CHAPTER 1

General Introduction and Review of Literature

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1.1. Snakebite: A global perspective

Snake envenomation is a life-threatening public health hazard which is included in Category A of "neglected tropical diseases" by World Health Organization (WHO) [1]. Like other neglected tropical diseases, snake envenomation mostly affects the rural population of tropical and sub-tropical underdeveloped countries causing significant morbidity and mortality [2, 3], however, the number of deaths due to snakebite is much higher than other neglected tropical diseases depicting the severity of snakebite problems [3]. According to a report by Kasturiratne et al. in 2008, an upper estimate of about 1.8 million envenoming occur globally due to snakebite with about 94000 deaths per year [4]. The highest burden of snakebite is faced by rural communities of South and Southeast Asia, sub-Saharan Africa & Central and South America [4, 5]. Poor and underprivilege groups, mostly agriculture practitioners working in crop fields, tea planters, fisherman, cattle herders, people living in poorly constructed houses etc. face utmost risk of encountering a snakebite making it an occupational hazard and a "disease of poverty" [6]. Poor governance, political and/or social conflict, economic instability, resource scarcity, poor healthcare facility, incompetent healthcare workers etc. altogether impose severe socio-economic burden to the snakebite victim and their family [6, 7]. As a consequence, World Health Organization (WHO) has recognized snakebite issue with high priority and collaborated with various researchers, medical fraternity, government and private stakeholders towards an integrative approach to achieve a global target to halve the number of deaths and disability due to snakebite envenomation by 2030 [8]. Given the scenario, it is crucial to improve access to appropriate medical treatment and preventive measures, along with facilitating state-of-the-art venom research to gain deeper insights on venom dynamics which would ultimately aid in the better management of snakebite.

1.2. Snakebite in India

India, being an agriculture-based tropical country, incidences of snakebite have large impact on rural communities. Global epidemiological data for snakebite envenomation reports about 35000 to 50000 deaths every year due to snakebite in India [4, 7, 9, 10]. Recently, Suraveera et al. in 2020 estimated about 58000 annual deaths and about three times more permanent disabilities due to snakebite. The study was based on analysis of 2833 snakebite cases recorded from verbal autopsies in the Million Death Study (MSD) in India from 2001 to 2014 [9], along with a thorough literature review from 2000 to 2019 recording snakebites [11]. In that study, Bihar, Uttar Pradesh and Andhra Pradesh accounts for maximum number of deaths due to snakebite. The literatures reporting snakebite incidences is, however, mostly based on hospital-based surveys, providing a deviation from actual epidemiological scenario. The main reason for this eccentricity being the strong faith of rural victims on traditional healers as well as poor healthcare facility, as a result of which many victims fail to seek medical help on time, leaving many snakebite cases unregistered [12].

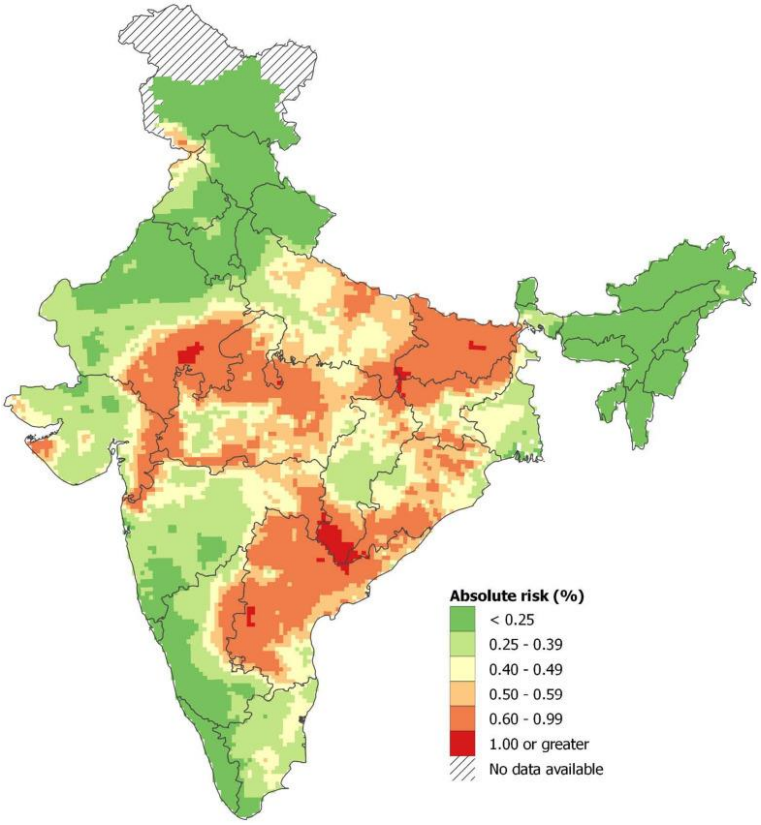

Figure 1.1. Distribution of snakebite mortality risk in India. (Adopted from *Suraveera et al, 2020* [11]).

One of the key reasons for the high incidences of snakebite in India is the large diversity and prevalence of venomous snakes resulting from country's rich vegetation and optimal climatic conditions. This leads to frequent encounters between humans and snakes, thereby, increasing the chances of snakebites. India is home to about 300 species of snakes, out of which about 60 are identified to be venomous [13]. With the continuous effort of taxonomists, the list is growing every year by identifying more and more species [14, 15]. The majority of the envenomation and death is reported to be caused by "Big-Four" snakes, which include *Daboia russelii*, *Naja naja*, *Echis carinatus* and *Bungarus caeruleus*, hence, considered as medically important snake [12, 16]. Other than Big-Four, various species of pit vipers, kraits, monocled cobra, oxus cobra etc. which are prevalent in different geographical provinces of India contribute about 21% of all snakebite incidences [11] [17]. For example, monocled cobra (*Naja koauthia*) causes the majority of deaths in north-eastern India and Bangladesh [18, 19]. Hump-nosed pit vipers (*Hypnale hypnale*) are reported to cause lethal envenoming in western ghats and the south-west coast of India and Sri Lanka [20, 21]. Likewise, the predominance of various pit vipers has been widely observed throughout the Indian subcontinent.

1.3. Antivenom therapy and its limitations

Antivenom is the only available antidote for the treatment of snakebite victims across the world. They are serum antibodies which is raised in large animals by hyperimmunization with snake venom. Since its inception over a hundred years ago, the intravenous administration of plasma-derived antivenom has successfully treated thousands of snakebite victims up until the present time [22, 23]. The first plasma-derived antivenom was developed by Phisalix and Bertrand, and Calmette simultaneously in 1894 [24, 25]. Large animals like sheep and horses are repeatedly immunized with snake venom, which elicit immune-response in the host body, thereby producing antibodies specific for various components of venom [26, 27]. The serum containing antibodies is purified and fractionated to collect the IgG molecules [27]. The formulated antivenoms might contain whole IgG molecules or their enzymatically (by pepsin and papain) digested fragments [28, 29]. The antivenom produced in this manner is highly specific and possesses high affinity towards venom toxins used in the immunization mixture. Moreover, antivenom might also show cross-reactivity with the antigens of heterologous venom having conformational similarities with the immunogenic epitopes [30, 31].

The immunization mixture might either contain venom from a single snake species or a pooled venom of multiple snakes, giving rise to monovalent or mono-specific and polyvalent or poly-specific antivenom [26, 28]. The polyvalent antivenom is widely used antivenom, as it contains antibodies against multiple venom toxins, making them useful for snakebite treatment envenomated by a broad range of snakes. On the other hand, the use of monovalent antivenom is restricted to the cases when the identity of snakes is confirmed, which is a rare scenario. In India, polyvalent antivenom (PAV) raised against an immunization mixture containing a venom pool of the Big-Four snakes viz. *Naja naja*, *Daboia russelii*, *Echis carinatus* and *Bungarus caeruleus* is used for treatment of all the venomous snake bites in India [17]. Additionally, Indian polyvalent antivenom is also used by various neighbouring countries including Nepal, Bangladesh and Sri Lanka for snakebite treatment as the prevalence of venomous snakes show similarities across the region [32].

Although being universally used for snakebite treatment, the antivenom therapy poses certain limitations as well. The first and foremost issue is the inadequate supply of antivenom and poor storage condition of hospitals which makes antivenom inaccessible at affordable price to the victims in many rural communities [33-36]. Secondarily, large number of antibodies in polyvalent antivenom are non-specific and are not directed towards relevant toxins, leading to reduced efficacy of antivenom [37]. Consequently, very high dose of antivenom is required to treat the clinical symptoms exhibited by the patients. For instance, about 35 vials of Indian polyvalent antivenom is administered to treat a patient bitten by *Naja kaouthia* [38]. However, administration of high dose of antivenom additionally elevates the level of non-specific antibodies in the body which might act as antigen to elicit immune response [39, 40]. As a result of which several side effects like pyrogenic reactions, serum sickness, anaphylactic shock is observed frequently in snakebite victims [39, 41, 42]. Further, one of the major disadvantages of antivenom is the inability to reverse the venom induced tissue damage and coagulopathy leading to significant morbidity in patients [43, 44].

Moreover, inefficient neutralization efficacy of polyvalent antivenom with heterologous venom as well as homologous venom from different geographical locations has often been well documented [45-48]. The para-specific inefficacy of antivenom is largely attributed to variation in immunogenic properties of various toxins [41] owing to

the variability of venom composition at both inter- and intra- specific levels [49, 50]. As such, pre-clinical evaluation of efficacy of existing polyvalent antivenom in neutralizing venom toxins of various regionally prevalent medically important snakes is of utmost significance [51]. With the continuous efforts of researchers and clinicians, standardization of antivenom production along with quality control and safety profiles has been enhanced immensely over the years [52-54]. Nevertheless, the reliability of antivenom therapy for snakebite management remains a pressing issue till date. Moreover, shortcomings of current antivenom treatment presents an urgent need for extensive research utilizing modern technologies to explore alternative approaches to address the critical concern of snakebite management.

1.4. Venomics and Proteomics

The advancement in omics-based technology in the past decade has incited a paradigm shift in the investigation of venom dynamics. With the introduction of "venomics" which encompasses -omics technological tools, like proteomics, genomics, transcriptomics, a rapid expansion of the knowledge of venom composition has taken place [55, 56]. In particular, the term "venom proteomics" denoting complete characterization of venom using multidimensional decomplexation strategies has gained immense popularity and therefore, used interchangeably with "snake venomics" [56]. Inspired by initial venomic workflow [57], most analysis include the fractionation of the crude venom through protein separation techniques, such as gel electrophoresis and/or chromatography followed by mass spectrometry analysis coupled with bioinformatics [56]. The utilization of proteomic tools in snake venom studies has led to a revolutionary enhancement in the understanding of overall venom composition [58] along with shedding lights on venom variation, evolutionary relationships, therapeutic potentials, toxicological profiles and their correlation with clinical manifestations of various snake venoms [59-62]. Further, immunological profiling of antivenom towards various homologous and heterologous venom can be assessed employing a proteomics-based tool antivenomics [51, 63].

The proteomic profiles of more than 200 snake species have been reported in the literature worldwide, with a continuously raising trend [55]. Proteomics tools have been employed for exploring the venom composition of medically significant snakes of south-east Asian countries which has tremendously helped in investigating venom complexity

and immuno-reactivity of crude venom with existing antivenom. For instance, proteomics of *Daboia russelii* venom from different geographical locations within Indian subcontinent has revealed significant variation in venom composition emphasizing the impact of geographical variation on clinical manifestation in victims [64-66]. Based on immuno-blotting and antivenomics approach, differential immuno-reactivity of venom toxins of geographically distant *Daboia russelii* towards antivenoms was revealed [67-69]. Further, phylovenomics studies combining venomics and antivenomics of Russell's viper from India, Pakistan, Sri Lanka and Bangladesh revealed Pakistan population to be evolutionary origin with a bi-routed radiation in Indian subcontinent; one towards north-eastern India and Bangladesh and other towards south India and Sri Lanka [48]. Further, the intra-specific venom variation as a result of geographical distribution in *Naja naja* [70-74] as well as inter-specific venom variation with closely related species like *Naja kaouthia* [75] has been unveiled using proteomics approach. Venomics and antivenomics of *Naja kaouthia* venom sourced from north-east India has also suggested inclusion of lethal toxic components of medically relevant snakes in available polyspecific antivenom for better management of snakebite [76]. A recent study deciphering bio-geographic venom variation in *Naja naja* species of Indian subcontinent also suggests pressing need of pan-India efficient antivenom [47]. Furthermore, proteomic analysis of south Indian *Bungarus caeruleus* and *Echis carinatus* revealed complex venom profile correlating with pathophysiological manifestations along with suggesting low molecular weight proteins responsible for poor immunoreactivity of Indian polyvalent antivenom [77-81]. Apart from Big-Four snakes, the proteomics of other venomous snakes of India like *Naja kaouthia*, *Hypnale hypnale*, *Calloselasma rhodostroma*, *Craspedocephalus malabaricus* has also been reported [76, 82-86]. These proteomics data along with cross-reactivity with antivenom suggests the need for species-specific or region-specific antivenom for the treatment of envenomation from medically relevant snakes. Moreover, employing proteomics tools for understanding venom composition and immuno-reactivity with antivenom would also help in investigating the range of existing antivenom for clinical use. Additionally, unveiling venom composition of phylogenetically closer species also contributes in identification of taxonomic and diagnostic marker [87]. Thus, understanding the snake venom complexity employing proteomics approach provides vast range of information which helps to comprehend various aspects of venom dynamics aiding in proper management of snakebite.

1.5. Asian pit vipers

Asian pit vipers or oriental pit vipers (Reptilia: Serpentes: Viperidae: Crotalinae) comprises a large number of venomous snakes having "lance-headed" appearance which were considered congeneric and assigned to *Trimeresurus sensu lato (s.l.)* for many years. Multiple taxonomic revision over the years has solicited at least four genera commonly recognized for Asiatic pit vipers viz. *Trimeresurus sensu stricto (s.s)*, *Ovophis*, *Protobothrops* and *Tropidolaemus* [88-93]. Among them, genus *Trimeresurus* Lacépède, 1804, comprises the largest group of venomous pit vipers in tropical and sub-tropical Asia. Due to high morphological resemblance in colouration pattern and sexual dimorphism in many arboreal species [94, 95], the genus *Trimeresurus* has always been one of the most challenging taxonomic groups of venomous snakes to identify [96, 97]. Based on four mitochondrial gene regions, Malhotra and her group in 2004 revised the taxonomic arrangement of *Trimeresurus sensu stricto (s.s.)* which was further amended by David et al, 2011 [98, 99]. They described eight genera/subgenera under *Trimeresurus* complex viz. *Trimeresurus*, *Craspedocephalus*, *Himalayophis*, *Parias*, *Peltopelor*, *Popeia*, *Sinovipera* and *Viridovipera* consisting of 46 species of Asian pit vipers [98]. With the continuous efforts of taxonomists, several new species have been identified in the last decade [14, 15, 100-103]. Currently, this group consists of 59 species enlisted under *Trimeresurus* and *Craspedocephalus* in www.reptile-database. A large number of species of this group exhibit a typical bamboo green to yellow hue in their bodies, making them difficult to differentiate by appearance, and are collectively referred to as "**Green Pit Vipers**".

1.6. Green pit vipers

The label "green pit viper" encompasses all pit vipers with green coloured body in the *Trimeresurus* complex, regardless of taxonomic distinctions. Besides having a characteristic green colour, some species possesses yellow, black, red, or gold markings on their bodies (Figure 1.2). They are relatively small, primarily arboreal species with small bodies and prehensile tail. A heat sensing pit organ is present between nostrils and eyes on both sides of their brain which help them to sense infrared radiation emitted from warm blooded animals, thereby, enabling them to detect and locate prey [104].

Figure. 1.2. Various species of green pit vipers showing different colouration pattern on their body. (A) *Trimeresurus albolabris,* **(B)** *Popeia popeiorum,* **(C)** *Viridovipera medoensis,* **(D)** *Parias malcomi,* **(E)** *Trimeresurus venustus,* **(F)** *Craspedocephalus trigonocephalus*. Photographs adopted from www.reptile-database.

The distribution of green pit vipers ranges from the Indian subcontinent throughout south-east Asia, southern China and the Indo-Malayan to Philippine archipelagos. Various hospital records reporting green pit viper bites in south-east Asian countries suggest their significant medical importance [105-109]. The epidemiological data from various south-east Asian nations including Thailand, Myanmar, Vietnam, Bangladesh, Sri Lanka etc. suggests high frequency of bite incidences instigated by green pit vipers, highlighting their prominent role in global snakebite epidemiology [110-115]. For instance, green pit vipers was responsible for about 84% of all identified envenomation cases in nine district and one central hospital in central Vietnam [111]. A prospective case data enlisting 3803 snakebite cases recorded from 5 hospitals in the Mandalay region of Myanmar over 3 years reported 355 cases as “green-snake” bites [114]. Further, 288 green pit viper bites were reported between 2016 and 2018 to the Ramathibodi Poison Center, Thailand [115].

Green pit viper venom is reported to be haemotoxic in nature, causing extensive swelling of bite site spreading considerably in severe cases, mild dyspnoea, nausea, bleeding and significant pain (Figure 1.3). Along with local injuries, the clinical manifestation is also characterized by systemic toxicity including tissue necrosis and haematological abnormalities such as hypofibrinogenemia, consumptive coagulopathy leading to incoagulable blood for many days, hypotension, low platelet count leading to thrombocytopenia and acute kidney injury (AKI) [105, 107, 116, 117]. Also, aphasia,

hemiplegia, speech disturbance, local erythema, ecchymosis and acute cerebral infarction have also been reported following green pit viper bite [108]. Although, death is rare, their bite can lead to severe morbidity in patients if untreated. Organ amputation as a result of severe tissue necrosis post green pit viper bite has been reported from Karnali Province, Nepal [118]. Further, incoagulable blood for multiple days and thrombocytopenia requires prolonged stay of patients in hospital for treatment. In most cases, the victim is the sole bread-earner of the family or hails from economically deprived group in society who cannot bear the costs and time commitment for hospital stay and therapeutic procedures, thereby, imposing severe socio-economic burdens to the family.

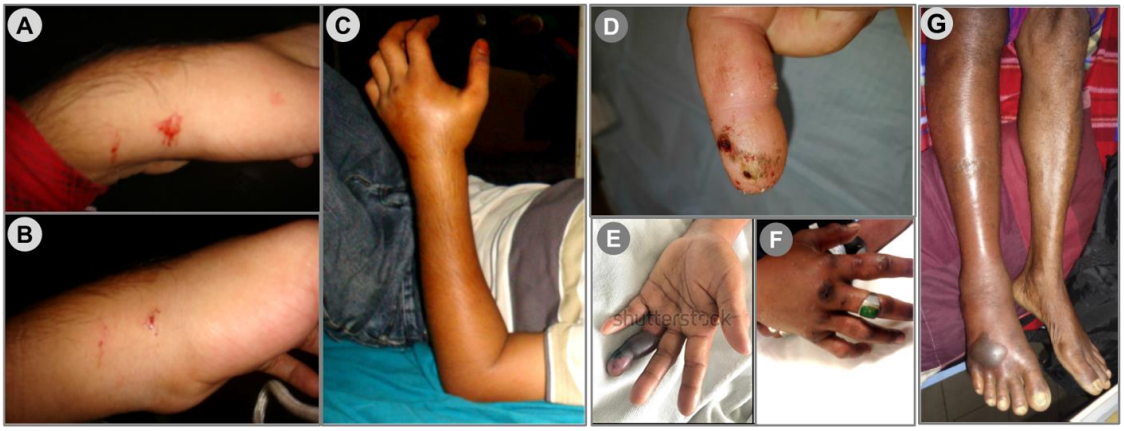

Figure. 1.3. Local symptoms in green pit viper envenomated patients. (A) Bleeding immediately after bite, **(B)** Swelling of wrist after 1.5 hour of bite, **(C)** Progression of swelling up to elbow, **(D)** Bite mark shortly after envenomation, **(E)** Visible clot in the bite site, **(F)** Swelling of hand. (G) Edema induced bite site. Photos adopted from *Pandey et al, 2019; Fuchs, 2019, Mong and Tan, 2016, Ratnayaka et al, 2017*.

Green pit viper bite leads to haemostatic aberrations in patients which is often treated conservatively in mild envenomation, however, in many cases antivenom therapy is employed to treat the patients [106, 108, 115, 116]. In later cases either polyvalent or monovalent antivenom is administered. The polyvalent antivenom is country specific and is raised against the venom of most prevalent snakes of particular country. The monovalent antivenom is raised against *Trimeresurus albolabris* (GPVAV) in Queen Saovabha Memorial Institute, Red cross society, Thailand [119] and is used in Thailand and other south-east Asian countries such as Vietnam, China, Myanmar etc. for green pit viper envenomation. However, early adverse reactions (EARs) including anaphylactic reactions of GPVAV has been reported in green pit viper envenomated patients [120]. Also, complications of polyvalent antivenom administration have been previously

discussed (mentioned in section 1.3) presenting the need for pre-clinical evaluation of antivenom efficacy in neutralizing venom toxins of snakes predominant in particular region. Various techniques used to assess immuno-reactivity of venom toxins with available antivenom include *in vitro* and *in vivo* neutralization studies, ELISA, immunoblotting, antivenomics etc. Many a times, a combination of them is used to obtain confirmatory results.

A number of studies have been conducted to assess the neutralizing potential and cross-reactivity of Thai green pit viper monovalent antivenom compared to country-specific polyvalent antivenoms towards various green pit viper venoms. Tan et al in 2019 studied the venom toxicity as well as cross reactivity of *Popeia nebularis* venom towards Thai Green Pit Viper monovalent antivenom (GPVAV) and reported efficient neutralization [121]. Similar results were shown with *Trimeresurus albolabris*, *Trimeresurus purpureomaculatus, Craspedocephalus wiroti* and *Craspedocephalus puniceus* venom from Malaysia [122, 123], while GPVAV was shown to have 1.4 times more neutralization potential than Myanmar Russell's viper antivenom in neutralizing Myanmar green pit viper *Trimeresurus erythrurus* [124]. In India, polyvalent antivenom (PAV) raised with an immunization mixture containing a venom pool of "Big-Four" snakes is used for treatment of green pit viper envenomation as it is the only available antivenom. The unavailability of specific antivenom for green pit vipers in India suggests the need to assess the effectiveness and toxin recognition profile of existing Indian polyvalent antivenom as well as Thai green pit viper monovalent antivenom.

The venom composition of various green pit vipers has been unveiled with the help of a proteomic approach e.g., *Viridovipera stejnegeri*, *Trimeresurus albolabris*, *Popeia nebularis*, *T. insularis* and *T. purpureomaculatus* [121, 123, 125, 126]. These studies have established a correlation between venom composition and the clinical toxicity presented by snakebite victims, thereby adding to the knowledge of venom-induced pathophysiology. For instance, predominance of snake venom metalloproteases (SVMPs) in the venom of *Popeia nebularis* has been found to be responsible for acute edema and haemorrhage in the envenomated victims [121]. Likewise, abundance of SVMPs has also been observed in the venom of *Trimeresurus albolabris* from Thailand and west Java Island of Indonesia, *Trimeresurus insularis* from east Java Island of Indonesia and *Trimeresurus purpureomaculatus* from Malaysia and Indonesia [123,

127]. Other dominant protein families of green pit vipers include snake venom serine protease (SVSPs), snake c-type lectin like proteins (Snaclecs) and phospholipase A_2 (PLA_2s). These protein families are widely known to possess prominent role in targeting various components of haemostatic system of prey. Further, proteome profile illustrated by SDS-PAGE and Rp-HPLC of phenotypically variable white-lipped Island Pit viper, *Trimeresurus insularis* across eight islands of Lesser Sunda Archipelago, Indonesia shows remarkable similarities demonstrating significant conservation in venom composition [125]. However, venomics of *T. insularis* from Sumbawa Island reveals phospholipase A_2 as the most abundant protein (30.5%) unlike other green pit vipers of south-east Asia [125]. Such disparities in venom composition of morphologically similar snakes might cause diverse clinical toxicity in patients arising the problem of mistreatment of patients. Unveiling the snake venom complexity of various unstudied green pit vipers would help to comprehend the venom variation among closely related species, their pharmacological profile and finding the toxins responsible for the pathophysiological abnormalities in victims.

1.7. Green pit vipers of India

India harbors a large variety of pit vipers which are distributed across diverse regions within the political boundaries of the country. Various pit vipers include species belonging to *Hypnale*, *Ovophis*, *Protobothrops*, *Glodius* and the green and non-green pit vipers of *Trimeresurus* complex. The distribution of green pit vipers has, however, remained blur for centuries due to the unjustified synonyms and misidentification as a result of phenotypic alikeness [97]. For example, *T. albolabris*, *T. erythrurus* and other recently identified taxa were kept under the name *Trimeresurus gramineus* which was considered to be widely distributed across Tropical Asia [97]. However, multiple taxonomic investigations undertaking redescription of type specimens and extensive herpetological surveys has led to inclusion, exclusion and range extension of certain species from the list of green pit vipers inhabiting Indian subcontinent. The green pit vipers of Indian subcontinent are distributed across the foothills of Himalayan and Tibetan plateau, spreading in the mesic forests of Indo-Burma region extending up to Sundarbans and Nicobar archipelago. Within the political boundaries of India, various species of green pit vipers inhabiting northern and north-eastern states include *Trimeresurus septentrionalis* Kramer, 1977 (Northern white-lipped pit viper),

Trimeresurus erythrurus Cantor, 1839 (spot-tailed or red-tailed pit-viper), *Popeia popeiorum* Smith, 1937 (Pope's pit viper) and *Viridovipera medoensis* Djao in Djao & Jiang, 1977 (Medo pit viper) (Figure 1.4). The distribution of *Trimeresurus septentrionalis* ranges from Siwalik and Himalayan range in Nepal and India [97, 128]. *Trimeresurus erythrurus* is distributed in Indo-Burma region, encompassing Bhutan, Nepal, Bangladesh, Myanmar and north-east India [97]. Apart from north-eastern states, *T. erythrurus* encounter in coastal mangrove forests in the Sunderbans of West Bengal, Odisha and as far south as Kakinada in Andhra Pradesh has been reported [129]. *Popeia popeiorum* and *Viridovipera medoensis* is also prevalent in north-eastern states of India [99, 130]. Further, three green pit viper species have been recently identified as *Trimeresurus salazar*, *Trimeresurus mayaae* and *Trimeresurus davidi* from Arunachal Pradesh, Meghalaya and Car Nicobar Island [14, 15, 100].

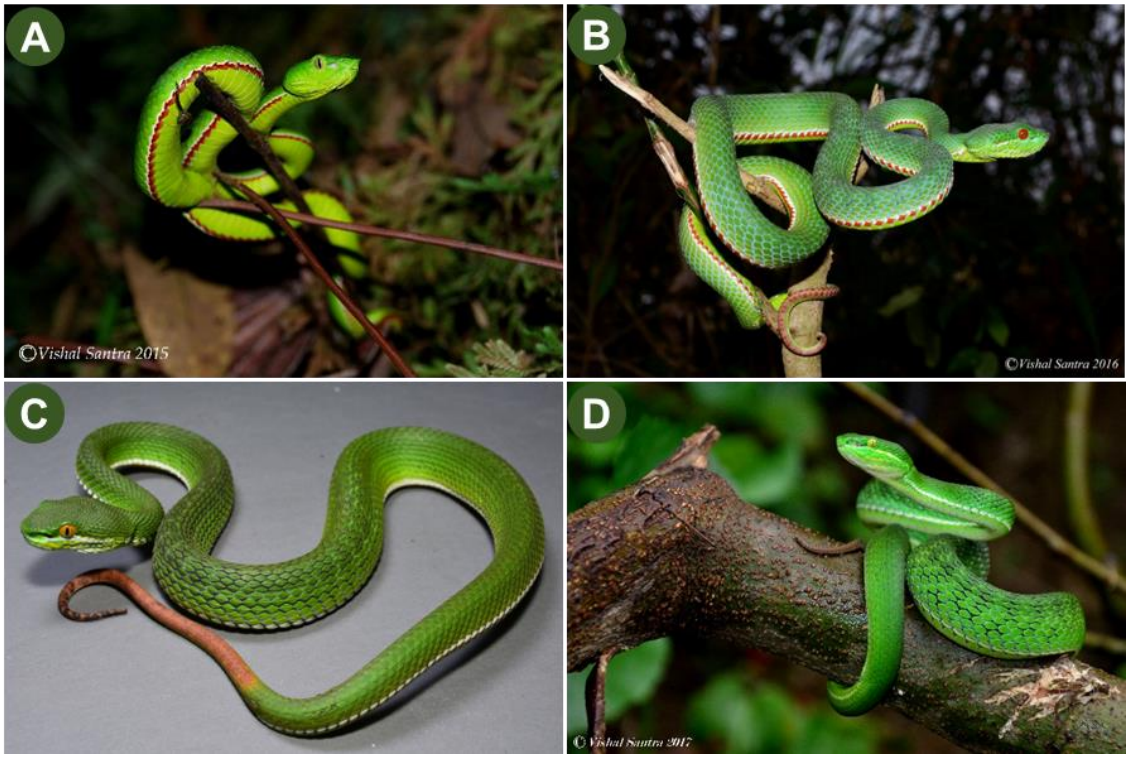

Figure 1.4. Photographs of Green pit vipers under study. (A) *Viridovipera medoensis*, **(B)** *Popeia popeiorum*, **(C)** *Trimeresurus erythrurus*, **(D)** *Trimeresurus septentrionalis* (Photographs by Vishal Santra).

Envenomation reports of Indian green pit vipers are scarce in the literature, partly because of the morphological similarity among several species of green pit viper that are prevalent in the same area. In most cases, the snake is identified as a "green snake" by snakebite victims, a description that could include any green pit viper found in the area.

However, a few epidemiological studies report haematological abnormalities in patients post green pit viper bite causing morbidity leading to socio-economic burden to victim and their family. In Solan district, Himachal Pradesh, green pit vipers were implicated in 70% of cases where the biting snake was identified, and coagulopathy was reported in about 50% of cases, with swelling in about 70% of cases [131]. A review of 30 paediatric snakebite cases in Shimla, Himachal Pradesh [132] also reported a high incidence of haemolytic envenomation (87.5%) involving thrombocytopenia (observed in 26.7% of cases), prolonged PT/INR (76.6%) and prolonged APTT (36.7%), as well as swelling in 80.9% of cases. Further, organ amputation and socio-economic impact of green pit viper prevalent in Karnali province in Nepal has been reported [118]. Based on these collective clinical manifestations and knowledge of distribution of species, these envenomation cases are most likely to be caused by *T. septentrionalis*, which is the only green pit viper present in Siwalik and Himalayan range of Himachal Pradesh and Nepal. A few envenomation reports further points out the medical importance and bite-associated pathophysiology of green snakes assumed to be *T. erythrurus* based on their predominance in a particular geographical area [114, 133]. Along with local injuries, the clinical manifestation is also characterized by haematological abnormalities, including prolonged coagulopathy leading to thrombocytopenia, hypotension and shock in a few patients [133, 134]. A recent study from Myanmar reports Acute kidney Injury (AKI) in a patient bitten by a confirmed *T. erythrurus* snake in Yangon area, along with shedding light on overall significance of "green snake" bite in Myanmar [114]. Some of these clinical complications might be associated with morphological misidentification of snakes, thereby leading to incorrect treatment causing further complications in patients.

Among the pit vipers of *Trimeresurus* complex, only the venom of *Craspedocephalus malabaricus* (Malabar pit viper), endemic to the Western Ghats, has been studied for pharmacological profiling as well as identification and isolation of functional peptides. Gowda and his team from University of Mysore, Karnataka isolated three different venom proteins: Trimarin [135] and Malabarin [136] belonging to the metalloprotease family, and Malabarase [137] belonging to the serine protease family. Immunoreactivity assays of *C. malabaricus* venom towards Indian Polyvalent Antivenom showed weak neutralization potential [82]. Nevertheless, the green pit vipers inhabiting the northern and north-eastern states of India, *Trimeresurus erythrurus*, *Viridovipera medoensis*, *Popeia popeiorum* and *Trimeresurus septentrionalis*, which

have morphological similarities with their southeast Asian cousin have remained unstudied. Previously, Mitrakul (1973) reported *Trimeresurus erythrurus* and *Popeia popeiorum* venoms to possess clotting activity by thrombin-like action, fibrinolytic activity and direct aggregating effect on platelets [138]. However, it was not followed by studies regarding characterization of crude venom to understand pathophysiological manifestations observed in victims, cross-reactivity with antivenom or isolation of peptides/proteins to understand structure-function relationship.

1.8. Haemostatic system

Haemostasis is a complex and tightly regulated process of homeostasis that maintains the regular blood flow under normal physiological conditions and prevents extensive blood loss resulting from vascular injury [139, 140]. The phenomenon of haemostatic balance, as first described by Astrup in 1958, involves the tendency of blood to clot and the clot to dissolve to maintain blood fluidity [141], however, the crucial role of antithrombotic agents in prevention of clot formation under normal physiological conditions was established much later [141, 142]. The haemostatic system includes a repertoire of various cellular and sub-cellular moieties, ions, enzymes and pro-enzymes which upon proteolytic cleavage activates a cascade of reaction facilitating the formation of a clot, thereby, preventing the blood loss following an injury. Any glitch in this vital array of inter-dependent reactions leads to pathophysiological conditions like thrombosis and haemorrhage.

The process of haemostasis includes three crucial steps: a) primary haemostasis involving the role of platelets, b) secondary haemostasis involving coagulation factors and their regulatory components, and c) tertiary haemostasis involving the process of fibrinolysis or clot dissolution. Primary haemostasis starts with the onset of a vascular injury resulting in the exposure of an adhesive protein von Willebrand factor (vWF) present between endothelial and sub-endothelial membrane [143, 144]. The free platelets in the blood interacts with the exposed collagen and vWF to form a loose platelet plug at the site of injury causing an initial arrest of bleeding [145, 146]. Secondary haemostasis refers to the blood coagulation process which can be broadly divided into extrinsic, intrinsic and common pathway. In response to vascular injury, a cell surface receptor tissue factor (TF) comes in contact of blood commencing a complex cascade of overlapping chemical reactions involving more than a dozen of coagulation factors [147,

148]. This results in the formation of a complex of various activated substances called prothrombin activator which in presence of Ca^{2+} catalyses the conversion of prothrombin into thrombin, a multi-functional enzyme [147, 149]. Thrombin then catalyses the degradation of fibrinogen releasing fibrinopeptides and fibrin monomers which then polymerizes over the loose platelet plug leading to formation of the clot/thrombus [149]. Thrombin also activates factor XIII which further aids in cross-linking of fibrin molecules, thereby stabilizing the clot and completing the coagulation process [149]. Tertiary haemostasis or fibrinolysis involves the process of clot dissolution when the injured blood vessel is healed. The vascular endothelium releases tissue plasminogen activator (t-PA) which converts plasminogen to plasmin [150]. Subsequently, the plasmin cleaves fibrin mess into fibrin degradation products, thereby, dissolving the clot and restoring back the haemostasis [147, 150].

1.9. Snake venom protein targeting haemostatic system

The haemostatic system is the most susceptible targets for various proteins/peptides of Viperidae snakes. These proteins/peptides are highly specific toxins which can either induce or inhibit various components of the coagulation cascade or platelet aggregation [151]. The venom toxins specifically targeting coagulation pathway can be broadly classified into procoagulants, anticoagulants and those affecting fibrinolysis [152]. Procoagulant toxins reduces the clotting time either by activating blood coagulation factors or converting fibrinogen to fibrin clot such as Factor V and Factor X activators, prothrombin activators and thrombin-like enzymes (TLEs) [152, 153]. Conversely, the anticoagulants delay the process of coagulation by interacting with clotting factors such as protein C activators, FIX/X binding proteins, thrombin inhibitors [152, 154]. These venom toxins belong to diverse array of snake venom protein families which might act enzymatically such as phospholipases A_2, serine proteases, metalloproteinases, l-amino acid oxidases etc. or non-enzymatically like c-type lectins and disintegrins [151, 155]. The procoagulant proteins which specifically catalyses fibrinogen clotting belongs to snake venom thrombin-like enzymes (SVTLEs), a subgroup of snake venom serine proteases (SVSPs).

1.10. Snake venom thrombin-like enzymes (SVTLEs)

Snake venom thrombin-like enzymes (SVTLEs) are the second most abundant protein family of Viperidae snakes which are functionally similar to thrombin [156].

However, they show better resemblance with trypsin when the structure and primary substrate specificity are considered [157]. They are mostly monomeric with molecular weight ranging from 26-67 kDa constituting 232-236 amino acid residue [158]. Frequent *N*-linked glycosylation along with a few *O*-linked glycosylation has been reported in many SVTLEs [156, 159]. The catalytic triad is composed of Asp102, His57 and Ser195, which is conserved in all SVTLEs, thrombin and trypsin. Further, unlike 5 disulphide bonds in trypsin and 3 in thrombin, SVTLEs consists of 12 conserved cysteine residues facilitating the formation of 6 disulphide bonds [156].

Snake venom thrombin-like enzymes (SVTLEs) show functional similarity with thrombin in catalysing the conversion of soluble fibrinogen to insoluble fibrin clot. However, unlike thrombin, SVTLEs either cleave α or β chain releasing fibrinopeptide A and B respectively [157]. The resulting fibrin monomers spontaneously polymerizes forming a tenuous thrombus, thereby, showing procoagulant effect. However, SVTLEs do not usually activate FXIII which functions as clot stabilizer by transglutaminase catalysed cross-linking of fibrin monomers [155]. This results in formation of an unstable and weak fibrin clot composed of short polymers which gets readily dissolved by fibrinolytic system [152, 155, 160]. The repeated formation and simultaneous dissolution of tenuous thrombus leads to consumption of most of the fibrinogen causing hypofibrinogenemic syndrome [161], resulting in an overall anticoagulant effect in patients commonly known as consumptive coagulopathy [157]. As a result of this pseudo-procoagulant property, SVTLEs are widely used as defibrinogenating agent for treating patients with some thrombo-embolic syndromes like ischemic stroke and peripheral artery diseases [162, 163]. Some of the SVTLEs used as therapeutic drugs and diagnostic agents include ancrod from *Calloselasma rhodostoma*, batroxobin from *Bothrops atrox*, reptilase from *Bothrops jararaca*, etc. Ancrod is used for indication of heparin-induced thrombocytopenia and thrombosis (HITT) in United states [164]. Further, the commercial formulation of ancrod, Viprinex™ is at phase III clinical trials as defibrinogenating agent for acute ischemic strokes [165]. Batroxobin (Defibrase®) is clinically used for acute cerebral and myocardial infarction, angina pectosis, peripherial arterial disease, pulmonary embolism, retinal vein thrombosis etc. [166]. Reptilase® is used for diagnosis of dysfibrinogenemia in a patient experiencing hypercoagulability or bleeding tendency [157].

Snake venom thrombin-like enzymes (SVTLEs) are widely distributed in several pit viper genera of Crotalinae family including various species of green pit vipers [167]. Green pit vipers are known to instigate haemostatic alterations, particularly, hypofibrinogenemia leading to prolonged coagulopathy for multiple days in envenomated victims. The venom induced consumptive coagulopathy (VICC) is one of the most important systemic clinical syndromes which can lead to serious complications including life-threatening haemorrhage [168]. Such venom induced coagulopathies mostly results from the pathological activities of the thrombin-like enzymes [158, 169]. Further, due to their defibrinating properties, the thrombin-like enzymes have been used as therapeutic drugs for treating a myriad of human disorders, such as ischemic strokes, deep vein thrombosis, myocardial and cerebral infarctions [161]. As such various thrombin-like enzymes targeting the haemostatic system has been isolated from green pit vipers across south-east Asian countries; GPV-TL1 and GPV-TL2, chitibrisin, albofibrase, purpurase etc. are to name a few [170-173]. However, specific toxins of Indian green pit vipers causing haemostatic aberrations in envenomated patients has not been explored yet. A thorough investigation of Indian green pit viper venoms for thrombin-like proteins might aid in identification of specific toxins targeting haemostatic system. Exploration of such toxins as therapeutic agents and diagnostic markers might aid in a prototype for designing a suitable antivenom or quality improvement of existing antivenom, eventually minimizing the venom-induce morbidity.

1.11. Aim of the study

The above review of literature highlights the notable role of Indian green pit vipers in global snakebite epidemiology, particularly emphasizing on their pathophysiological manifestations and venom-induced morbidity. However, despite their prevalence and envenomation, there exists a dearth of knowledge regarding the venomics and antivenomics of green pit vipers of Indian subcontinent. Owing to the medical relevance, there is an urgent need to characterize the Indian green pit viper venoms in light of inter-specific venom variation, identification of toxins imparting clinical manifestations and cross-reactivity with available monovalent and polyvalent antivenom. Therefore, the present study has been aimed to understand the venom composition of crude venoms of Indian green pit vipers, their immuno-reactivity with antivenom and characterization of haemostatically active proteins. The Indian green pit vipers undertaken in the current

study include *Trimeresurus erythrurus*, *Viridovipera medoensis*, *Popeia popeiorum* and *Trimeresurus septentrionalis* from northern and north-eastern India, however *Trimeresurus salazar* and *Trimeresurus mayaae* was not considered in the study as they were discovered after undertaking the study.

1.12. Objectives

1. Comparative proteomic and biochemical profiling of Indian green pit viper venoms.
2. Immunological cross reactivity of green pit viper venom with Indian polyvalent antivenom and Thai Green pit viper monovalent antivenom.
3. Proteomics of *Trimeresurus erythrurus* venom from Mizoram, India.
4. Characterization of a haemostatically active protein from *Trimeresurus erythrurus* venom.