ABSTRACT

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Snake envenoming is an occupational health hazard that primarily affects rural agricultural workers in developing countries of Africa, Asia, Latin America, and Oceania. Recently (2017), the World Health Organization (WHO) has recognized snake envenoming as a neglected tropical disease. Conservative estimates suggest globally at least 421,000 snakebites occur annually and approximately 20,000 of these result in death. The estimations of snakebite in India are even more poignant, with approximately 81,000 envenoming per year resulting in 11,000 deaths; these numbers are the highest for any country.

Russell's Viper (*Daboia russelii*) (RV) is a category I medically important snake that is responsible for a heavy toll of mortality in the India sub-continent. RV venom (RVV) is hemotoxic in nature and causes haemostatic disturbances that result in consumption coagulopathy and incoagulable blood. Notably, apart from these common clinical symptoms, RV bite patients from Sri Lanka, southern India (SI), and to some extents, western India (WI) exhibits neurotoxic symptoms. These differences and severity of neurotoxicity are associated with variation in RVV composition due to geographic location of this snake.

Administration of antivenom is the only choice of treatment against snake envenomation. Due to the frequency and severity of envenomation by the 'Big Four' snakes (Indian Cobra, Common Krait, Russell's Viper, and Saw-scaled Viper), Indian polyvalent antivenom (PAV) is raised against a cocktail of venoms of these four species. However, safety and efficacy of equine antivenom are of immense concern for successful hospital management of bite victims.

For easy understanding, this thesis is divided into following six chapters -

Chapter I - This chapter provides an introduction to the global burden of snakebite including the Indian scenario, introduction of Russell's Viper, clinical manifestations of RV bite, antivenoms for the treatment of snake envenoming, and proteomic tools to study venom proteomes. The aims and objectives of the study are described in this chapter.

Chapter II - This chapter reviews the published literature on snake venom proteomics and antivenomics with special reference to Russell's Viper from different parts of the Indian sub-continent and its neighbouring country Myanmar.

Chapter III - This chapter enlists the materials used in the study and the methods employed for performing various experiments.

Chapter IV and V - These two chapters include results and discussion.

Chapter IV - This chapter describes the proteomic characterization of RVV from three different geographical locations - eastern India, western India, and southern India. Further, the correlation between RVV composition and clinical manifestation of RV-envenomation is also discussed. This chapter also focuses on the immunological characterization of different RVV samples against commercial antivenom by using the techniques of ELISA, western blot, neutralization assays, and antivenomics studies.

Chapter V - This chapter deals with the purification and characterization of a novel apyrase (Ruviapyrase) enzyme from Indian RVV.

Chapter VI - This chapter includes the major findings of this work and contemplates the future perspectives of the findings of the present study.

In the present study, Russell's Viper venom proteomes from three different geographical locations of India were de-complexed using multidimensional chromatography and SDS-PAGE followed by trypsin digestion and subsequent mass spectrometry analysis. ESI-LC-MS/MS analysis of the gel filtration and ion-exchange chromatographic fractions or SDS-PAGE protein bands of the RVV samples against Viperidae protein entries of the NCBI database led to the identification of 55, 66, 73, and 69 proteins in western India (WI) RVV, southern India (SI) RVV, eastern India RVV from Burdwan [EI RVV (B)], and Nadia [EI RVV (N)], respectively. These proteins were clustered into 14 to 15 enzymatic and non-enzymatic protein classes. Phospholipase A_2 (PLA_2) was the most abundant enzymatic component of all the RVV samples, whereas Kunitz-type serine protease inhibitor (KSPI) and snaclec were most abundant non-enzymatic components of WI and EI RVV (B and N), and SI RVV, respectively. A comparison of these RVV proteomes revealed significant differences in the number of toxin isoforms and relative toxin abundances, highlighting the impact of geographic location on RVV composition.

The enzymatic activities exhibited by WI, EI, and SI RVV were consistent with the proteomic analyses of the RVV samples. RVV samples from all the three regions of India were found to be pro-coagulant under *in vitro* conditions, the potency being dictated by the distribution and abundance of snake venom metalloprotease (SVMP)

and snake venom serine protease (SVSP), more specifically, RVV-X and RVV-V. It induced indirect hemolysis of erythrocytes, aggregation of platelets and reduction in platelet count (thrombocytopenia). The variation in pharmacological properties exhibited by RVV from different parts of India was also well corroborated by the proteomic findings.

The common clinical features of RV bites, such as rapid swelling and extreme pain of the bitten body part, local ecchymosis and hemorrhage, and intense blebs over the affected extremities are primarily caused by the abundant SVMPs in RVV. Largely due to the activities of SVSPs, RVV initially affects the vascular system by provoking hemostatic disturbances, including rapid thrombosis and hypofibrinogenemia that ultimately results in consumptive coagulopathy and incoagulable blood. Subsequently, abundant anti-coagulant RVV proteins such as PLA_2s, KSPIs, and snaclecs exert anti-coagulant action by inhibiting various blood factors to promote incoagulable blood. Notably, RV bite patients from SI, Sri Lanka (SL), and occasionally from WI are also reported with neuroparalytic symptoms. These differences in severity of neurological symptoms can be explained on the basis of variation in the relative abundances of neurotoxic PLA_2 isoforms in RVV samples from SL (>30%), SI (15.7%), and WI (3.2%).

ELISA and Western blotting (WB) analyses of WI, EI, and SI crude RVV and/or gel filtrations fractions unequivocally pointed out the poor immunogenicity of low molecular mass components (<20 kDa) of these venoms. Using an antivenomics approach, the major PAV unbound toxins in WI, EI, and SI RVV were identified as PLA_2 and KSPI. In addition, PAVs also lacked sufficient antibodies against proteases (SVMP and SVSP) of EI RVV. The above poorly immunogenic components are hemostatically active toxins in RVV that play a pivotal role in RVV-induced toxicity and exhibit diverse pharmacological effects in bite victim. Therefore, poor recognition of these RVV toxins by commercial PAVs is likely the cause of poorly effective antivenom therapy.

The neutralization potency of PAVs toward various properties of WI, EI and SI RVV were found to vary significantly. The enzymatic activity and pharmacological properties of SI RVV were neutralized by PAVs with exceptions of PLA_2 and indirect hemolytic activity, while the PAVs were extremely poor in neutralizing several

enzymes such as fibrin(ogen)olytic, TAME, BAEE, and pro-coagulant properties of WI and EI RVV.

In this study, a high molecular weight (~79.4 kDa) apyrase enzyme was purified from WI RVV by using multi-dimensional chromatography technique. The enzyme was named Ruviapyrase (Russell's Viper apyrase). The presence of a putative conserved domain of apyrase superfamily in Ruviapyrase suggested that the purified enzyme belongs to apyrase family (EC 3.6.1.5). Ruviapyrase demonstrated dose-dependent preferential hydrolysis of ATP over ADP with a ~1.6 fold higher specific activity towards the former substrate. Transient stability of Ruviapyrase under physiological conditions indicated that this class of enzymes may have evolved in snake venoms to rapidly accomplish its task of depletion of their cellular harmony (ATP). The Michaelis-Menten constant (*Km*) of Ruviapyrase against ATP and ADP were calculated at 2.5 and 5.8 μM, respectively and the *Vmax* values of ATP and ADP hydrolysis by Ruviapyrase were determined at 615.0 and 372.8 μM of Pi released min per min, respectively. Further, Ruviapyrase contained neutral carbohydrates and significant amounts of N-linked oligosaccharide.