biochem. 1980; 112(3):493–9. https://doi.
org/10.1111/j.1432-1033.1980.tb06112.x PMID: 7460933
23. Chowdhury MA, Miyoshi S, Shinoda S. Purification and characterization of a protease produced by Vib-
río mimicus. Infect Immun. 1990; 58(12):4159–62. https://doi.org/10.1128/IAI.58.12.4159-4162.1990
PMID: 2254038
24. Kishimoto M, Takahashi T. A spectrophotometric microplate assay for L-amino acid oxidase. Anal Bio-
chem. 2001; 298(1):136–9. https://doi.org/10.1006/abio.2001.5381 PMID: 11673909
25. Gerceker D, Karasartova D, Elyurek E, Barkar S, Kiyam M, Ozsan TM, Calgin MK, Sahin F. A new, sim-
ple, rapid test for detection of DNase activity of microorganisms: DNase Tube test. J Gen Appl Micro-
biol. 2009; 55(4):291–4. https://doi.org/10.2323/jgam.55.291 PMID: 19700923
26. Teng CM, Ouyang C, Lin SC. Species difference in the fibrinogenolytic effects of alpha- and beta-fibrino-
genases from Trimeresurus murogrammatus snake venom. Toxicon. 1985; 23(5):777–82. https://
doi.org/10.1016/0041-0101(85)90008-x PMID: 4089873
27. O’Leary MA, Isbister GK. A turbidimetric assay for the measurement of clotting times of coagulant
venoms in plasma. Journal of pharmacological and toxicological methods. 2010; 61(1):27–31. https://
doi.org/10.1016/j.vascn.2009.06.004 PMID: 19615454
28. Tan CH, Liew JL, Tan NH, Ismail AK, Khomvilai S, Sitprija V. Cross reactivity and lethality
neutralization of venoms of Indonesian Trimeresurus complex species by Thai Green Pit Viper Anti-
venom. Toxicon. 2017; 140:32–7. https://doi.org/10.1016/j.toxicon.2017.10.014 PMID: 29051104
29. Maisano M, Trapani MR, Parrino V, Parisi MG, Cappello T, D’Agata A, Benenati G, Natalotto A, Mauceri
A, Cammarata M. Haemolytic activity and characterization of nematocyst venom from Pelagia noctiluca
(Cnidaria: Scyphozoa). Italian Journal of Zoology. 2013; 80(2):168–76.
30. Casewell NR, Cook DA, Wagstaff SC, Nasidi A, Durfa N, Wuster W, Harrison RA. Pre-clinical assays
predict pan-African Echis viper efficacy for a species-specific antivenom. PLoS Negl Trop Dis. 2010; 4
(10):e851. https://doi.org/10.1371/journal.pntd.0000851 PMID: 21049058
31. World Health Organization (WHO). World Health Organisation guidelines for the production, control and
regulation of snake antivenom immunoglobulins. World Health Organisation; 2018.
32. Finney D. Probit Analysis. 3rd ed. London: Cambridge University Press; 1971.
33. Ainsworth S, Menzies SK, Casewell NR, Harrison RA. An analysis of preclinical efficacy testing of anti-
venoms for sub-Saharan Africa: Inadequate independent scrutiny and poor-quality reporting are barriers
to improving snakebite treatment and management. PLoS Negl Trop Dis. 2020; 14(8):e0008579.
https://doi.org/10.1371/journal.pntd.0008579 PMID: 32817682
34. Mendonca-da-Silva I, Magela Tavares A, Sachett J, Sardinha JF, Zaparolli L, Gomes Santos MF,
Lacerda M, Monteiro WM. Safety and efficacy of a freeze-dried trivalent antivenom for snakebites in the
Brazilian Amazon: An open randomized controlled phase IIb clinical trial. PLoS Negl Trop Dis. 2017; 11
(11):e0006068. https://doi.org/10.1371/journal.pntd.0006068 PMID: 29176824
35. QGIS Development Team (2019). QGIS Geographic Information System. Open Source Geospatial
Foundation Project. http://qgis.osgeo.org.
36. Shelke RR, Satish S, Gowda TV. Isolation and characterization of a novel postsynaptic/cytotoxic neur-
otoxin from Daboia russelli russelli venom. J Pept Res. 2002; 59(6):257–63. https://doi.org/10.1034/j.
1399-3011.2002.02969.x PMID: 12010516
37. Venkatesh M, Prasad N, Sing T, Gowda V. Purification, characterization, and chemical modification of
neurotoxic peptide from Daboia russelli snake venom of India. J Biochem Mol Toxicol. 2013; 27(6):295–
304. https://doi.org/10.1002/jbt.21486 PMID: 23629951
38. Mukherjee AK, Kalita B, Mackessy SP. A proteomic analysis of Pakistan Daboia russelli russelli venom and
assessment of potency of Indian polyvalent and monovalent antivenom. J Proteomics. 2016; 144:73–86.
https://doi.org/10.1016/j.jprot.2016.06.001 PMID: 27265321
39. Toivanen PI, Nieminen T, Laakkonen T, Heikura T, Kaikkonen MU, Yla-Herttuala S. Snake venom
VEGF Vammin induces a highly efficient angiogenic response in skeletal muscle via VEGFR-2/NRP
specific signaling. Sci Rep. 2017; 7(1):5525. https://doi.org/10.1038/s41598-017-05876-y PMID: 28717175
40. Yamazaki Y, Matsunaga Y, Tokunaga Y, Obayashi S, Saito M, Morita T. Snake venom Vascular Endo-
thelial Growth Factors (VEGF-Fs) exclusively vary their structures and functions among species. J Biol
Chem. 2009; 284(15):9885–91. https://doi.org/10.1074/jbc.M809071200 PMID: 19208624
41. Kini RM. Excitement ahead: structure, function and mechanism of snake venom phospholipase A2
enzymes. Toxicon. 2003; 42(8):827–40. https://doi.org/10.1016/j.toxicon.2003.11.002 PMID: 15019485
42. Soares AM, Giglio JR. Chemical modifications of phospholipases A$_2$ from snake venoms: effects on catalytic and pharmacological properties. *Toxicon*. 2003; 42(8):855–68. https://doi.org/10.1016/j.toxicon.2003.11.004 PMID: 15019487

43. Stabeli RG, Amui SF, Sant’Ana CD, Pires MG, Nomizo A, Monteiro MC, Romao PR, Guerra-Sa R, Vieira CA, Giglio JR, Fontes MR, Soares AM. *Bothrops moojeni* myotoxin-II, a Lys49-phospholipase A$_2$ homologue: an example of function versatility of snake venom proteins. *Comp Biochem Physiol C Toxicol Pharmacol*. 2006; 142(3–4):371–81. https://doi.org/10.1016/j.cbpc.2005.11.020 PMID: 16442348

44. Andriao-Escarso SH, Soares AM, Fontes MR, Fuly AL, Correa FM, Rosa JC, Greene LJ, Giglio JR. Structural and functional characterization of an acidic platelet aggregation inhibitor andhypotensive phospholipase A(2) from *Bothrops jararacussu* snake venom. *Biochem Pharmacol*. 2002; 64(4):723–32. https://doi.org/10.1016/s0006-2952(02)01210-8 PMID: 12167491

45. Slagboom J, Kool J, Harrison RA, Casewell NR. Haemotoxic snake venoms: their functional activity, impact on snakebite victims and pharmaceutical promise. *Br J Haematol*. 2017; 177(6):947–59. https://doi.org/10.1111/bjh.14951 PMID: 28233897

46. Ferraz CR, Arrahman A, Xie C, Casewell NR, Lewis RJ, Kool J, Cardoso FC. Multifunctional Toxins in Snake Venoms and Therapeutic Implications: From Pain to Hemorrhage and Necrosis. *Frontiers in Ecology and Evolution*. 2019; 7(218).

47. Cortelazzo A, Guerranti R, Bini L, Hope-Onyekwere N, Muzzi C, Leoncini R, Pagani R. Effects of snake venom proteases on human fibrinogen chains. *Blood Transfus*. 2010; 8 Suppl 3:S120–5. https://doi.org/10.2450/2010.019S PMID: 20606742

48. Yamashita KM, Alves AF, Barbaro KC, Santoro ML. *Bothrops jararaca* venom metallopeptinases are essential for coagulopathy and increase plasma tissue factor levels during envenomation. *PLoS Negl Trop Dis*. 2014; 8(6):e2814. https://doi.org/10.1371/journal.pntd.0002814 PMID: 24831016

49. Lu Q, Clemetson JM, Clemetson KJ. Snake venoms and hemostasis. *Journal of Thrombosis and Haemostasis*. 2005 Aug; 3(8):1791–9. https://doi.org/10.1111/j.1538-7836.2005.01358.x PMID: 16102046

50. Guo C, Liu S, Yao Y, Zhang Q, Sun MZ. Past decade study of snake venom L-amino acid oxidase. *Toxicon*. 2012; 60(3):302–11. https://doi.org/10.1016/j.toxicon.2012.05.001 PMID: 22579637

51. Ande SR, Kommoju PR, Draxl S, Murkovic M, Macheroux P, Ghisla S, Ferrando-May E. Mechanisms of cell death induction by L-amino acid oxidase, a major component of ophidian venom. *Apoptosis*. 2006; 11(8):1439–51. https://doi.org/10.1007/s10495-006-7959-9 PMID: 16770529

52. Costa TR, Burin SM, Menaldo DL, de Castro FA, Sampao SV. Snake venom L-amino acid oxidases: an overview on their antitumor effects. *J Venom Anim Toxins Incl Trop Dis*. 2014; 20:23. https://doi.org/10.1186/1678-9199-20-23 PMID: 24940304

53. Paloschi MV, Pontes AS, Soares AM, Zuliani JP. An Update on Potential Molecular Mechanisms Underlying the Actions of Snake Venom L-Amino Acid Oxidases (LAAOs). *Curr Med Chem*. 2018; 25(21):2520–30. https://doi.org/10.2174/0929867324666171109114125 PMID: 29119915

54. Swethakumar B, NaveenKumar SK, Girish KS, Kemparaju K. The action of *Echis carinatus* and *Naja naja* venoms on human neutrophils; an emphasis on NETosis. *Biochim Biophys Acta Gen Subj*. 2020; 1864(6):129561. https://doi.org/10.1016/j.bbagen.2020.129561 PMID: 32068016

55. Setubal Sda S, Pontes AS, Nery NM, Bastos JS, Castro OB, Pires WL, Zaqueo KD, Calderon Lde A, Stabeli RG, Soares AM, Zuliani JP. Effect of *Bothrops bilineata* snake venom on neutrophil function. *Toxicon*. 2013; 76:143–9. https://doi.org/10.1016/j.toxicon.2013.09.019 PMID: 24080356

56. Katkar GD, Sundaram MS, NaveenKumar SW, Swethakumar B, Sharma RD, Paul M, Vishalakshi GJ, Devaraja S, Girish KS, Kemparaju K. NETosis and lack of DNase activity are key factors in *Echis carinatus* venom-induced tissue destruction. *Nat Commun*. 2016; 7:11361. https://doi.org/10.1038/ncomms11361 PMID: 27093631

57. Stackowicz J, Babino B, Todorova B, Gordan O, Iannascalii B, Jönsson F, Bruhns P, Reber LL. Evidence that neutrophils do not promote *Echis carinatus* venom-induced tissue destruction. *Nature Communications*. 2018; 9(1):2304. https://doi.org/10.1038/s41467-018-04688-6 PMID: 29899337

58. Yamazaki Y, Morita T. Snake venom components affecting blood coagulation and the vascular system: structural similarities and marked diversity. *Curr Pharm Des*. 2007; 13(28):2872–86. https://doi.org/10.2174/138161207782023775 PMID: 17979732

59. Maduwage K, Isbister GK. Current treatment for venom-induced consumption coagulopathy resulting from snakebite. *PLoS Negl Trop Dis*. 2014; 8(10):e3220. https://doi.org/10.1371/journal.pntd.0003220 PMID: 25340841

60. Saikia D, Thakur R, Mukherjee AK. An acidic phospholipase A$_2$ (RVVA-PLA$_2$-I) purified from *Daboia russelli* venom exerts its anticoagulant activity by enzymatic hydrolysis of plasma phospholipids and by non-enzymatic inhibition of factor Xa in a phospholipids/Ca$^{2+}$ independent manner. *Toxicon*. 2011; 57(6):841–50. https://doi.org/10.1016/j.toxicon.2011.02.018 PMID: 21356226
61. Alangode A, Reick M, Reick M. Sodium oleate, arachidonate, and linoleate enhance fibrinolysis by Russell’s viper venom proteinases and inhibit FXIIIa; a role for phospholipase A₂ in venom induced consumption coagulopathy. *Toxicon*. 2020.

62. Tokunaga F, Nagasawa K, Tamura S, Miyata T, Iwanaga S, Kisel W. 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*. 1988; 263(33):17471–81. PMID: 3053712

63. Prasad NB, Uma B, Bhatt SK, Gowda VT. Comparative characterisation of Russell’s viper (Daboia/Vipera russelli) venoms from different regions of the Indian peninsula. *Biochim Biophys Acta*. 1999; 1428(2–3):121–36. https://doi.org/10.1016/s0304-4165(99)00053-7 PMID: 10434030

64. Warrell DA. Snake venoms in science and clinical medicine. 1. Russell’s viper: biology, venom and treatment of bites. *Trans R Soc Trop Med Hyg*. 1989; 83(6):732–40. https://doi.org/10.1016/0035-9203(89)90311-8 PMID: 2533418

65. Kendre PP, Jose MP, Varghese AM, Menon JC, Joseph JK. Capillary leak syndrome in Russell’s viper (Daboia russelli) envenomation. *Toxicon*. 2014; 99:68–83. https://doi.org/10.1016/j.toxicon.2014.01.013 PMID: 24463169

66. Saikia D, Bordoloi NK, Chattopadhyay P, Choklingam S, Ghosh SS, Mukherjee AK. Differential mode of action on membrane phospholipids by an acidic phospholipase A2 (RVVA-PLA2-I) from Daboia russelli venom. *Biochim Biophys Acta*. 2012; 1818(12):3149–57. https://doi.org/10.1016/j.bbamer.2012.08.005 PMID: 22921758

67. Fry BG, Winkel KD, Wickramaratchi JC, Hodgson WC, Wüster W. Effectiveness of snake antivenom: species and regional venom variation and its clinical impact. *Journal of Toxicology: Toxin Reviews*. 2003; 22(1):23–34.

68. Silva A, Johnston C, Kuruppu S, Kneisz D, Maduwage K, Smith AI, Siribaddana S, Buckley NA, Hodgson WC, Isbister GK. Clinical and Pharmacological Investigation of Myotoxicity in Sri Lankan Russell’s Viper (Daboia russelli) Envenoming. *PLoS Negl Trop Dis*. 2016; 10(12):e0005172. https://doi.org/10.1371/journal.pntd.0005172 PMID: 27911900

69. Warrell DA, Gutierrez JM, Calvette JJ, Williams D. New approaches & technologies of venomomics to meet the challenge of human envenoming by snakebites in India. *Indian J Med Res*. 2013; 138:38–59. PMID: 24056555

70. Menon JC, Joseph JK. Complications of Hemotoxic Snakebite in India. In: Gopalakrishnakone P, Faiz A, Fernando R, editors. Clinical Toxicology in Asia Pacific and Africa. Dordrecht: Springer Netherlands; 2015. p. 209–32.

71. Silva A, Johnston C, Kuruppu S, Kneisz D, Maduwage K, Kleifeld O, Smith AI, Siribaddana S, Buckley NA, Hodgson WC, Isbister GK. Clinical and Pharmacological Investigation of Myotoxicity in Sri Lankan Russell’s Viper (Daboia russelli) Envenoming. *PLoS Negl Trop Dis*. 2016; 10(12):e0005172. https://doi.org/10.1371/journal.pntd.0005172 PMID: 27911900

72. Chanda A, Kalita B, Patra A, Senevirathne W, Mukherjee AK. Proteomic analysis and antivenomics of Western India Naja naja venom and its immunological cross-reactivity towards commercial antivenom. *Int J Biol Macromol*. 2020; 160:224–32. https://doi.org/10.1016/j.ijbiomac.2020.05.106 PMID: 32439440
79. Dutta S, Chanda A, Kalita B, Islam T, Patra A, Mukherjee AK. Proteomic analysis to unravel the complex venom proteome of eastern India *Naja naja*: Correlation of venom composition with its biochemical and pharmacological properties. *J Proteomics*. 2017; 156:29–39. https://doi.org/10.1016/j.jprot.2016.12.018 PMID: 28062377

80. Eapen CK, Chandy N, Joseph JK. A study of 1000 cases of snake envenomation. *Toxicon*. 1985; 23(4):621. PMID: 4060175