Abstract

Snake envenomation is one of the most severe yet underestimated health issues of the tropical and sub-tropical countries of the world which leads to large number of morbidity and mortality every year. India, being an agricultural based tropical country, encounters snakebite cases frequently especially in the rural areas causing severe loss to human lives. *Daboia russelii* commonly known as Russell's viper is one of the most venomous snakes of the Indian subcontinent responsible for most of the snakebite cases in the agricultural and tea plantation areas. Polyvalent antivenom produced against the "Big Four" snake venoms (*Naja naja*, *Daboia russelii*, *Bungarus caeruleus* and *Echis carinatus*) is the only therapy available to treat snakebite victims in India. However, its efficacy is often questioned due to various unforeseen medical conditions like anaphylactic reactions and late serum sickness which make the management scenario far more critical. These side effects mostly occur due to the large repertoire of non-specific antibodies in the polyvalent antivenom along with the unique venom proteins from venomous snakes other than the "Big Four". Moreover, the prevalence of venom variation in the snakes from different geographical locations challenges the efficacy of the polyvalent antivenom still more. Although a number of individual proteins have been purified and characterized from this Indian viper but most these are only partially characterized in terms of sequence and mechanism. Moreover, there is a dearth of knowledge regarding its proteomic and transcriptomic profile. Besides this, coagulopathy is one of the most manifested pathological conditions observed in Russell's viper envenomation, however; no major anticoagulant protein is reported so far from this Indian viper which could explain its role in pathology post envenomation.

As such the present study illustrates the venom variation of *Daboia russelii* from four different geographical regions of India (Tamil Nadu, RvTN; Karnataka, RvKA, Kerala, RvKE and West Bengal, RvWB). The electrophoretic and chromatographic profiles of the four crude venoms revealed considerable variation in venom composition. The four crude venoms also exhibited differential level of PLA_2 (RvWB > RvKA > RvKE > RvTN) and procoagulant activity (RvKE > RvWB > RvTN > RvKA) revealing variation at the functional level. The commercially available Indian

polyvalent antivenom could differentially neutralize the PLA_2 and procoagulant activity of the four venoms. It was most effective in neutralizing the PLA_2 activity of RvTN followed by RvKE, RvWB and RvKA. On the other hand, it could potentially neutralize the procoagulant effect of RvTN and RvKA while was least effective against RvKE and RvWB. Antivenomics study revealed the presence of non-immunodepleted toxins in all the four venoms and partly immunodepleted toxins in RvTN and RvKA. This suggest the inefficacy of the polyvalent antivenom to neutralize and immunodeplete the venom proteins of the four crude venoms due to variation in venom composition. Thus, this observation emphasizes the need to design regiospecific antivenom to confront the challenges of envenomation more effectively.

Subsequently, the study was focused towards explicating the proteome of the crude venom of *Daboia russelii* from Irula by high throughput proteomics approach. Gel filtration chromatography resolved the crude venom into 8 peaks which were trypsinized and subjected to tandem mass spectrometry. Analysis of the MS/MS spectra revealed the presence of 63 different proteins belonging to 12 snake venom protein families namely phospholipase A_2, serine proteases, metalloproteases, L-amino acid oxidase, snake C-type lectins, phosphodiesterase, nucleotidase, cysteine rich secretory proteins, kunitz-type serine protease inhibitor, disintegrin, vascular endothelial growth factor and vascular nerve growth factor. This is the first report of the complete proteome of Indian *Daboia russelii* venom and the presence of nucleotidase, cysteine rich secretory proteins, kunitz-type serine protease inhibitor, disintegrin, vascular endothelial growth factor and vascular nerve growth factor in the Indian Russell's viper. The observed proteome profile correlates well with the experimental activities which are in agreement with some of the pathological effects of Russell's viper envenomation.

Succeeding proteomics study, the study was focussed towards the identification of a major anticoagulant protein from this proteome. Gel filtration peak P6 constituted 32% of the total protein of the crude venom and exhibited highest anticoagulant and PLA_2 activity. It was purified to homogeneity by ion-exchange chromatography followed by Rp-HPLC. The purity of the protein, named daboxin P was confirmed by SDS-PAGE which displayed a single protein band at ~14 kDa. It has a molecular

mass of 13,597 Da and constituted 42.73% of α-helix and 12.36% of β-sheet. It is stable at acidic as well as neutral pH and thermostable up to 70^{o}C with a Tm value of 71.59 ± 0.4^{o}C. Its primary structure is composed of 121 amino acids with 14 cysteine residues. The presence of His48-Asp49 pair revealed its calcium dependent catalytic nature. Daboxin P exhibited anticoagulant activity upstream of the common pathway (recalcification time, prothrombin time, activated partial thromboplastin time and stypven time) of blood coagulation in a dose dependent manner but did not inhibit the amidolytic activity of the serine proteases (FXIIa, FXIa, FXa, FIXa, FVIIa) involved in those pathways. However, it inhibited the activation of FX to FXa by the extrinsic and intrinsic tenase complexes both in the presence and absence of phospholipids as well as after alkylation with p-BPB. This suggests its anticoagulant activity by both enzymatic and non-enzymatic mechanism. Moreover, it inhibited the activity of the prothrombinase complex when pre-incubated with FXa followed by the addition of FVa. Fluorescence emission spectra and affinity chromatography study indicate the presence of protein-protein interaction of daboxin P with both FX and FXa. *In-silico* docking studies shows the interaction of the heavy chain of FXa (Thr132, Arg165, Lys169, Asn166, Leu170, Tyr225 and Arg125) with the Ca^{+2} binding loop (Trp31, Gly32, Gly33); helix C (Asp49, Tyr52, Gly53); anticoagulant region (Asn67) and C-terminal region (Phe124) of daboxin P. This is the first report of an anticoagulant PLA_2 enzyme, daboxin P (*Daboia russelii* FX inhibitor PLA_2 enzyme) from the Indian *Daboia russelii* venom which targets both FX and FXa for exhibiting its anticoagulant activity. Thus, this study unveiled one of the crucial pathological functions of a major anticoagulant protein during Russell's viper envenomation.