CHAPTER 1

Introduction and Review of Literature

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1.1 Venomous snakes and snakebite

Snakes are limbless, carnivorous reptiles that throughout history have fascinated and drawn the interest of human curiosity owing to their distinctive characteristics. Over the ages, snakes have evoked a mix of fear and intrigue, cementing a significant position within various cultures and societies. As of 2022, there are more than 3700 known species of snakes out of which more than 650 species possess a dangerous chemical called venom which are used to incapacitate as well as kill their prey [1,2]. Snake venom has evolved and diversified over millions of years in different snake families and is stored in specialized glands of the mouth called venom glands, which they use as a means of offence and defence. The taxonomic classification of venomous snakes categorizes them into three distinct families, namely, Elapidae, Viperidae and Atractaspididae (Burrowing Asps). The Viperidae lineage undergoes further classification into two subfamilies: the True Vipers are categorized as Viperinae, and the Pit Vipers are categorized as Crotalinae [3].

The venom apparatus present in the venomous snakes is used for both predation and defence. In Viperids and Elapids, although some morphological and size differences are observed in the venom glands, however, they all share a similarity of design in which the principal venom gland through a primary duct releases its contents into an auxiliary gland, which in turn expels venom via a secondary duct to the root of the hollow fangs. Depending on the arrangement of the fangs in the upper jaw, snakes can be classified as front-fanged or rear-fanged. Evolutionary studies using marker genes like the sonic hedgehog gene, which is expressed in dental epithelium during dentition, have been utilized to understand the origin of front- and rear-fangs in snakes [4]. It has been observed using three dimensional reconstruct of maxillary dentition that both front-fangs and rear-fangs have a similar morphogenesis and develop from the posterior region of

the upper jaw. The sonic hedgehog gene is not expressed in anterior portion of upper jaw in front-fanged snakes which suggest ontogenic development moved the fangs from posterior origin to its adult anterior position. Although, in rear-fanged snakes, the fangs emerge from a posterior dental lamina, which develops independently in the posterior position, it is suggested that the tooth-forming posterior sub-region separated during evolution, as a result of which, the posterior and anterior teeth evolved independently along with the venom gland. This resulted in the emergence of different lineages and the vast expansion of advanced snake species [5]. Although Elapids are known for their neurotoxic venom, and Viperids for their hemotoxic venom, however, there are exceptions within these families which challenge this definitive contrast.

Snakebite envenomation is responsible for thousands of death and permanent disability every year in victims worldwide. It is estimated that more than three times of the survivors face long-term disabilities hugely impacting their quality of life [6]. For instance, a population-based study in Sri Lanka examined the long-term consequences which developed within a month of snakebite envenoming in 816 victims and persisted for an average 12.7 years. The recorded disabilities included musculoskeletal complications such as swelling of lower limbs, reduced movement, lowered muscle power, wasting of muscles, balance impairment, chronic ulcers, walking disorder, deformity of limbs and amputations [7]. Other long-term complications included migraine-like-syndrome, sensitivity to sunlight, impairment of vision, renal injury, blistering of skin and paresthesia at the bite site, right facial nerve palsy, hemiplegia, general shivering, mental stress and some nonspecific somatic complications as well [8].

According to the World Health Organization (WHO) every year 450,000 to 540,000 people suffer from snakebites out of which envenomation occurs in 180,000 to 270,000 who suffer from its ill-effects [6]. It has been estimated that 81,000 to 138,000 fatalities occur annually worldwide as a result of snakebites out of which around 58,000 death occur only in India, and hence, India is also known as the "*Global capital of snakebite deaths*" [9,10]. WHO data reports that more than 3,700 snake species are identified globally, out of which only 650 are venomous and 250 of them may be considered as medically relevant species [6]. In India, reports indicate the existence of more than 273 snake species, with 43 identified as venomous, and 38 as possessing a mild venom [11]. Among the venomous snakes, *Bungarus caeruleus* (Common krait), *Naja naja* (Indian

cobra), *Daboia russelii* (Russell's viper) and *Echis carinatus* (Indian saw-scaled viper), which are the primary source of snakebite incidents in India, are called the "Big-Four" snakes [12,13].

There are also other groups of medically important snakes in India potentially inducing deadly or less severe envenomation in bite victims. For instance, the snake *Naja oxiana* (Central Asian cobra) is a highly venomous most frequently encountered in north-western region of India such as Jammu and Kashmir, and Himachal Pradesh [14]. The Hump-nosed pit viper (*Hypnale hypnale*) present in the South-west coast and Western Ghats are responsible for numerous lethal snakebites in this region [15]. Pit vipers like *Trimerusurus salazar* (Salazar's pit viper) and *T. albolabris* (Green pit viper) commonly found in North-Eastern states of India are responsible for numerous snakebites in this region [16]. *N. kaouthia* (Monocled cobra) and krait species like *Bungarus niger* (Greater black krait) common in Eastern and North-East India are also responsible for various snakebite cases [14,17]. Another venomous krait species *B. lividus* (Lesser known krait) is common in North-East India for which bites have also been reported in humans [14].

1.2 *Bungarus fasciatus*

Kraits constitute a crucial subset of medically significant elapids classified under the genus *Bungarus* [18]. Kraits are active at the night and their venom contain two types of neurotoxins namely, presynaptic neurotoxins and postsynaptic neurotoxins [19–21]. The kraits belong to the genus *Bungarus* and 12 to 14 species are reported from various regions of South Asia and South-East Asia [22–25]. The kraits which are reported from India include *B. niger* (Greater black krait) [26], *B. fasciatus* (Banded krait) [27], *B. caeruleus* (Common krait) [13], *B. lividus* (Lesser black krait) [28], *B. bungaroides* (North-Eastern hill krait) [29], *B. sindanus* (Sind krait) [30], *and B. andamanensis* (South Andaman krait) [31].

The medically significant venomous snake *B. fasciatus*, also called the Banded krait belong to the kingdom Animalia, phylum Chordata, class Reptilia, order Squamata, sub-order Serpentes and family Elapidae. They are found in South and South East Asia including India and have been recorded at a maximum height of 5000 metres above sea level in Myanmar [32]. The origin of the generic name *Bungarus* finds its roots in the

Telegu (and Kannada) term "*Bungarum*" which literally means 'gold', referring to the yellow rings encircling the body [33]. It is known by various local names in Assam (India) such as '*Xokha*', '*Xongkhosur*', '*Gowala*', '*Bagraj*', '*Okaoki*' and '*Bandphora'* [34–36].

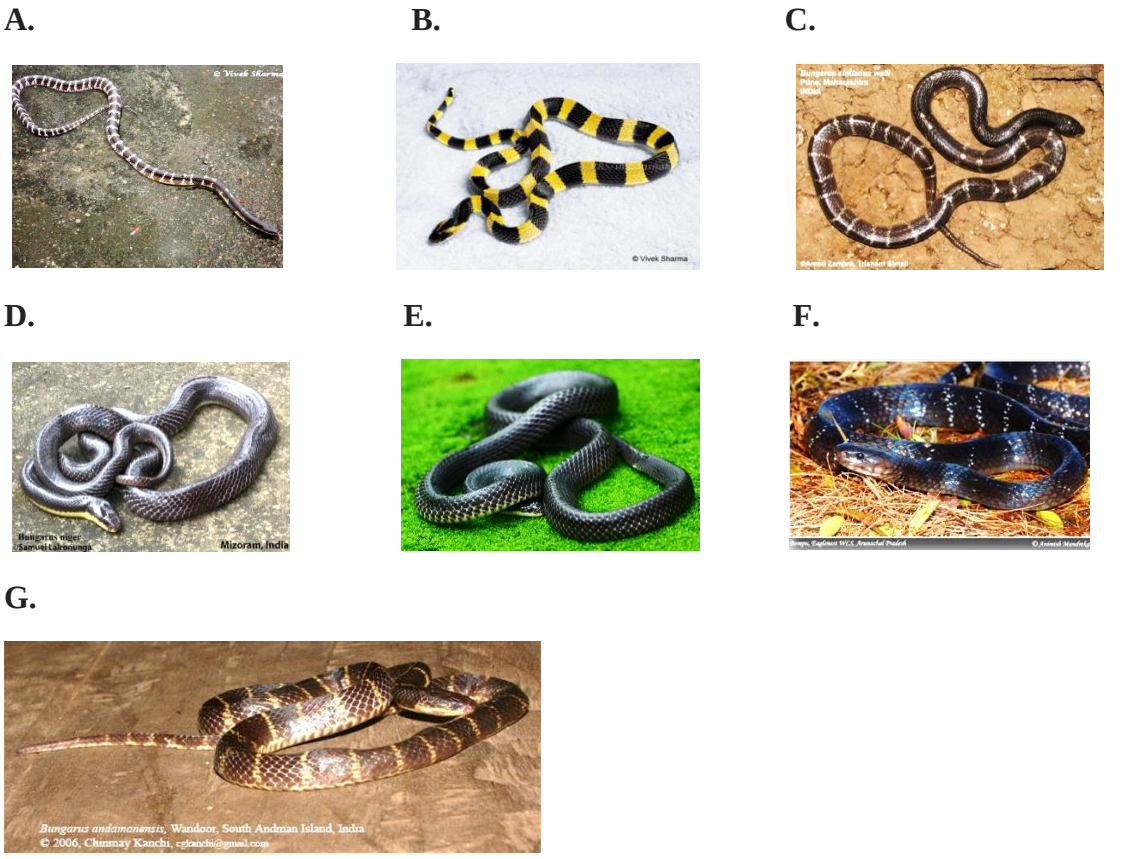

Figure 1.1: Krait species found in India. A. *Bungarus caeruleus;* B. *Bungarus fasciatus* (Photo Credits: Vivek Sharma); C. *Bungarus sindanus* (Photo Credits: Trishant Simali); D. *Bungarus niger* (Photo Credits: Samuel Lalronunga); E. *Bungarus lividus* (Photo Credits: Prajjwal Ray); F. *Bungarus bungaroides* (Photo Credits: Animish Mandrekar); G. *Bungarus andamanensis* (Photo Credits: Chinmay Kanchi). Photo Source: www.reptile-database.org (for A., B., C., D., F., and G.) and www.inaturalist.org (for E.).

1.2.1 Distribution of *B. fasciatus*

B. fasciatus has a geographical distribution in Asia and extends from India [27] to Bangladesh [22], Nepal [37], Myanmar [38,39], Southern China [22], Brunei, Cambodia, Indonesia, Malaysia, Lao People's Democratic Republic, Singapore, Thailand, and Vietnam [41]. In India, *B. fasciatus* has been reported from various regions such as the states of North-East India *i.e.*, Arunachal Pradesh, Assam, Meghalaya, Manipur, Tripura, Mizoram, and Nagaland; four states from Eastern India *i.e.*, Odisha, Bihar, Jharkhand and West Bengal; one state of central India *i.e.*, Chattisgarh; two states from Northern

India *i.e.*, Uttar Pradesh and Uttarakhand; one state of western India *i.e.*, Maharashtra; and two states of Southern India *i.e.*, Andhra Pradesh and Telangana [41–44].

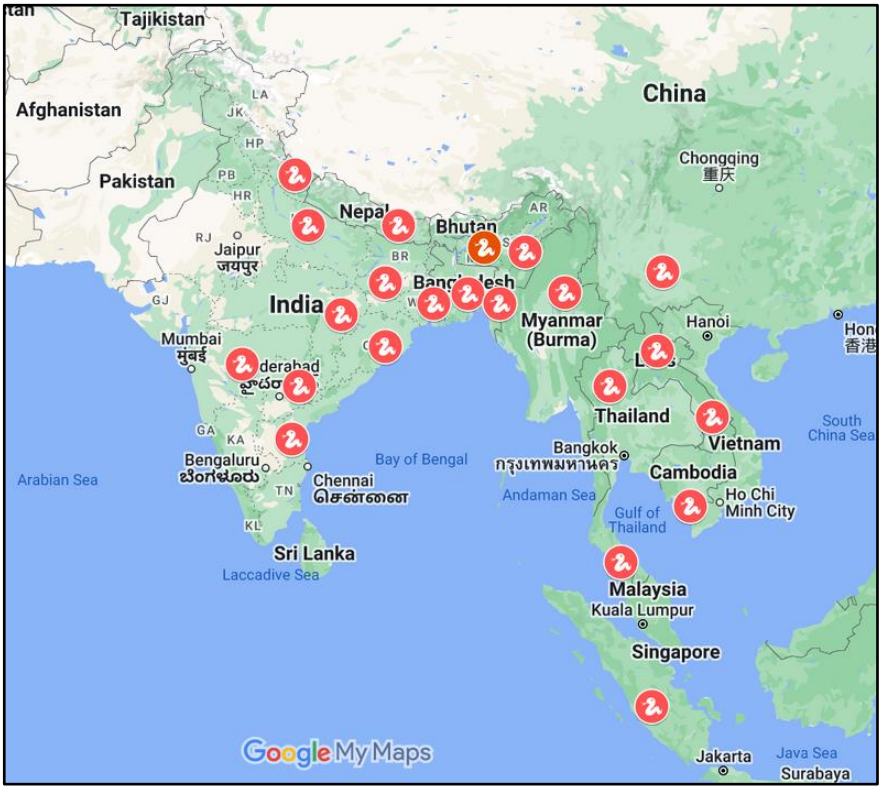

Figure 1.2: Distribution map of *B. fasciatus* (Created in: Google My Maps).

1.2.2 Morphology of *B. fasciatus*

The average length of the snake varies from 44.4 to 122.0 cm with a maximum recorded length of 2.25 m. The tail is small, blunt and around one-tenth of the total length, it is recorded to be 4.7 to 13.3 cm in length [45]. The head is black in color and has yellow lips. There is no clear distinction of the neck from the head and the body. There is an elongate black marking on the neck which extends towards the head and finally merges with the black color of the head. The body shape exhibits a triangular form and the dorsal surface of the body features black and yellow (or white) bands that alternate in the same width, due to which it is commonly known the Banded krait [46,47].

1.2.3 Habitat and Ecology of *B. fasciatus*

B. fasciatus occupies diverse environments, spanning from humid and arid deciduous forests, temperate broadleaf forests and tropical scrublands, to cultivated areas like rice fields [41,48]. They also inhabit areas adjacent to human settlements and other habitats

that have been altered. They can also be located in expansive plains, grassy terrains, burrows of rodents, and in the vicinity of permanent water sources like ponds and canal edges [41,48]. Nest attendance while sharing a common burrow complex has been reported among females of the species which provides preliminary suggestions of parental care and social behaviour in *B. fasciatus* [48].

1.2.4 Food habits of *B. fasciatus*

The food habit of *B. fasciatus* is partially ophidian *i.e.*, it hunts and eat a wide range of snake species including congeneric kraits due to its wide distribution. It feeds on kraits like *B. walli* (Wall's krait), *B. lividus* (Lesser black krait) and *B. caeruleus* (Common krait) [49], as well as on various other snakes such as *Amphiesma stolatum* (Buff Striped keelback), *Xenochrophis pisacator* (Checkered keelback), *Xenopeltis unicolor* (Sunbeam snake), *Boiga trigonata* (Common cat snake), *Ptyas korros* (Indo-Chinese rat snake), *P. mucosa* (Indian rat snake), *Cylindrophis ruffus* (Red-tailed pipe snake), *Enhydris enhydris* (Rainbow water snake), *Daboia russelii* (Russel's viper), *Ovophis tonkinensis* (Tonkin pit viper), and *Trimerusurus erythrurus* (Red-tailed pit viper) [33,50,51]. *B. fasciatus* have also been reported to scavenge on dead snakes like *C. ruffus* and *E. enhydris* [52]. Apart from snakes they also prey on fish, frogs, skinks and eggs of snakes.

1.3 *B. fasciatus*'s pathophysiological landscape

Bungarus fasciatus is a nocturnal snake and was designated as a "Category 2" medically important snake by the WHO in 2019 [6]. Encounter of this snake with humans are rare in daylight, however, accidental encounter or occupational exposure may result in occasional snakebite cases, which may lead to fatalities as well [16,45,53]. The venom of *B. fasciatus* contains both pre-synaptic and post-synaptic neurotoxins, however, the pre-synaptic neurotoxins are more significant in envenomated victims as they cause damage to the motor nerve termini, resulting in loss of neurotransmitters from the synaptic vesicle [54]. *B. fasciatus* bites may cause little to no pain in humans along with minimal local tissue damage at bite site which may provide a false reassurance to the victims initially. Gradually systematic symptoms appear which include a rapid or delayed progressive respiratory paralysis [55–57]. Previous instances of *B. fasciatus* bite in humans have reported neurotoxic symptoms. The toxicity of *B. fasciatus* is considerably less potent when compared to other kraits like *B. caeruleus*, *B, sindanus* or

B. multicinctus which has a median lethal dose (LD_{50}) < 0.2 µg/g, whereas, *B. fasciatus* venom from different countries have exhibited a higher LD_{50} of 0.45-2.55 µg/g [58]. In a case study from Bardhaman, West Bengal (India), 3 deaths due to bites from a single *B. fasciatus* have been reported. Autopsy findings and histological analysis of kidneys have revealed nephrotoxicity in such patients which exhibited cortico-medullary hemorrhage and infiltration of inflammatory cells. The duration from bite to death was estimated to be around 6 h during which the patients did not receive any medical treatment and were declared dead on arrival at the hospital [53]. Similarly, a retrospective analysis of snakebites from April 2018 to August 2022 at Demow Model Hospital of Sivasagar district in Assam revealed two incidences of *Bungarus fasciatus* bite in patients. The patients exhibited neuroparalytic clinical features such as ptosis, dysarthria, dysphagia, lethargy, blurring of vision and dizziness, however, no death was reported [16]. In another case report, *B. fasciatus* envenomation was reported from southern Myanmar where a 13-year-old boy was bitten on the right-hand middle finger while climbing a tree. He was taken to a hospital where onset of neurotoxic symptoms was observed after 2 h post-bite. The patient had drooping swollen eyelids and struggled to keep the eyes open, exhibited drowsiness and inability to speak, difficulty to open mouth or swallow saliva. The patient remained drowsy and developed severe ptosis, and a slight swelling was observed in the bite site. Since *B. fasciatus* venom specific antivenom was not available, monospecific Russell's viper antivenom was administered but it did not show any improvement in the neurotoxic symptoms. After 14 h of envenomation the patient died of respiratory failure [39].

A retrospective study from Ramanthibodi Poison Centre in Thailand over a period of 9 years (2008-2016) revealed 9 patients to be bitten by *B. fasciatus* out of which 3 were dry bites. The average time for onset of neurological symptoms in envenomated bite victims was 3 h. Apart from neurotoxic symptoms following envenomation, some other clinical effects (extra-neurological symptoms) such as high-blood pressure and tachycardia were observed in some patients. However, no death occurred in any of the envenomated patients and all of them recovered fully as they received antivenom treatment along with assisted ventilation during the recovery period [59]. From the above case studies, it can be inferred that *B. fasciatus*'s pathophysiological landscape in envenomated victims is associated with systemic symptoms such as neuromuscular

paralysis, blurry vision, drowsiness, ptosis, muscle pain, myotoxicity, respiratory failure, and death due to suffocation in absence of timely and appropriate treatment.

1.4 Antivenom therapy: Present scenario and limitations

Snakebite is mostly considered as a hazard associated with certain occupations predominantly affecting the vulnerable section of the society such as cultivators, herders, field labourers, irrigation workers, fish harvesters, people living in villages, huts and small thatches, migrating communities, and snake charmers. Often professionals like snake rescuers, scientists and snake-house workers also become victim to snakebites due to improper handling or while milking venoms [60]. The WHO has included snakebite in the list of "*Neglected* Tropical Diseases" in 2017 and decided to form a working committee which will design a detailed framework for resolving this problem globally [61]. The WHO estimated ~4.5 to 5.4 million snakebites annually out of which ~1.8-2.7 million individuals experience envenomation leading to development of severe symptoms resulting in mortality of 81,000 to 138,000 victims and around 400,000 people are left with long-lasting complications and disabilities [6]. India is one of the major snakebite hotspot of the world with an estimated 1.1 to 1.7 million bites annually of which 50-70% victims are envenomated, leading to ~58,000 deaths and 140,000 disabilities [9]. The actual number of snakebites is much more as a large fraction of population in developing and under developed countries depend on traditional medicines and healers for snakebite treatment and these numbers remain unreported in any database [16]. The death or permanent disability of a young member or sole bread earner of the family due to snakebite has a devastating impact on the whole family.

The most effective and common strategy to treat snakebite victims is the administration of antivenom which are whole (IgGs) or fragments of immunoglobulin ($F(ab')_2$) obtained after pepsin digestion, derived from serum of hyperimmunized animals (horses, sheep, mules, etc.) injected with the venom of an individual snake or the collective pool of venoms from several medically relevant snake species [62]. The antivenom's specificity plays an important role in its efficacy against venom toxins. Antivenom are mostly monovalent (species-specific), bivalent or polyvalent. Within the Indian subcontinent, polyvalent antivenom produced to target the venom of the "Big-Four" snakes are employed to treat most of the snakebite cases [63]. Monovalent antivenom are also available against *Daboia russelii* and *Naja kaouthia* venoms in India which are

manufactured by VINS Bioproducts Ltd. [64,65]. Bivalent antivenom are manufactured to target the venoms from two distinct snake species. In Taiwan, bivalent freeze-dried hemorrhagic antivenom (FHAV) target the venom of *Viridovipera stejneri* and *Protobothrops mucrosquamatus,* and Freeze-dried neurotoxic antivenom (FNAV) target the *Bungarus multicinctus*, and *N. atra* venoms [66]. The quantity of antivenom vials to be injected is determined by the amelioration of clinical manifestations exhibited by the patients [16]. The potency of the Indian polyvalent antivenom is extremely poor, with reconstituted antivenom being claimed to neutralize a minimum 0.6 mg of *D. russelii* and *N. naja,* and 0.45 mg of *B. caeruleus* and *E. carinatus* venom respectively. Thus, patients need numerous vials of antivenom injection to recover completely. It has been reported that on average, 51 antivenom vials were utilized for the treatment of elapid envenomation and 31 vials when envenomated by viperid snakes, from a hospital in Northern India [67]. Furthermore, the route of antivenom administration is also a crucial factor in determining the efficacy of antivenom therapy. The World Health Organization (WHO) has recommended the intravenous (*i.v*) route of antivenom administration to envenomated victims due to the speedy distribution of antibodies and greater bioavailability when compared to other routes [68].

The production of polyvalent antivenom should involve a series of complex processes followed by its distribution and subsequent administration in envenomated patients (Figure 1.3). According to the WHO (2017), the process involves 1. Collection of snake venom from healthy snakes which should adequately represent diverse snake populations such as sex and life-stage; 2. Preparation of the venom pool to be used for injection in animals; 3. Hyperimmunization of selected healthy animals (mostly equines) using the venom pool and subsequent monitoring of their health parameters; 4. Collection of plasma/blood from the animal when sufficient antibodies are produced due to immune response; 5. Preparation of the plasma pool; 6. Separation of immunoglobulins from plasma pool; 7. Formulation of immunoglobulins and aseptic processing; 8. Testing of antivenom purity (biochemical characterization, enzymatic and toxicological activity), and determination of in vivo efficacy; 9. Next step involves labelling and packaging of antivenom; 10. Finally, distribution to regions where the snakes, whose venom pool was used in step 1, are prevalent [69].

Numerous limitations are associated with currently available antivenom and antivenom therapy. Variations in venom composition of snakes belonging to same species, due to factors such as geographical locations, diet, sex, age and season, are responsible for variations in toxic manifestations in bite victims as well as batch variations in antivenom. Owing to such variable factors differences in the antigenicity of venom is observed which may be responsible for low efficacy of antivenom [70]. In India, there is documented evidences which indicate variations in venom constituents of *D. russelii* and *N. naja* from different regions, however, more than 80% of the venom used for the Indian polyvalent antivenom is sourced from a single place in Tamil Nadu (Mahabalipuram) via the Irula Snake Catchers' Industrial Co-operative Society (ISCICS).

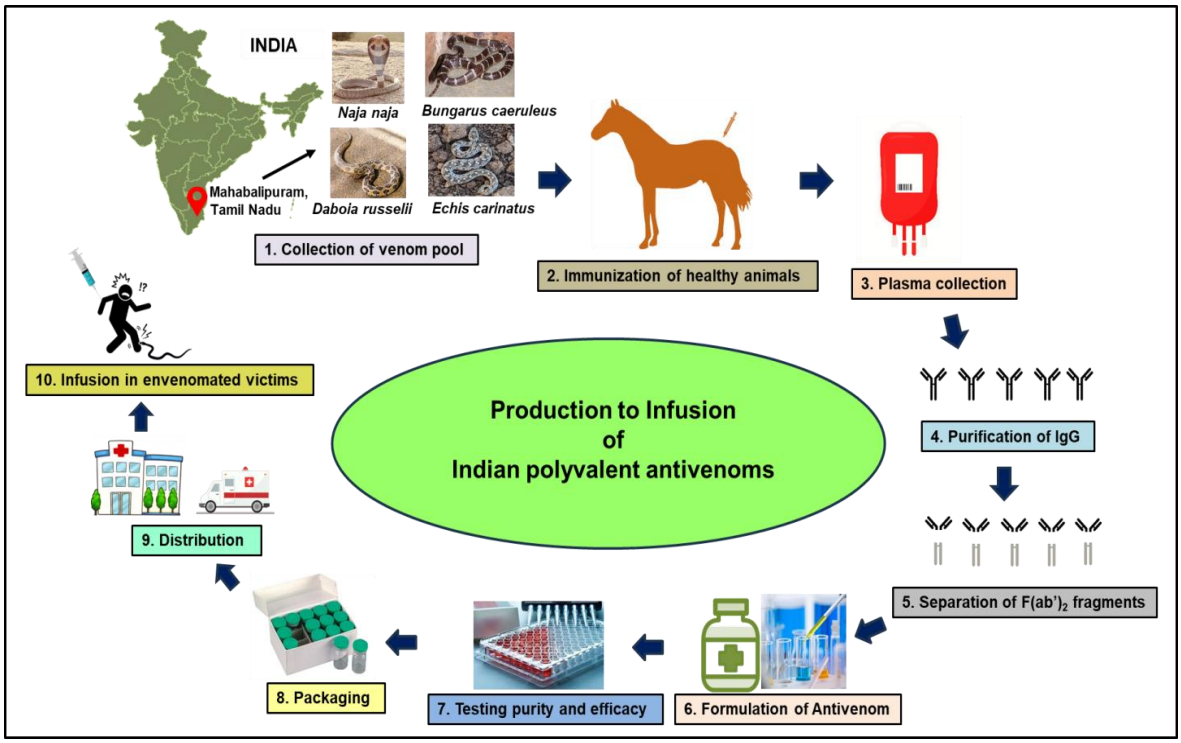

Figure 1.3: Critical steps involved in manufacturing of safe and effective polyvalent antivenom followed by its subsequent infusion in envenomated patients in India.

The quality of venom historically obtained from this traditional society (ISCICS) do not meet the requisite standards which typically apply to pharmaceutical raw materials, which may be an important factor that affects the overall efficacy of currently available antivenom [71]. There is also growing evidence which suggests that the Indian polyvalent antivenom becomes increasingly less potent with increasing distance from the source venom used for antivenom development. For instance, administration of the

standard 10-20 initial dose of antivenom failed to inhibit the capillary leak syndrome of *D. russelii* envenomation in Kozhikode, Kerala [72]. Another study by Sharma et al. on *D. russelii* venom from the South Indian states of Tamil Nadu, Karnataka and Kerala, and East Indian state of West Bengal, exhibited variation in the composition and its activity. Bharat Serums polyvalent antivenom differentially neutralized the venom induced coagulopathy and phospholipase A_2 activity. Moreover, the study also identified partially immunodepleted and non-immunodepleted proteins from the venom using antivenomics approach, which reflects that Indian polyvalent antivenom are not effective in recognizing all the proteins of a single snake species from different locations [73]. Similarly, monovalent antivenom (NNEV-IgG) developed against venom of *N. naja* from East India was effective in neutralizing the venom induced toxicity in mice at 1:25 ratio, however, the venom from Southern and Western India were not neutralized at this ratio, although the survival time was prolonged. When Indian polyvalent antivenom was used for the venom from Western India it could neutralize its effects at 1:60 ratio, although it could only slightly increase the survivability of mice infused with venom from Eastern and Southern India [74]. Although in vitro analysis using antivenomics approach, ELISA and Western Blotting reflect the binding of venom proteins to antibodies, it does not reflect the in vivo neutralization efficacy of antivenom. The antivenom may contain contaminants such as non-neutralizing antibodies alongside the toxin-neutralizing antibodies, leading to a potential competition between them [75]. The venom pool used during antivenom development may have one or more immunodominant epitope apart from the neutralizing epitope, due to which although the immunoglobulins raised against these venom proteins are able to detect similar proteins from other snakes in vitro but these antibodies are ineffective in vivo as the antivenom fails to bind to the neutralizing epitopes [76].

It has been observed that the venom toxins of the "Big-Four" snake species are not equally immunogenic. The venom toxins (mostly cytotoxins and neurotoxins) belonging to 3FTx superfamily (7 kDa) mostly present in elapid snakes are weakly immunogenic, whereas, the high molecular weight toxins (23-30 kDa) which are mostly hematotoxic, belonging to the SVMPs, SVSPs and PLA_2 superfamily are highly immunogenic [77]. As a result, the antivenom produced contains more immunoglobulins for the viperid venoms than the elapid venom. This variably affects their venom neutralization potencies with the antivenom exhibiting a higher potency against viperid venom than elapid venom

[78,79]. Moreover, when snakebite victims bitten by elapid snakes are treated with these polyvalent antivenom, the anti-viper immunoglobulins or their fragments remain unused and vice-versa, which may elicit adverse side-effects such as pyrogenic reactions [80].

Antivenom are not always effective against some associated complications of snakebite such as Venom induced consumption coagulopathy (VICC), which occurs as a result of rapid absorption of venom toxins [81,82]. Also, adverse reactions to antivenom therapy are triggered due to the entry of foreign proteins into the human body which necessitates prior allergy testing before antivenom therapy which leads to a loss of valuable time in such potential life-threatening situations [83,84]. The anaphylactic reactions can be classified as early reactions and late reactions. The early reactions appear from 10 to 180 min of antivenom infusion which may include anaphylaxis and pyrogenic fever. The anaphylactic reactions are induced due to the recognition of foreign antibodies by the immune system, and pyrogenic fever is induced due to the presence of microbial components in the antivenom such as lipopolysaccharides. For late reactions, a type III hypersensitivity reaction occurs 1 to 2 weeks after antivenom administration due to the circulating IgGs or antigen-antibody complexes which further activates the complement system and triggers inflammatory response [85–87]. Furthermore, the high cost, ideal storage conditions (4 ^{o}C) and lack of availability, associated with the requirement of multiple vials of antivenom for effective neutralization of venom toxins, pose a serious challenge to the people living in several low- and middle-income countries of Africa and Asia. It also lowers the confidence of the bite victims and doctors towards the treatment mode [88]. In a country like India, where the per capita income is estimated to be around 170,000 INR in 2022-23, a 10 ml vial of polyvalent antivenom costs around 500 to 935 INR, and although two vials are recommended for initial administration, a patient typically requires a minimum of 10 vials which puts the price of antivenom therapy to 1,000 to 9,350 INR. Some studies have also showed that some snakes such as cobras may inject up to 742 mg of venom during a single bite. The current Indian polyvalent antivenom can neutralize only 0.6 mg/ml of antivenom which means 6 mg cobra venom per vial. This means to neutralize 742 mg of venom, theoretically 124 vials of antivenom are required which would cost around 62,000 to 115,000 INR. This puts an unmanageable financial burden to the victim and their family who mostly belong to low income groups [89–91].

1.5 Alternative approaches to antivenom therapy

Owing to the limitations of anti-venom therapy, alternative therapeutic approaches are explored by researchers which may complement the current antivenom therapy. Various approaches explored in this regard include plant extracts or compounds isolated from plant extracts including their derivatives, components isolated from snake or other animal sera, small synthetic inhibitors including repurposed drugs, nanoparticles and oligonucleotide aptamers. The use of plant extracts to treat snakebites is followed by several tribes and communities of the tropical and subtropical countries as a traditional knowledge. The use of such medicinal plant extracts is the only available option for such communities where antivenom is not available. Plant extracts and compounds isolated from plant extracts have exhibited anti-inflammatory, anti-hemorrhagic, anti-neurotoxic, anti-myotoxic, and anti-edemic activity in various studies [92–95]. The active components of the plant species have also been able to reduce inflammation, reverse local tissue damage and pain [70]. However, the scientific validation for such plant extracts are limited and in their early stage due to the complex composition of such chemical compounds [95]. Inhibitors of snake venom toxins have been isolated from the plasma and serum of venomous as well as non-venomous snakes [96]. In venomous snakes, these inhibitors provide protection from their own venoms [97]. The Habu serum factor (HBS, 70 kDa) is a glycoprotein isolated from *Trimerusurus flavoviridis*. It was the first inhibitor isolated from snake sera belonging to cystatin superfamily and could inhibit HR-1 and HR-2 hemorrhagins from its own venom [98]. Several protease and anti-hemorrhagic inhibitors from snake species such as *Crotalus atrox*, *Vipera palestinae*, *Natrix tessellate*, *Waglerophis merremii*, *Bothrops asper* and *T. muscrosquamatus* are subsequently isolated and characterized [99,100]. Similarly, PLA_2 inhibitors have been isolated from the serum and plasma of snakes belonging to genera *Elaphe*, *Laticauda*, *Crotalus*, *Naja*, *Bothrops* and *Agkistrodon* [101].

Although natural inhibitors have exhibited neutralization of various toxins of snake venom, however, their large-scale production remains a challenge. As a result, 'Small synthetic inhibitors' (SSIs) are explored as therapeutics against snake venom toxins due to their specificity and low toxicity [102]. These synthetic molecules and their analogues target snake venom toxins like PLA_2, SVSPs and SVHYs. Some of the compound which exhibited inhibition of snake venom PLA_2 activity include ethyl 3-coumarincarboxylate,

8-methoxy-coumestrol, 4-methoxysalicylaldehyde analogues, vanillic acid, and ethyl (4-chlorobenzoyl) thio-acetate to name a few [63]. Similarly, drug repurposing is another approach in which approved or clinical trial drugs originally designed and developed for treating other medical conditions are explored for their efficacy in some other condition such as anti-snake venom property. For instance, drugs like batimastat and marimastat which are developed for treating arthritis and rheumatoid cancer have exhibited anti-SVMP and anti-coagulant activity against the venom of *Bothrops. asper*, *Echis ocellatus* and *Crotalus atrox* venom [63,103].

Another approach for detection of venom and neutralizing its effects is the use of oligonucleotide aptamers, an alternative to conventional antibodies, which are produced through a method named Systematic Evolution of Ligands by Exponential enrichment (SELEX). However, development of oligonucleotide pool and selecting specific aptamers is a time intensive and costly method due to which *in silico* designing of aptamers has gained popularity among researchers [104,105]. For instance, an aptamer α-Tox-FL which is developed to detect the α-Toxin of *B. multicinctus* have exhibited its capability to detect the congeneric *B. caeruleus* venom as well [106]. Similarly, *in silico* designed aptamers developed against a snake venom PLA_2 from *D. russelii* (Daboxin P) exhibited in vitro inhibition of $sPLA_2$ and anticoagulant activity [107]. The only US FDA-approved aptamer is Macugen (Pegaptanib sodium injection), which was first approved in 2005, and is used for the treatment of an eye condition called wet age-related macular degeneration (AMD). A number of other aptamers are in different phases of clinical trials for treating medical conditions like coronary artery disease, hemophilia, cancer and diabetes [108].

Similar to aptamers, another approach to target venom toxins is the use of synthetic nanoparticles which are designed and developed from a library of nanoparticles through a process called directed chemical evolution (DCE) which is followed by an optimization step [109]. The snake venom toxins bind to these nanoparticles through a combination of weak interactions like ionic, hydrogen bonding, dipole-dipole and hydrophobic interactions [110]. For instance, a nanoparticle-based hydrogel has been engineered to mitigate the progression of local tissue damage due to elapid snake bite by binding and inhibiting the activity of PLA_2 and 3FTx toxins. The nanoparticle based hydrogel demonstrated a dose-dependent in vitro and in vivo suppression of the toxic effects of

Naja nigricolis venom [111]. The use of aptamers and nanoparticles for detection and mitigation of snake venom toxins are still in their early phase of research and development, and currently no such aptamer or nanoparticle-based treatment has been approved for therapeutic usage in snakebite patients.

1.6 *B. fasci*atus venom variation and antivenom cross-reactivity

1.6.1 *B. fasciatus* venom composition and intra-species venom variation

Snake venom comprises a complex blend of proteins and peptides that are pharmacologically active and comprise ~90-95% of the total dry weight. The remaining 5-10% of the venom composition comprises of nucleic acids, carbohydrates, amino acids, metal ions, lipids, citrate, organic molecules and metal ions [112–114]. Based on their primary sequence and molecular structure the snake venom proteins are broadly classified under enzymatic and non-enzymatic superfamilies [115,116] (Table 1). Numerous proteins and peptides have previously been extracted from *B. fasciatus* venom sourced from diverse geographical locations. The potent neurotoxin named Ceruleotoxin was isolated from *B. fasciatus* venom originating in Miami, USA. It has a molecular weight of 13 kDa, amino acid composition similar to phospholipases, and blocks postsynaptic transmission irreversibly [117,118]. Two protease inhibitors which are analogous namely VIIb (BF8b) and IX (BF9) consisting of 62 and 65 amino acid residues respectively was isolated from the venom of *B. fasciatus.* The inhibitors had a molecular weight of 6.2 kDa and inhibited Chymotrypsin [119,120].

Table 1.1: Superfamilies of snake venom.

Enzymatic Superfamilies	Non-Enzymatic Superfamilies
Phospholipase A_2 (PLA_2)	Three Finger Toxins (3FTx)
Snake Venom Metalloprotease (SVMP)	Kunitz-type serine protease inhibitor (KSPI)
Snake Venom Serine Protease (SVSP)	Sarafotoxins
Nucleases, Nucleotidases, Phosphomonoesterases	Fasciculins
Acetylcholinesterase (AChE)	Cysteine-Rich Secretory Proteins (CRiSP)
L-Amino Acid Oxidases (LAAO)	Disintegrins
Snake Venom Hyaluronidase (SVHY)	Snake C-Type Lectins (CTLs) and Snake C-type lectin-like proteins (Snaclecs)
Phosphodiesterase	Vascular Nerve Growth Factor (VNGF)
Nucleotidase ($5^{/}$-NUC)	Vascular Endothelial Growth Factor (VEGF)

A factor X activator was purified from the venom of *B. fasciatus* having a molecular weight of 70 kDa which activated factor X into factor Xaα and exhibited amidolytic activity against chromogenic substrate S-2266 and S-2302 [121]. BFPA which is a group-I secretory phospholipase A_2 ($sPLA_2$-I) of 118-amino acid length isolated from the venom of *B. fasciatus* exhibited antimicrobial properties against *Escherichia coli* and *Staphylococcus aureus* [122]. A cathelicidin like-peptide named Cathelicidin-BF with antimicrobial properties was isolated from the venom of *B. fasciatus* from China. The Cathelicidin-BF was effective in killing bacterial and some saprophytic fungal species [123]. A KSPI was isolated from *B. fasciatus* venom from China and was named as Bungaruskunin having 59 amino acid sequences. The protein had structural similarity and a highly conserved signal peptide sequence similar to β-Bungarotoxin which suggest their possible common ancestry [124]. A 13 kDa protein BF-CT1 isolated from *B. fasciatus* venom from Kolkata, India exhibited cytotoxicity in U937 (histiocytic lymphoma) cell lines as well as in BALB/c mice model of Ehrlich ascites carcinoma [125]. Another Kunitz-type serine protease named Fasxiator, which inhibits Factor XIa have been extracted from the venom of *B. fasciatus* from Paris, France. It exhibited its anticoagulant activity by prolonging the activated partial thromboplastin time (APTT) [126]. A venom fraction named BF-F47 was isolated from the *B. fasciatus* venom from Kolkata by ion exchange chromatography and HPLC. It exhibited anti-osteoarthritic activity in male Wistar rats by preventing bone destruction and promoting partial restoration of joints [127].

The proteome of *B. fasciatus* venom from various countries like China, Vietnam, Malaysia, India, Thailand, Myanmar and Indonesia have been elucidated using LC-MS/MS analysis by different authors over the years [58,78,128–130]. Variation in the presence of different venom superfamilies and their corresponding proportion was observed among all the *B. fasciatus* venom samples (Figure 1.4).

Variation in the amount of venom proteins belonging to the PLA_2, 3FTx, KSPI, AChE and SVMP superfamily were observed, which was present in every venom samples, moreover, the remaining venom superfamilies were present in some and absent in other samples (Figure 1.4). For instance, the *B. fasciatus* venom from India contained PLA_2 superfamily as the major component (74.39%), similar to Vietnam (66.83%), Malaysia (44.2%), Thailand (65.82%), Myanmar (76.18%) and China (91.88%), however, the

venom from Indonesia consisted of the KSPI superfamily (48.96%) as the major component with the PLA_2 component being only 29.47%. Similarly, proteins belonging to LAAO superfamily were absent for the samples from Thailand and Myanmar, 5′-NUC and phosphodiesterase were absent for samples from Thailand and China, CRiSP superfamily was present only in the samples from Vietnam and Malaysia, VNGF superfamily was absent in samples from Malaysia and Indonesia, Snaclecs and Phospholipase B were present only in the samples from Vietnam and India, VEGF were present only in the samples from Vietnam and Malaysia, and Serine protease superfamily was present only in the samples from Malaysia and India. Moreover, the Complement depleting factors were reported only from the sample from Vietnam. The diversity of venom composition within individuals of *B. fasciatus* and other snakes in general is due to the interplay of various factors such as geographical location, habitat, diet, sex, age and season [131,132].

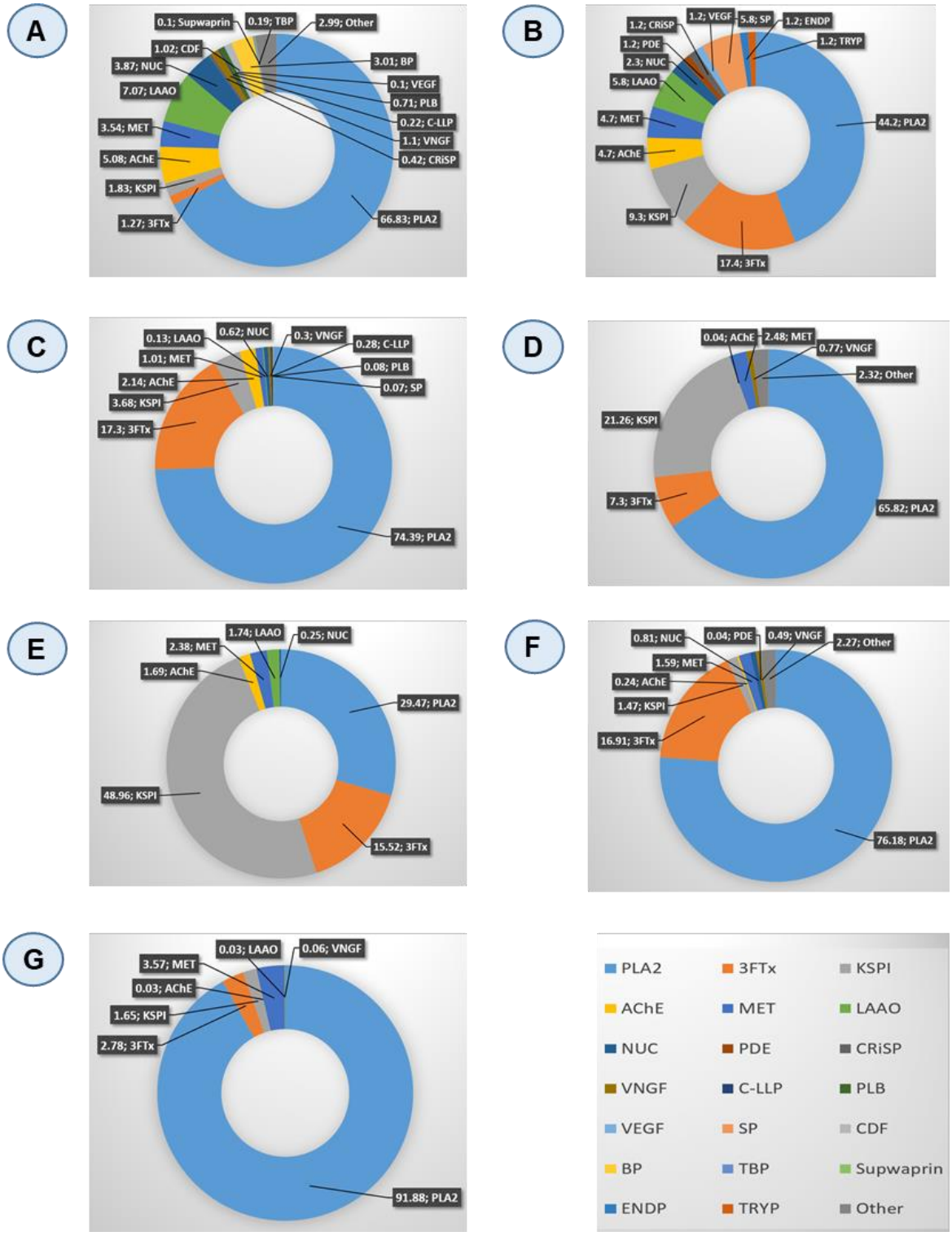

Figure 1.4: Intra-specific venom variation of *B. fasciatus* from seven different countries. A. Vietnam, B. Malaysia, C. India, D. Thailand, E. Indonesia, F. Myanmar, and G. China. (A. adapted from Ziganshin et al. [128]; B. adapted from Rusmili et al. [129]; C. adapted from Senji Laxme et al. [78]; D., E., F. and G. adapted from Hia et al. [58]).

1.6.2 *B. fasciatus* venom and antivenom cross-reactivity

Variation in intra-species venom composition may be responsible for the differential manifestation of pathophysiological symptoms in envenomated victims and the effectiveness of antivenom therapy [133]. The efficacy of the antivenom used (either monovalent or polyvalent) for treating envenomated victims depends on the composition of the venom/venom pool utilized in the immunization concoction to raise antibodies. Monovalent antivenom are superior to polyvalent antivenom in treating bite victims due to specificity to the snake venom used for development of the antivenom, although prior identification of the snake species by the clinician is necessary before the administration of monovalent antivenom [134]. Monovalent antivenom against *B. fasciatus* venom has been developed in Thailand (Queen Saovabha Memorial Institute) known as the *Bungarus fasciatus* Monovalent antivenom (BFMAV) is found to have differential median effective concentration (EC_{50}) in binding activity to *B. fasciatus* venom from 5 countries, *i.e.*, Malaysia (EC_{50} = 13.90 ± 0.63 µg/ml), Indonesia (EC_{50} = 13.86 ± 0.94 µg/ml), Myanmar (EC_{50} = 12.19 ± 0.94 µg/ml), China (EC_{50} = 11.43 ± 0.80 µg/ml), and Thailand (EC_{50} = 11.31 ± 1.11 µg/ml). Differential potency (mg/ml) of BFMAV against the *B. fasciatus* venoms injected in mice were also observed where the quantity of venom fully neutralized by a single antivenom unit has been highest from Thailand (1.61 mg/ml), followed by Myanmar (0.99 mg/ml), Indonesia (0.90 mg/ml), China (0.45 mg/ml) and Malaysia (0.19 mg/ml) [58]. The amount of venom typically injected by *B. fasciatus* in one venomous bite may range from 20-114 mg [135,136]. Hence, the number of BFMAV vials (total volume 10 ml per vial) required to completely neutralize the *B. fasciatus* venom in the victim from these five countries can be theoretically estimated to be 1-8 vials for Thailand, 2-12 vials for Myanmar, 3-13 vials for Indonesia, 5-26 for China and 10-60 vials for Malaysia.

Rusmili et al. studied the effectiveness of monovalent BFMAV, monovalent *Bungarus candidus* antivenom (BCAV), and Neuro-polyvalent antivenom (NPAV) from Thailand, for neutralizing the venom toxicity of *B. fasciatus* from Malaysia by inhibiting indirect twitches in chick biventer cervicis nerve-muscle preparation [18]. It was observed that a minimum of 3-fold the recommended concentration of BFMAV and NPAV was needed to counteract the inhibition of indirect twitches. The monovalent BCAV failed to inhibit the effects of *B. fasciatus* venom indicating differences in venom composition of

between *B. fasciatus* and *B. candidus* and an absence of congeneric cross-neutralization by the antivenom. Moreover, the antivenom were not effective even at high titre to prevent the inhibition of twitches when added at the t_{90} time-point *i.e.*, the time required for 90% inhibition of the twitch height. This could be attributed to the irreversible nature of the pre-synaptic neurotoxins present in the venom [18]. This failure of antivenom to considerably reverse the snakebite associated systemic toxicity following a definite time interval may have significant consequences for the patient's health and life. Hence, early presentation of the patient to the hospital using rapid transportation followed by rapid diagnosis and identification of snake species may enable early species-specific monovalent antivenom administration and necessary supportive care [137].

Antivenom manufacturing companies do not produce monovalent antivenom against the venom of *B. fasciatus* in the Indian subcontinent, hence, Indian polyvalent antivenom are commonly used for victims envenomated by the snake. Senji Laxme et al. studied the efficacy of four commercially available polyvalent antivenom in India produced by Premium Serums and Vaccines Pvt Ltd., Bharat Serums and Vaccines Pvt. Ltd., Haffkine Bio-Pharmaceuticals Co. Ltd., and VINS Bioproducts Ltd. targeting the *B. fasciatus* venom from Eastern India [78]. All the four Indian polyvalent antivenom performed poorly in recognizing the venom of *B. fasciatus*, the detection titre being extremely low (1:100) for Premium Serums and low (1:500) for the remaining three antivenom. In preclinical in vivo neutralization study using Premium Serums polyvalent antivenom in rats revealed a neutralization efficacy of 0.64 mg/ml, which suggests its potential application to treat bite victims of *B. fasciatus* [78]. However, there is a rising demand to enhance the effectiveness of the Indian polyvalent antivenom so that they can identify and counteract the harmful pathophysiological manifestations of different venom proteins from medically important snakes like *B. fasciatus*.

1.7 Need for the study

Previous studies reported considerable variations in the composition of *B. fasciatus* venom from South and South-East Asian countries like India, Myanmar, Malaysia, Indonesia, Vietnam and China [58,78,128–130]. Although *B. fasciatus* holds significance as a medically important snake species of Eastern and North-East India, there has been no comprehensive study on the compositional differences between the venoms from these locations. Indian polyvalent antivenom are administered to patients envenomated by *B. fasciatus* as monovalent antivenom are not manufactured by antivenom manufacturing companies in India against this venom. Hence, there is a need to understand the compositional variation in venom and their cross-neutralization by Indian polyvalent antivenom which may help to develop more effective antivenom.

1.8 Research Hypothesis

Understanding the venom compositional variation of B. fasciatus from different regions of India will help in isolating unique/non-immunodepleted proteins which may be utilized to enhance the effectiveness of Indian polyvalent antivenom.

1.9 Aim of the study

To understand the intra-specific venom variation of *B. fasciatus* from Eastern and North-East India by analyzing their profile and biochemical activities, and their immuno-crossreactivity with Indian polyvalent antivenom to isolate and characterize unique/non-immunodepleted protein/s.

1.10 Objectives of the study

1. *Comparative analysis of biochemical activities of crude B. fasciatus venom from different locations of Eastern and North-East India.*
2. *Understanding the immuno-cross reactivity of B. fasciatus venom from different locations of Eastern and North-East India with commercially available Indian polyvalent antivenom.*
3. *Isolation and characterization of unique/non-immunodepleted protein/s from crude B. fasciatus venom.*

1.11 Experimental approach

The experimental approach to fulfil the above-mentioned objectives has been described in the form of a flow chart as follows:

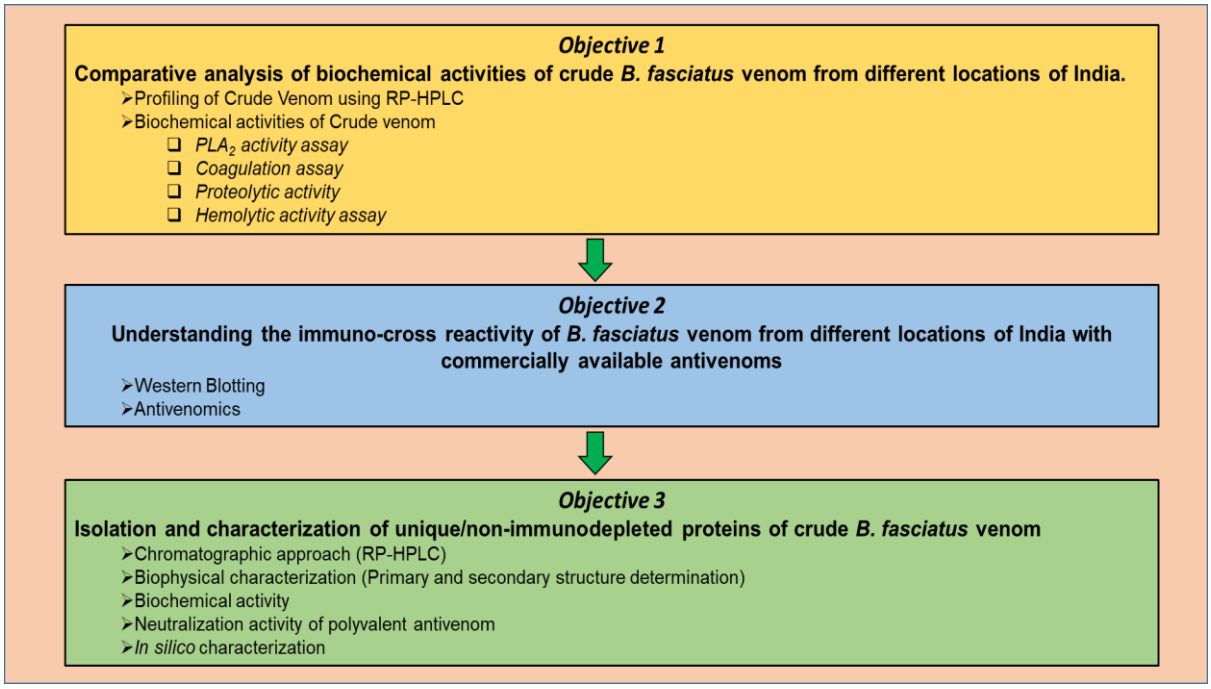

Figure 1.5: Flow chart of Research objectives.