ABSTRACT

Green pit vipers comprise a large group of Asiatic venomous snakes having lance-head appearance and a typical bamboo-green coloured body. They are distributed from Indian subcontinent throughout south-east Asia, southern China and the Indo-Malayan to Philippine archipelagos. The venom of green pit viper is haemotoxic in nature, typically causing painful local swelling accompanied by haematological abnormalities such as incoagulable blood for several days and thrombocytopenia. In India, green pit vipers are distributed across the northern and north-eastern states of India extending to Sundarbans and Central India. Although envenomation reports are scarce in literature, partly because of morphological misidentification, the few available epidemiological data highlights the prevalence of Indian green pit vipers. Despite of the predominance of green pit vipers in the Indian subcontinent, there exists a dearth of knowledge regarding their venom composition and pharmacological profile, clinical features of envenomation and the causative toxins, venom variation among closely related species etc.

The only available antidote for the treatment of all the venomous bites including green pit viper bites in India is the polyvalent antivenom (PAV) which is raised against the “Big-four” snakes. However, inefficient neutralization efficacy of Indian polyvalent antivenom with heterologous venom as well as homologous venom from different geographical locations has been reported. Moreover, the antivenom therapy also presents other challenges such as anaphylactic reactions, serum sickness, inability to combat tissue damage etc. leading to significant morbidity in victims. The unavailability of specific antivenom for green pit vipers in India presents an urgent need to assess the effectiveness and toxin recognition profile of existing Indian polyvalent antivenom towards the venom of Indian green pit vipers.

In this study, an attempt was made to characterize the crude venom of Indian green pit vipers viz. *Trimeresurus erythrurus*, *T. septentrionalis*, *Popeia popeiorum* and *Viridovipera medoensis* in terms of venom composition and biochemical activities along with emphasizing the interspecific venom variation among them. Firstly, profiling of crude venoms was performed using a combination of SDS-PAGE and Rp-HPLC. The electrophoretic and chromatographic profiles revealed presence of multiple clusters of low to high molecular weight proteins which showed considerable variation in the

expression across the species. The crude venoms also showed differential activities in the tested *in vitro* enzymatic assays suggesting variation at the functional level. Crude venoms showed variable level of procoagulant activity, PLA_2 activity, thrombin-like activity, protease activity and haemolytic activity which indicates the presence of proteins targeting various components of haemostatic system of prey.

Further, the immuno-reactivity of Indian green pit vipers was evaluated with Indian polyvalent antivenom and compared with Thai green pit viper monovalent antivenom. Immuno-blotting experiments with Indian polyvalent antivenom showed consistent recognition of high molecular weight proteins and poor cross-reactivity with low molecular weight proteins in all the crude venoms. On the other hand, Thai green pit viper monovalent antivenom showed differential recognition of venom proteins across the species. Further, neutralization potential of antivenom was evaluated against various biochemical activities and the results displayed poor neutralization of thrombin-like activity and fibrinogenolytic activity by Indian polyvalent antivenom. Furthermore, the toxin recognition profile of Indian polyvalent antivenom towards Indian green pit vipers was assessed using immuno-depletion studies which demonstrated poor immuno-capturing efficacy of antivenom. Moreover, clinical features of green pit viper envenomation and their treatment protocol was recorded for 100 patients from Demow community health centre, Assam. The retrospective observational study confirmed the inefficacy of Indian polyvalent antivenoms available in hospitals in reversing the envenomation mediated pathology caused by Indian green pit viper bites. The experimental findings along with clinical data emphasizes the need for a suitable antivenom or an alternative of antivenom for the treatment of Indian green pit viper envenomated patients.

Furthermore, using a combination of chromatography and mass spectrometry approach, proteomics of *Trimeresurus erythrurus* was performed to explicate the detailed venom composition. Crude venom was firstly fractionated using Rp-HPLC into 10 peaks, each of which was digested by trypsin and subjected to LC-MS/MS. Analysis of MS/MS spectra revealed presence of 53 proteins/peptides belonging to 10 snake venom families viz. Phospholipase A_2 (PLA_2), snake venom serine protease (SVSPs), snake c-type lectin like proteins (Snaclecs), snake venom metalloprotease (SVMPs), l-amino acid oxidases, disintegrins, cysteine-like secretory proteins (CRiSPs), c-type lectins, bradykinin

potentiating c-type natriuretic peptides and glutaminyl-peptide cyclotransferases. PLA_2 (31%), SVSPs (22%) and snaclecs (19%) occupied the majority of proteome of *T. erythrurus*. The study reports the first proteome profile of *T. erythrurus* venom which was correlated with *in vitro* biochemical activities of crude venom and pathophysiological manifestations observed in the green pit vipers envenomated victims. Moreover, the proteome of *T. erythrurus* shows a very distinctive pattern of venom composition with PLA_2s as dominant family, unlike other green pit vipers of southeast Asia, thereby, providing additional information on inter-species venom variability.

The study was subsequently aimed towards characterizing a haemostatically active protein from *Trimeresurus erythrurus* venom. Using a two-step chromatographic method, erythrofibrase was purified to homogeneity from Rp-HPLC Peak 7. SDS-PAGE of erythrofibrase showed a single band of ~30 kDa in both reducing and non-reducing condition. The primary structure of erythrofibrase was determined by ESI LC-MS/MS and multiple sequence alignment depicted 77% sequence similarity with other snake venom thrombin-like enzymes (SVTLEs). Homology modelling showed typical β/β hydrolase fold similar to SVTLEs with 12 conserved cystein residues and His57, Asp102 and Ser206 as active site residues. Functionally, erythrofibrase showed direct fibrinogenolytic activity by degrading Aα chain of bovine fibrinogen, thereby causing hypofibrinogenemia and incoagulable blood for several days in envenomated patients. Further, erythrofibrase also showed dose dependent thrombin-like and plasmin-like activity along with mild plasminogen activation, fibrinolytic and haemolytic activity. Moreover, the inability of Indian polyvalent antivenom to neutralize the thrombin-like and plasmin-like activity of erythrofibrase can be correlated with clinical inefficacy of antivenom therapy. This is the first study reporting an α-fibrinogenase enzyme erythrofibrase from *T. erythrurus* venom which is crucial for the pathophysiological manifestations observed in envenomated victims.

Keywords: Green pit viper, Asian pit viper, *Trimeresurus*, Snakebite, Haemostatic system, Snake venom thrombin-like enzymes, Chromatography, Electrophoresis, mass spectrometry, Antivenomics, venom variation, Indian polyvalent antivenom, Immuno-reactivity, Antivenom efficacy, Antibodies, Proteomics, *Trimeresurus erythrurus*.