CHAPTER I

INTRODUCTION

1.1 Introduction

Snakes, also called serpents, are a class of limbless reptiles that are grouped under the suborder Serpentes. They are believed to have diverged from terrestrial lizards (order Squamata) in the Cretaceous period which dates back to 70 to 140 million years [1], and they flourished in the Neogene period, which is appropriately known as the 'Age of Snakes' [2]. On the basis of their ability to produce a highly specialized toxic secretion known as venom, snakes are broadly classified into two categories, venomous and non-venomous. There are nearly 3000 species of snakes distributed throughout the globe with exceptions to Antarctica and several islands, including Greenland, Iceland, and Ireland, and among them only one-fifth are venomous [3,4].

Every venomous organism, including snakes, are equipped with venom glands whose primary function is the synthesis and storage of a suite of toxins for instant availability and to provide protection to the snake itself against its own toxins [5-7]. Snake venom contains a myriad of proteins and peptides that have accelerated through positive Darwinian evolution to aid in immobilizing, killing, and digesting prey [8-11]. Bestowed with high affinity and selectivity, these zootoxins primarily affect the cardiovascular and neuromuscular systems, and blood coagulation of prey or victim [12-18]. Nevertheless, this spectrum of medically important toxins, with meticulous characterization, also holds good promise as candidate lifesaving drugs [18-20].

India has a unique relationship with serpents, which are both respected and feared. This country is the home to more than 250 species of snakes, out of which about 60 are highly venomous [21,22]. Among the venomous species, the 'Big Four' snakes namely Russell's Viper (*Daboia russelii*) (RV), Spectacled Cobra (*Naja naja*), Common Krait (*Bungarus caeruleus*), and Saw-scaled Viper (*Echis carinatus*) are distributed throughout the country and are responsible for most cases of envenoming, morbidity and mortality [23,24]. Apart from these deadly species, Monocelleate Cobra (*N. kaouthia*), Wall's Krait (*B. walli*), Sind Krait (*B. sindanus*), King Cobra (*Ophiophagus hannah*), and several species of Pit Vipers (*Hypnale hypnale*, *Protobothrops* spp.) also inhabit in different parts of the country and can cause fatalities albeit incidence of snakebite by these species of snakes, except *N. kaouthia*, is much less compared to Big Four venomous snakes [14,25-27].

1.2 Families of venomous snakes

Among the 3000 species of snakes distributed worldwide, about 600 species are known to be venomous and are classified under the four families namely Colubridae, Viperidae, Elapidae and Atractaspididae [4,6,28]. The members of Colubridae, the largest snake family with almost 1,800 species, are distributed throughout the globe [6,28-30]. It is further sub-divided into 7 subfamilies that includes several medically relevant species such as the Boomslang (*Dispholidus typus*), the Red-necked Keel-back (*Rhabdophis subminiatus*), and Brown Treesnake (*Boiga irregularis*) [31,32]. The family Viperidae with around 310 species is classified into three subfamilies - Azemiopinae (with only one recognized member, *Azemiops feae*), Viperinae (true Vipers belonging to genus *Bitis, Cerastes, Echis,* and *Daboia*), and Crotalinae (Pit Vipers belonging to *Crotalus, Agkistrodon,* and *Bothrops* genera). These venomous snakes are primarily terrestrial and widely distributed in Asia, Africa, Europe, and America [4,31,32]. About 350 species of the Elapidae family are distributed throughout Africa, America, Middle East, Asia, and Australasia. The family is further sub-divided into two sub-families - Hydrophiinae (sea snakes belonging to genus *Laticauda*, *Enhydrina, Hydrophis,* and *Microcephalophis*) and Elapinae (belonging to genus *Naja*, *Bungarus*, *Dendroaspis*, *Oxyuranus*, *Calliophis*, and *Micrurus*) [31,32]. The members of the family Atractaspididae are distributed in Africa and Middle-east and contain only a few medically important snakes including burrowing asp (*Atractaspis engaddensis*), and southern African stiletto (*Atractaspis bibronii*) [32,33].

Notably, members of all the four families of snakes produce venoms containing an arsenal of toxin classes, several of which share significant sequence homology across the family. However, with marked differences in amino acid sequence and relative toxin abundance, venoms from each family exhibit distinguishing biological properties [4,28,34]. For example, Viperidae venoms are usually rich in enzymatic proteins that affect the haemostatic system of victims or prey [13,35-38]. On the contrary, snakes from the Elapidae family produce venoms that contain high quantity of neurotoxic proteins [39-43]. Further, Atractaspididae venoms harbour several peptide toxins that affect the blood vascular system [4,44], and Colubridae venoms exhibit activities similar to both Viperidae and Elapidae snakes [30,45,46].

1.3 The global burden of snake envenomation: a neglected tropical disease

Snake envenoming is an occupational health hazard that primarily affects the rural agricultural workers in developing countries of Africa, Asia, Latin America, and Oceania [47]. Snakebite victims in these regions are inflicted with high morbidity and mortality rate due to poor access to health services, and in many instances because of the scarcity of antivenom, the only choice of treatment for snakebite [27,48]. Regardless of its physical, psychological and socio-economic impacts, the problem of snakebite has received scant attention from regional and national health authorities as well as research funding agencies throughout the world. Recently (March, 2018), the World Health Organization has recognized snake envenoming as a neglected tropical disease [49] and subsequently, interests in understanding and addressing this grave problem have gradually emerged among health authorities, non-governmental organizations, and several antivenom manufacturers and research groups throughout the globe.

A recent global epidemiological study on snake envenoming conducted in 227 countries by Kasturiratne and her co-workers reported that globally at least 4, 21,000 snakebites occur annually and approximately 20,000 envenoming results in deaths [50]. However, these estimates can be as high as 1, 841,000 envenomings and 94,000 deaths [50]. The highest burden of snake envenoming was found in south and southeast Asia, and sub-Saharan Africa (Fig. 1.1) with conservative estimates of 121,000, 111,000, and 43,000, respectively, while lower incidences of envenomings were estimated for central Europe and central Asia (100 to 200 cases). Further, the data suggested that highest number of deaths occur in South Asia (14,000), West sub-Saharan Africa (1,500), East sub-Saharan Africa (1,400), and Southeast Asia (790). In addition, country-wise distribution of snakebite suggests that highest incidences are reported from India (81,000), followed by Sri Lanka (33,000), Vietnam (30,000), Brazil (30,000), Mexico (28,000), and Nepal (20,000) [50]. Nevertheless, accurate numbers on snake envenoming and death are difficult to estimate due to inadequate reporting and record-keeping [27,50] (Mukherjee, A.K., unpublished data). Therefore, given the grave scenario of snake envenomation around the globe, the paucity of reliable snakebite data is both astonishing and alarming. Methods for reporting and record-keeping on morbidity and mortality due to snake envenomation in health facilities should be standardized and optimized in order to address and tackle this serious problem [50,51] (Mukherjee, A. K., unpublished data).

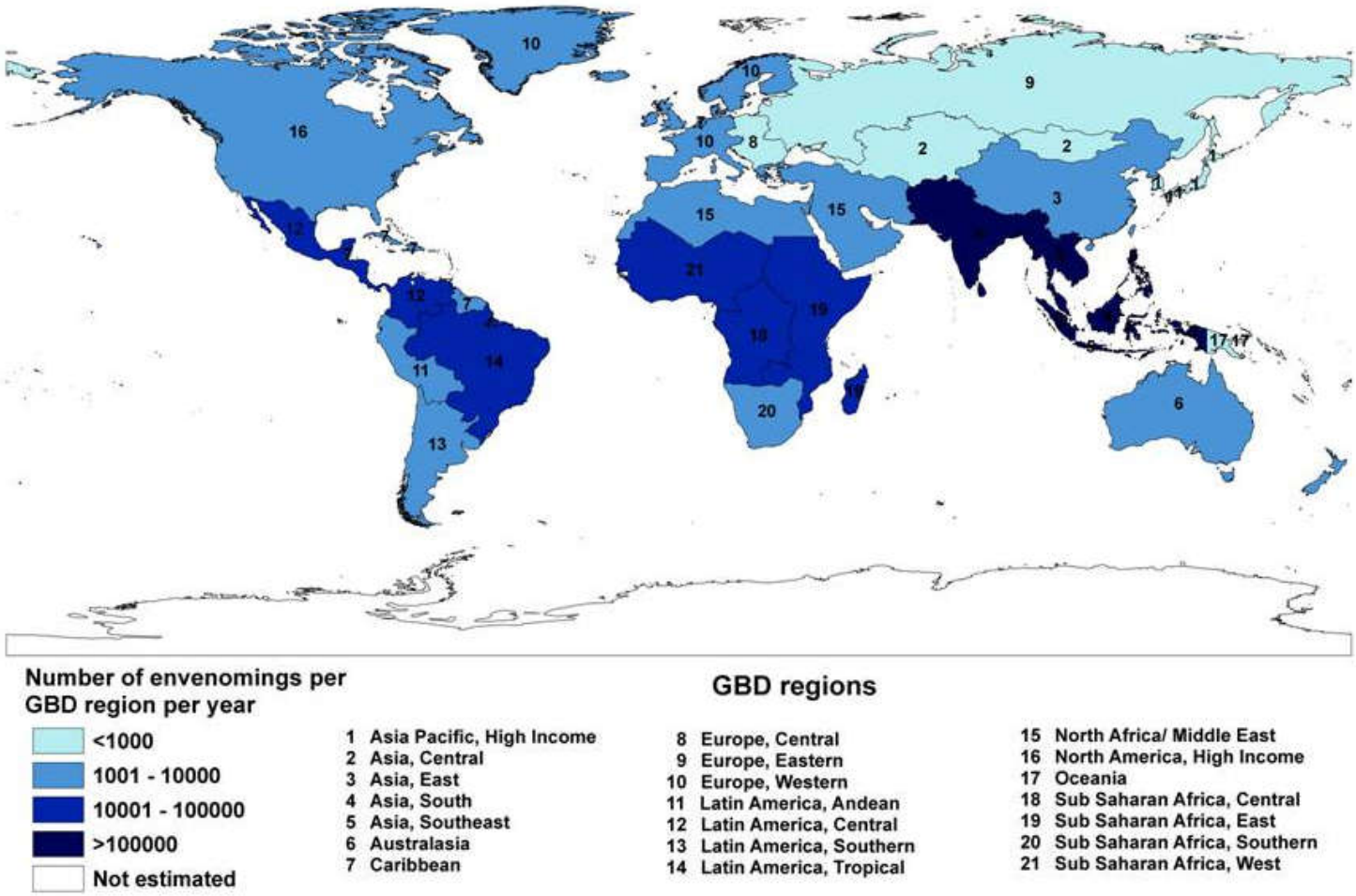

Fig. 1.1. Regional estimates of envenomings due to snakebite (low estimate) (adapted from Kasturiratne et al. [50]).

1.4 Epidemiology of snakebite in India

India is reported with the highest incidence of snake envenoming (81,000) as well as mortality (11,000) [50]. Mortality data from the Million Death Study (2001-03) conducted by the Registrar General of India and the Centre for Global Health Research provided the first ever direct estimate of 45,900 annual snakebite deaths in India [22].

The majority of snakebite deaths were reported from the rural areas (97%) during the monsoon months of June to September, were more frequent in males (59%) than females (41%), and highest in the age group of 15–29 years (25%) [22]. Further, annual mortality was highest in the state of Uttar Pradesh (8,700) followed by Andhra Pradesh (5,200) and Bihar (4,500) (Fig. 1.2). Other Indian states that were also inflicted with significant snakebite mortalities were Madhya Pradesh (4,000), Maharashtra (3,200) and West Bengal (3,000). However, it is very unfortunate that there is no snakebite mortality and morbidity data in the northernmost and north-eastern states of the country which are inhabited by several venomous snake species (Fig. 1.2).

In Asia, RV bites account for a heavy toll of mortality; envenomation by which is responsible for 70% and 40% of snakebite incidences in Myanmar and Sri Lanka, respectively. While in western India (WI) these numbers are 20.8%, the highest incidence of RV envenomation has been recorded in eastern India (EI) [13,52,53]. Nevertheless, snakebite data in India are fragmentary because less than 40% of snakebite patients attend to hospitals [52]. Regardless, the Russell's Viper is a major source of snakebites and is considered as a category-I medically important snake in the Indian subcontinent. Further, occurrence and mortality is only part of the picture: a significant (but unknown) number of envenomings result in permanent morbidities, such as loss of limbs, digits and/or function, and these effects can debilitate a victim for life.

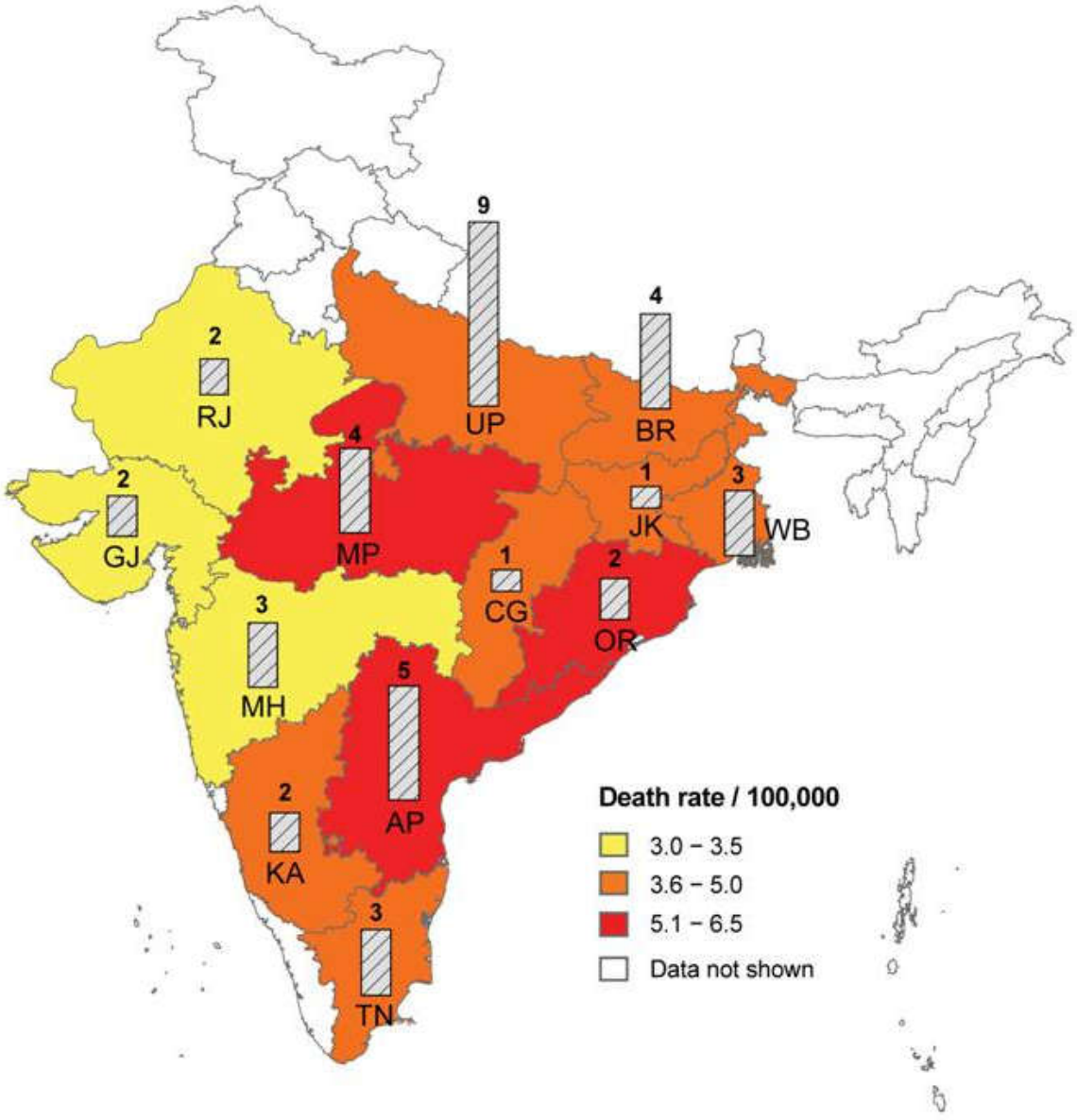

Fig. 1.2. Estimated deaths and standardized death rates in Indian states with high prevalence of snakebite mortality (adapted from Mohapatra et al. [22]). The vertical bars represent the state wise estimated deaths (in thousands). AP-Andhra Pradesh, BR-Bihar, CG-Chhattisgarh, GJ-Gujarat, JH-Jharkhand, KA-Karnataka, MP-Madhya Pradesh, MH-Maharashtra, OR-Orissa, RJ- Rajasthan, TN-Tamil Nadu, UP-Uttar Pradesh, WB-West Bengal.

1.5 The Indian RV (*Daboia russelii*) - a category I medically important snake

1.5.1 Systemic classification of RV

The following is the systemic classification of RV (*D. russelli*).

Kingdom: Animalia

Subkingdom: Bilateria

Infrakingdom: Deuterostomia

Phylum: Chordata

Subphylum: Vertebrata

Infraphylum: Gnathostomata

Superclass: Tetrapoda

Class: Reptilia

Order: Squamata

Suborder: Serpentes

Infraorder: Alethinophidia

Family: Viperidae

Subfamily: Viperinae

Genus: *Daboia*

Species: *Daboia russelii*

1.5.2 The distinctive features of RV

RV, commonly known as 'Daboia' (in Hindi) or 'Chain viper', is named after Dr. Patrick Russell, a Scottish surgeon and naturalist who described many of the Indian snakes. The genus name '*Daboia'* is adapted from a Hindi word that translates to 'that lies hidden' or 'the lurker'. The size of this snake varies from medium to large; RV can grow up to 180 cm and averages about 100 cm with a flattened and triangular head which is distinct from the neck and its snout blunt, and rounded (Fig. 1.3). The scales are keeled; several distinctive bright chain patterns, the body color is typically yellowish to brown and the pattern is composed of dark, round spots black and white edges which are important features helpful for their easy recognition [21,24]. The underside is white in the western India, partly dotted in the south-eastern India and deeply spotted in the north-eastern race of this species of snake.

Adults are usually slow and sluggish unless pushed beyond a certain limit, while the juveniles are very active [24,54].

Fig. 1.3. Photographs of adult Russell's Viper. Photographs by A.K. Mukherjee, copyright 2017.

1.5.3 Habitat, geographic distribution, and classification of RV

RV is found all over Asia and is distributed throughout the Indian subcontinent, to a great extent of Southeast Asia including Myanmar, Southern China, and Taiwan [55,56]. It is found up to 2756 m (9040 ft) above sea level [21,24]. It is abundant in southern, western and eastern States of India but very rare in the Ganges valley, Northern Bengal and Assam (Fig. 1.4). Generally, distribution of RV is not restricted to any particular type of habitat; however, it has a tendency to avoid intense forests. Its favourite habitat is the vicinity of the farmlands and rice fields where its prey such as rats and mice thrive. That is the reason why rice farmers are the major victims of RV [13]. They primarily feed on rodents, especially murid species and mostly inhabit open, grassy or bushy areas, but may also be encountered in forested plantations and farmland [24,54].

RV is classified into 5 sub-species based on the differences of coloration and markings, *Daboia russelii russelii* (India, Pakistan, Nepal, and Bangladesh), *Daboia russelii pulchella* (Sri Lanka), *Daboia russelii siamensis* (Thailand, Myanmar and China), *Daboia russelii formosensis* (Taiwan), and *Daboia russlii limitis* (Indonesia) [57]. However, based on morphological characteristics and mitochondrial DNA analysis, Thorpe et al. [58] classified RV into two species - *D. russelii* inhabiting the Indian sub-continent (Fig. 1.4) and *D. siamensis* endemic to parts of southeast Asia (other than the Indian subcontinent), southern

China, Indonesia, and Taiwan. In addition, Tsai et al. [59] reported the presence of two types of Russell's Viper based on the occurrence of either Asparagine (Asn, N) or Serine (Ser, S) at the N-terminus of the phospholipase A_2 (PLA_2) isoenzymes. The venoms of *D. r. formosensis* (Taiwan), *D. r. siamensis* (Thailand and Burma) and *D. r. russelii* (Pakistan) containing PLA_2s having an Asn residue at the N-terminus were classified under one type, while *D. r. pulchella* (southern India and Sri Lanka) represents the other type, whose venom contains PLA_2s with an N-terminal Ser residue [59].

Fig. 1.4. General distribution of the RV (*Daboia russelii*) on the Indian sub-continent (adapted with permission from ©World Health Organization, 2009; retrieved from http://apps.who.int/bloodproducts/snakeantivenoms/database/Images/SnakesDistribution/Large/map_Daboia_russelii.pdf).

1.5.4 Venom delivery apparatus in RV

The venom delivery apparatus of venomous snakes is comprised of a primary venom gland, a duct with an accessory gland, and fangs for venom delivery. Venom glands are modified parotid salivary gland which consist of three major cell types - basal cells, conical mitochondria-rich cells, and secretory cells, which produce the venom [60]. Venom flows from the venom gland to the fangs via a duct and an accessory gland. It has been reported that

the accessory gland is under voluntary control and snakes can and do control the amount of venom that is released during envenomation [60]. Nevertheless, the precise function of the accessory gland was debatable and believed to play a judicious role to prevent extraneous loss of toxin secretions [60]. However, a recent proteomic study has demonstrated that *Bothrops jararaca* accessory venom gland is not likely an important but an ancillary source of toxins such as phospholipase A_2, C-type lectin-like proteins, metalloprotease, serine protease, nerve growth factor, vascular endothelial growth factor, cysteine-rich secretory protein, and L-amino acid oxidase [61].

The venom delivery system of venomous snakes is divided into two categories - rear-fanged and front-fanged envenomation systems [62,63]. The rear-fanged (opisthoglyph) is found in most of the members of the Colubridae family [28]. The front-fanged system is further classified into two types - the proteroglyph system found in Elapidae snakes and the solenoglyph system present in members of the Viperidae family (Fig. 1.5). In the solenoglyph system as in the case of RV, the fangs are tubular, long and positioned on a short and highly movable maxilla. Further, the compressor muscles that originate distant to the ventral side of the braincase aids the movements of the palato-maxillary arches during a strike [64]. This particular design of the Viperid venom delivery system enables efficient venom delivery upon strike, thereby injecting large quantities of venom into the victim or prey.

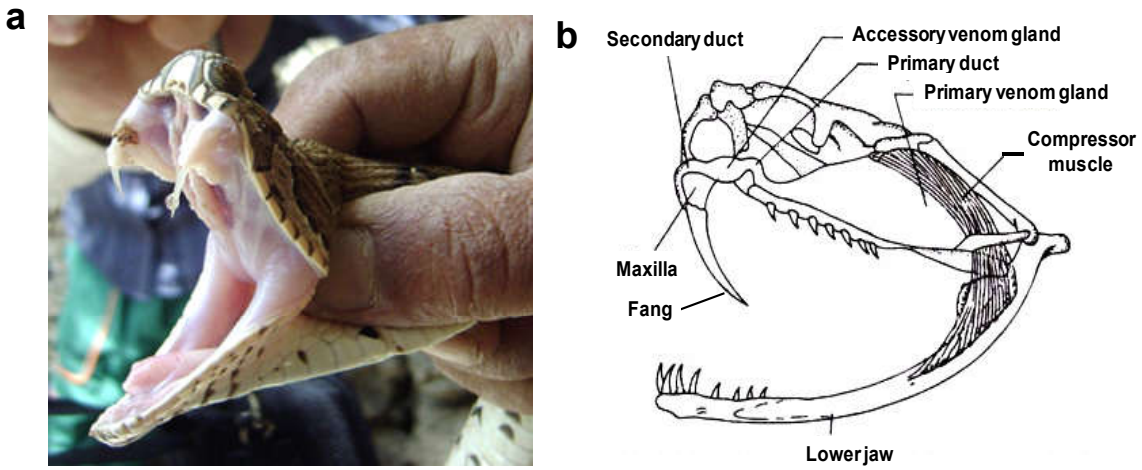

Fig. 1.5a. A live specimen of Russell's Viper with venom oozing out of its fangs (Photo © Usman Ahmad, 2009). **b.** Position of the venom gland with the accessory gland and their primary and secondary ducts, and fang in a Viperid snake (adapted from Deufela and Cundall [64]).

More than 90% of RV venom (RVV) is comprised of proteins and polypeptides, and it possesses a characteristic yellow color due to the presence of FAD, a cofactor of the L-amino acid oxidase enzymes. The pH of an aqueous solution of RVV is acidic (~5.8), while its specific gravity ranges from 1.03 to 1.07 [65,66].

1.5.5 Pathophysiology and clinical manifestations of RV envenomation

An adult RV possesses approximately 200-225 mg of venom in its glands, and so bites to prey or a victim can result in large amounts of venom being injected [67]. The LD_{50} values of RVV (in mice) ranges from 0.7 (*i.v.*) to 10 mg/kg (*i.p.*) depending upon the geographic source of the venom; the EI RVV being the most lethal [13,68]. At the onset of RV envenomation, victims experience extreme pain at the bite site, followed by rapid swelling, local ecchymosis, and intense blebs over the affected extremities [13,53,69,70]. Similar to other Viperid venoms, RVV is also hemotoxic in nature and affects the blood vascular system by provoking haemostatic disturbances, including rapid thrombosis and hypofibrinogenemia that ultimately results in consumption coagulopathy and incoagulable blood [13,53]. In addition to haemostatic disturbance, RV envenomed patients also manifests necrosis, blistering, capillary permeability, hypotension, and systemic bleeding including bleeding from gums, hematuria, and haemoptysis [13]. Further, acute renal failure (ARF) is a persistent clinical symptom of RV envenomation, and the management of RV-induced nephrotoxicity is a serious concern. Patients must undergo repeated dialysis, and antivenom therapy fails to mitigate the problem of nephrotoxicity in RV bite patients [71]. Notably, apart from these common clinical symptoms, RV bite patients from Sri Lanka, southern India, and to some extents, western India exhibits neurotoxic symptoms. Notably, clinical reports on RV envenomation in Sri Lanka clearly suggest the limited efficacy of Indian antivenom in reversing the neuro-myotoxic symptoms and systemic poisoning [72]. These differences in severity of neurotoxicity as well as antivenom efficacy are associated with variation in RVV composition due to geographic location.

1.6 Mass spectrometry - an integral tool in the field of snake venom proteomics and antivenomics

For several decades mass spectrometry was confined to the analysis of small and thermostable molecules due to the dearth of technological advancements in soft ionization and conversion of ionized molecules in condensed phase to gaseous phase with minimal

fragmentation [73,74]. However, the developments of matrix-assisted laser desorption/ionization (MALDI) [75] and electrospray ionization (ESI) [76] techniques in the late 1980s could overcome the constraints of ionization and made proteins accessible to mass spectrometry analysis. Since then, mass spectrometry has been an integral component in biological research and has evolved substantially to tackle the challenges presented by protein and proteome analysis.

Mass spectrometers are fascinating instruments that accurately measure the molecular mass of a protein and provide structural information such as amino acid sequence or the type of post-translational modifications (PTM), which ultimately results in the identity of the target protein. In the former case, intact proteins are ionized and analyzed, usually in a single-stage mass spectrometer, for determination of its molecular mass. On the contrary, for determination of amino acid sequence and PTM, the protein is first digested with a proteolytic enzyme, preferably trypsin that cleaves specifically at the C-terminal of lysine and arginine amino acid residues, to generate peptide fragments [77]. Thereafter, these tryptic fragments are ionized and analyzed in a multi-stage mass spectrometer. As depicted in Fig. 1.6, a typical mass spectrometer consists of three parts:

1. Ion source: that generates gaseous ions from the protein or the tryptic fragments being studied.

2. Mass analyzer: that resolves the generated ions according to their characteristics mass-to-charge ratio (m/z).

3. Detector: that detects the ions and records the relative abundance of the resolved ionic species.

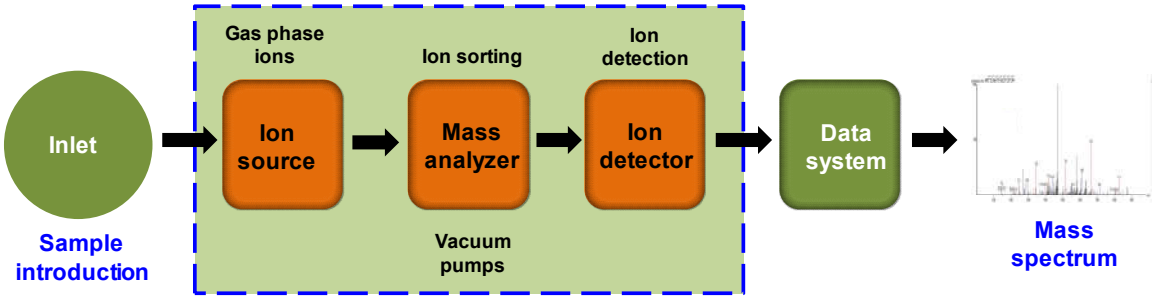

Fig. 1.6. A schematic diagram of a typical mass spectrometer.

Although several types of ion sources are used in mass spectrometry, nevertheless, soft ionization techniques such as matrix-assisted laser desorption/ionization (MALDI) and

electrospray ionization (ESI) are primarily opted for analyzing protein samples [74]. Typically, mass analyzers use either static or dynamic fields, and electric or magnetic fields; however, in mass spectrometers designed specifically for performing tandem mass spectrometry (MS/MS), two or more mass analyzers can also be fused. In biological research, mass spectrometers are generally equipped with time-of-flight, quadrupole mass filter, linear quadrupole ion-trap, orbitrap and hybrid (combination of two or more analyzers) mass analyzers [73,74]. The schematic diagram of an LTQ orbitrap hybrid mass spectrometer is shown in Fig. 1.7.

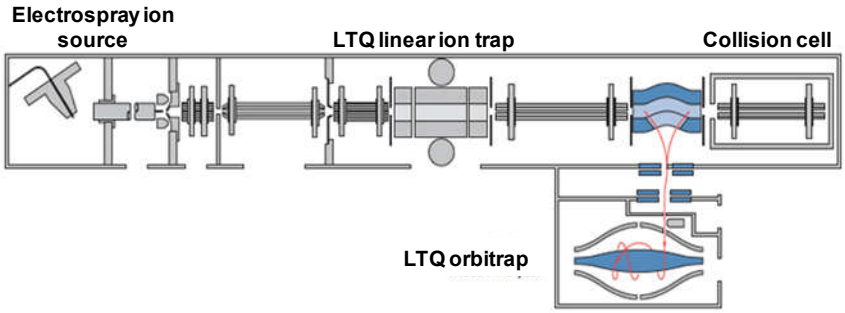

Fig. 1.7. Schematic diagram of an LTQ hybrid mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) containing linear ion trap and orbitrap mass analyzers (adapted from Gross [78]).

Detector, the final element of the mass spectrometer, is usually an electron multiplier. Nonetheless, other detectors such as ion-to-photon and Faraday cup detectors are also used. When an electron hits the surface of the detector, it records the charge or the current produced in the event and subsequently converts it into a mass spectrum, representing ions as a function of m/z [79]. Thereafter, the raw MS/MS data recorded by the mass spectrometer is fed into database search engines, for example, MASCOT, PEAKS, Proteome Discoverer, and Morpheus, to deconvolute the mass spectra and search the existing databases for establishing protein identity [80-83].

From the inception of modern snake venom research, which deals with intensive biochemical studies of venoms and dates back to the late 50's [84], it was known that venom toxin arsenal comprises of several proteins, polypeptides and proteinaceous components [85]. With the advent of biochemical assays and protein separation techniques the venom complexity was gradually fathomed [13,14,68,86-89]. However, this approach has a major limitation of identification and quantification of the non-enzymatic snake venom

components. Nevertheless, with the recent advancements in the field of mass spectrometry, robust database search algorithms, and powerful venom de-complexing strategies, proteomic studies have shed light on several snake venom proteomes. This vast array of data has helped to understand the complex organization as well as the pathophysiological role of snake venom components [4,90,91]. Further, the application of proteomic tools for identification of the poorly immunogenic venom toxins (antivenomics) has provided valuable and relevant information for the development of potent antivenoms [38,92,93].

1.7 Apyrase, a neglected class of nucleotide hydrolytic enzyme of snake venoms

RVV comprises a myriad of different enzymatic and non-enzymatic proteins [35,37] which primarily targets the blood vascular system of victim or prey [13,68,94]. However, the presence of enzymes in snake venoms which are acclaimed to cause distinct adverse effects other than the haemostatic imbalance is also reported [35,37]. Examples are nucleotidases responsible for catalyzing the hydrolysis of adenosine triphosphate (ATP), adenosine diphosphate (ADP) and adenosine monophosphate (AMP), termed as ATPase, ADPase and AMPase, respectively, to release inorganic phosphates [87,95]. Several nucleotidases of other than snake venom origin termed apyrase, can cleave both ATP as well as ADP in a decreasing order of preference. The apyrase, a Ca^{2+}-activated enzyme, sequentially catalyzes ATP to ADP and then ADP is further catalyzed to AMP [96]. Both ATPase and ADPase play important roles in many biological processes including the modulation of neural cell activities [97], prevention of intravascular thrombosis [96,98], and regulation of immune responses [99].

Purines, a class of multi-toxin plays a pivotal role in prey immobilization by inducing hypotension and vascular permeability via the activation of A1 (neuronal), A2 (vascular smooth muscle) and A3 (mast cell) receptors [100,101]. These multi-toxins are integral components of snake venoms [102] or are generated by hydrolysis of ATP, ADP and AMP by venom 5'-nucleotidase, endonucleases, ATPase, ADPase, apyrase, phosphodiesterase, phosphomonoesterase, and NADase [100,101,103]. Although biochemical analysis has unambiguously indicated the occurrence of ATPase and/or ADPase enzyme(s) in numerous snake venoms [13,37,104], there were no reports on purification and characterization of ATPase, ADPase or apyrase enzyme from snake venoms, hitherto they remained as an untapped RVV component. Their low abundance in venom, transient stability, and sensitivity

to denaturing agents are the major bottlenecks that have been encountered in the isolation and detailed biochemical and pharmacological characterization of apyrases and nucleotidases [96]. Therefore, knowledge of such enzymes from RVV will contribute to our understanding about the rationale of their presence in RVV as well as their role in the pathophysiology of RV bite.

1.8 Gap in the study

A few studies have provided valuable information on RVV composition and its variation across different geographic locales of India [13,68]. However, these studies were primarily based on biochemical analysis of RVV; therefore, they were associated with the major limitation of identification and quantification of the non-enzymatic components of RVV. In addition, several proteins and peptides belonging to diverse snake venom protein families were also purified and characterized extensively from India RVV [16,17,94,105-114]. Nevertheless, a comprehensive analysis of the venom proteome of RV from different geographic location of India by proteomic approach was pending. Further, assessment of neutralization potency and immunological cross-reactivity of commercial antivenom towards RVV and identification of poorly immunogenic toxins of RVV (antivenomics) from different regions of India was also not attempted. This is a very important prerequisite for assessing the potency of commercial polyvalent antivenom manufactured in one part of the country to treat the snake envenomation in another part of the country.

1.9 Aim and objectives of the present study:

The present study has been designed to investigate the venom proteomes of RV from different parts of India by proteomic analysis. Further, we tried to correlate the venom composition with the differences and severity of clinical manifestations post RV envenomation in different parts of the country. The present study also aims to assess the immunological profiles of commercial PAVs against the RVV samples. In addition, isolation, purification, and biochemical and pharmacological characterization of an apyrase enzyme from RVV were also undertaken in this study. Accordingly, the following objectives were set for the present investigation:

1. Proteomic analysis of Russell's Viper venom (RVV) from three different geographical locations of India.

2. Correlation of RVV proteomics data with biochemical and pharmacological properties of RVV and clinical manifestations of RV bite.

3. Determination of the degree of immunological cross-reactivity between RVV of different geographical location of India with commercial polyvalent / monovalent antivenom (antivenomics).

4. Biochemical and pharmacological characterization of an apyrase enzyme from RVV.

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