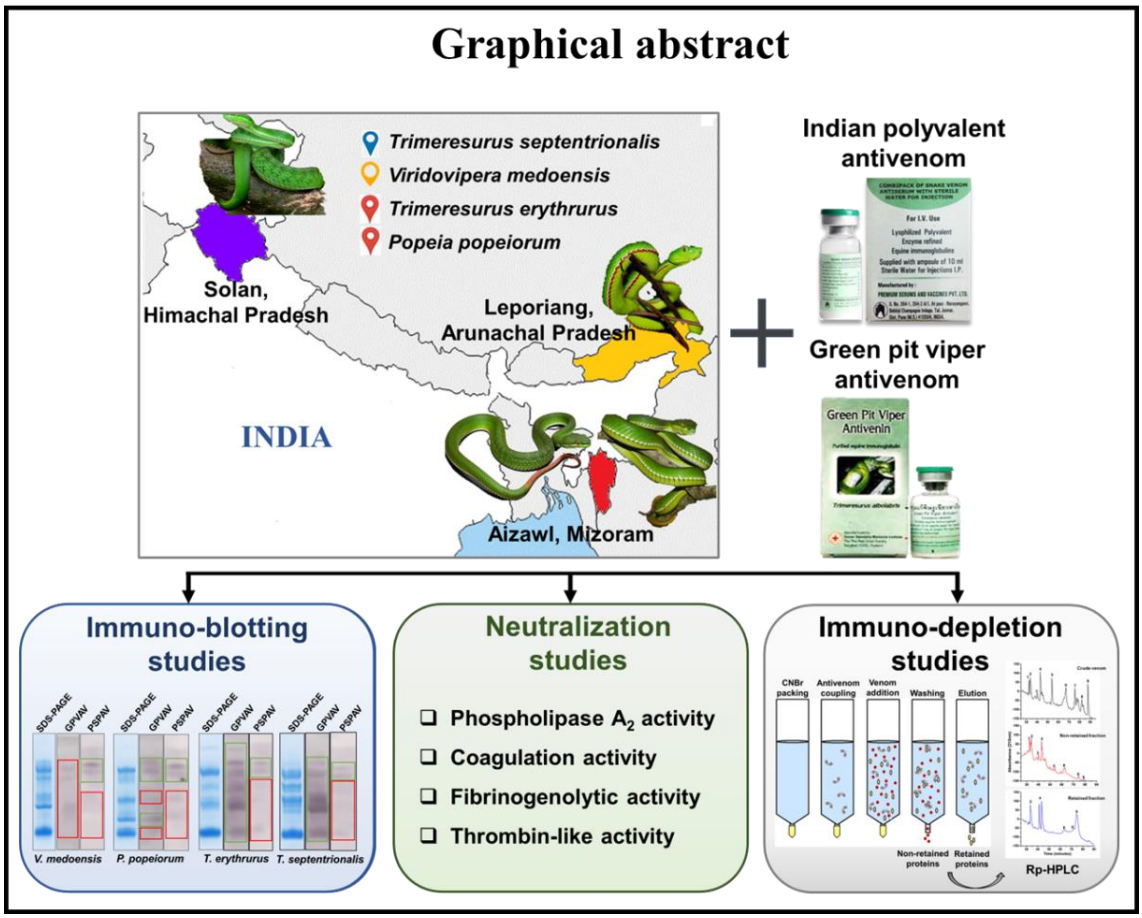
# CHAPTER 4

## Immunological cross-reactivity of Indian green pit viper venoms with Indian Polyvalent Antivenom and Thai Green pit viper monovalent antivenom

# Chapter 4

# Immunological cross-reactivity of Indian green pit viper venoms with Indian Polyvalent Antivenom and Thai Green pit viper monovalent antivenom




## 4.1. Introduction

Snakebite is a life-threatening disease, and the only available treatment for this disease is the intravenous administration of antivenom [52]. Antivenoms are purified IgG molecules or their enzymatically digestion fragments which is raised upon hyperimmunization of large mammals like horses and sheep with snake venom [205, 206]. The immunization mixture might either contain venom from a single snake species

or a mixture of venom collected from multiple species, giving rise to monovalent or polyvalent antivenom respectively [205]. The polyvalent antivenom is commonly used in many countries as it can be difficult to determine which snake caused the bite and a venom detection tool is not readily available. Although being the universal antidote for snakebite, antivenom therapy poses some serious limitations such as inefficient neutralization, inability to combat tissue damage, side effects like anaphylactic shock, serum sickness, pyrogenic reactions etc. leading to significant morbidity in victims [39, 42, 47, 184].

In India, polyvalent antivenom raised against an immunization mixture containing a venom pool of the "Big-Four" snakes is used for treatment of all the venomous snake bites including green pit vipers [17]. Although Indian polyvalent antivenom is largely effective against envenomation caused by the Big-Four, poor efficacy in mitigating the toxic effects of heterologous venom has been reported in literature [46, 76, 207]. The unavailability of specific antivenom for green pit vipers in India presents an urgent need to evaluate the effectiveness and toxin recognition profile of existing Indian polyvalent antivenom towards the venom of Indian green pit vipers. Moreover, a monovalent antivenom raised against *Trimeresurus albolabris* is administered for green pit viper envenomation in Thailand. Various studies have reported better neutralization of green pit viper venoms with Thai green pit viper monovalent antivenom compared to country-specific polyvalent antivenom [121, 122, 124, 208]. Thus, the present study was conducted to assess the immuno-reactivity of commercially available Indian polyvalent antivenom towards the crude venoms of Indian green pit vipers and comparison with immuno-reactivity of Thai green pit viper monovalent antivenom. Also, a case record highlighting clinical features of green pit viper bites and their treatment protocol with respect to effect of antivenom therapy was further investigated.

## 4.2. Results

### 4.2.1. Immunoblot analysis

Cross-reactivity of Indian polyvalent antivenom (PSPAV) and Thai Green Pit Viper monovalent antivenom (GPVAV) towards crude venoms were analysed by western blot assay (Figure 4.1). The immunoblot profiles show variable recognition and non-recognition of protein bands in comparison to the SDS-PAGE profile. GPVAV

recognized all the bands present in *Trimeresurus erythrurus* and *T. septentrionalis* venom, partially recognized a few bands in *Popeia popeiorum* venom (55-72 kDa and ~25 kDa), and did not recognize any of the bands in *Viridovipera medoensis* venom (Figure 4.1). However, Indian polyvalent antivenom (PSPAV) consistently recognized high molecular weight protein bands belonging to molecular weight range of 55-72 kDa but could not recognize any other bands in crude venoms tested.

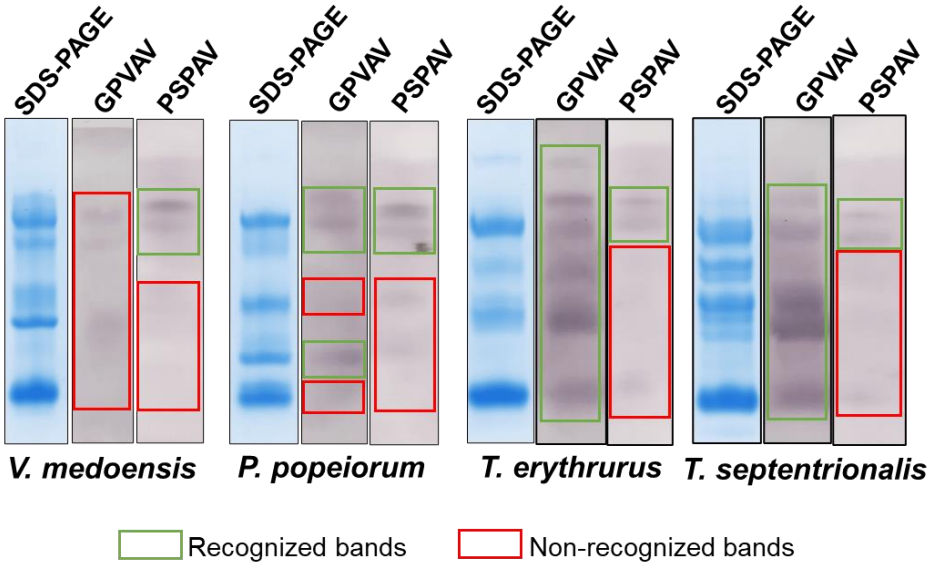



**Figure 4.1. Immunoblot profiles of crude venoms of green pit vipers against GPVAV and PSPAV antivenin. SDS-PAGE-** Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis, **GPVAV-** Green Pit-viper antivenom (monovalent) blot**, PSPAV-** Premium serum polyvalent antivenom blot. 20 µg of each crude venom was run on 10% Tris-Tricine gel followed by western blotting using GPVAV and PSPAV.

### 4.2.2. Neutralization of biochemical activities by antivenoms

Premium serum polyvalent antivenom (PSPAV) and green-pit viper antivenom (GPVAV) was evaluated for its ability to neutralize various biochemical activities exhibited by crude venoms. When neutralization of $PLA_2$ activity was assessed, it was observed that PSPAV could most efficiently neutralize *T. erythrurus* venom with 79% inhibition, while the least inhibited venom was *T. septentrionalis* with only 7% inhibition (Figure 4.2). GPVAV on the other hand shows excellent inhibition of $PLA_2$ activity (94-97%) in *P. popeiorum*, *T. erythrurus* and *T. septentrionalis* venoms, with slightly lower inhibition percent (88%) in *V. medoensis*.

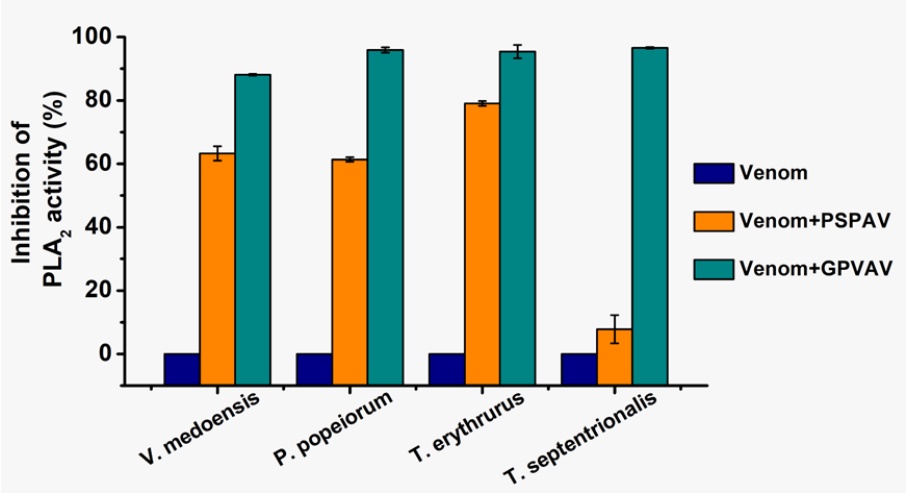



**Figure 4.2. Neutralization of $PLA_2$ activity of crude venom of green pit vipers.** PSPAV- Premium serum polyvalent Antivenom, GPVAV- Thai Green pit viper antivenom (monovalent). Percent inhibition was calculated by considering activity of crude venom as 100%. Each data represents mean ± SD of three independent experiments.

For thrombin-like activity, both GPVAV and PSPAV showed highest inhibition of *V. medoensis* venom, followed by decreasing inhibition in *T. septentrionalis*, *T. erythrurus* and *P. popeiorum* venoms respectively (Figure 4.3); however, efficacy of PSPAV was found to be considerably lower than GPVAV. GPVAV could inhibit all the crude venoms much efficiently in a range of 65-95%, whereas PSPAV showed a maximal inhibition of only 25% for *V. medoensis*.

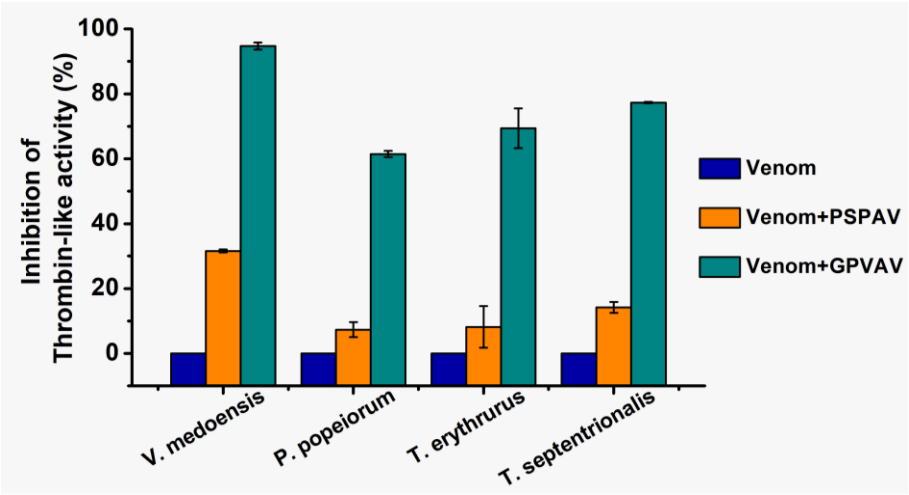



**Figure 4.3. Neutralization of thrombin-like activity of crude venom of green pit vipers.** PSPAV- Premium serum polyvalent Antivenom, GPVAV- Thai Green pit viper antivenom (monovalent). Percent inhibition was calculated by considering activity of crude venom as 100%. Each data represents mean ± SD of three independent experiments.

Both PSPAV and GPVAV showed some neutralization of the procoagulant activity of crude venoms, with effective inhibition of *T. erythrurus* and *T. septentrionalis* venom and feeble inhibition of *P. popeiorum* and *V. medoensis* venoms (Figure 4.4). The sharp decrease in the clotting time up to 40 sec and 50 sec compared to the NCT of 180 sec in case of *T. erythrurus* and *T. septentrionalis* respectively represent strong procoagulant nature of venom. However, clotting time of plasma treated with both PSPAV and GPVAV was observed to be similar to NCT indicating neutralization of recalcification time of crude venom by antivenoms.

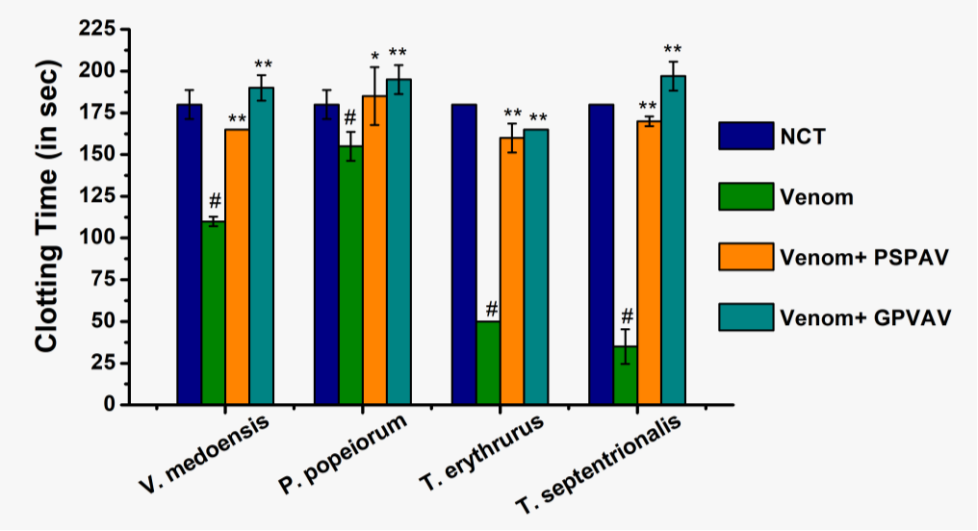



**Figure 4.4. Neutralization of procoagulant activity of crude venom of green pit vipers.** PSPAV- Premium serum polyvalent Antivenom, GPVAV- Thai Green pit viper antivenom (monovalent). Each data represents mean ± SD of three independent experiments. # *p* value < 0.01 with respect to NCT, * *p* value < 0.05 and ** *p* value < 0.01 with respect to crude venom.

The efficacy of antivenoms in neutralizing fibrinogenolytic activity was assessed by observing the bands of fibrinogen treated with venom and antivenom in SDS-PAGE (Figure 4.4). It was observed that fibrinogen treated with crude venoms showed complete degradation of Aα band of fibrinogen. Upon treatment with GPVAV, re-appearance of Aα band was observed for all the crude venoms suggesting that GPVAV could potently neutralize the fibrinogenolytic activity. Whereas, PSPAV showed partial neutralization of fibrinogenolytic activity in *V. medoensis* indicated by re-appearance of faint Aα band. Presence of prominent Aα band in PSPAV treated samples in all the other crude venoms suggests complete neutralization of fibrinogenolytic activity.

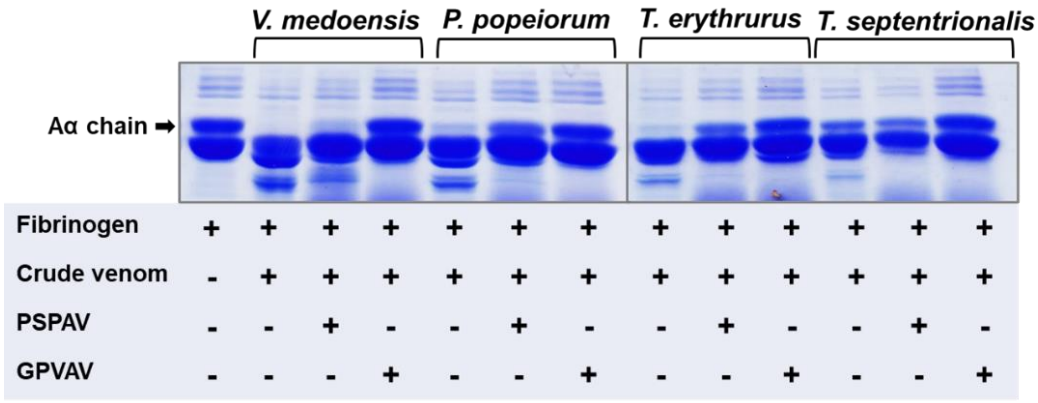



**Figure 4.5. Neutralization of fibrinogenolytic activities of crude venom of green pit vipers.** PSPAV- Premium serum Polyvalent Antivenom, GPVAV- Thai Green pit viper antivenom (monovalent). Presence or absence of each component in the reaction mixture has been represented by '+' and '-' respectively.

### 4.2.3. Immuno-depletion study

The immuno-capturing capacity of Premium serum polyvalent antivenom (PSPAV) towards the venom toxins of green pit vipers under study was performed by immuno-depletion experiments. Immuno-affinity columns immobilized with 4 mg of Indian polyvalent antivenom was allowed to capture the venom toxins of 50 µg crude venom of green pit vipers (antivenom: venom ratio was 80:1) and the retained and non-retained fractions were subjected to Rp-HPLC. A comparative chromatographic profile of crude, non-retained and retained fractions of each venom is shown in Figure 4.6 and 4.7. The immuno-depletion study of Indian green pit viper venoms towards Indian polyvalent antivenom shows presence of maximum peaks in non-retained fractions compared to retained ones, suggesting the low immune-capturing capacity of Indian polyvalent antivenom (Figure 4.6 and 4.7). Some of the peaks were observed to be present in both retained and non-retained fraction suggesting either their partial recognition by antivenom or speedy saturation of toxin specific epitopes. The inefficacy of antivenom in recognizing most of the venom toxins even at a high ratio of venom and antivenom (1:80) owes to the fewer number of toxin specific epitopes due to the para-specific nature of antivenom.

The Rp-HPLC profile of *V. medoensis* shows a total of 9 peaks in the crude venom, out of which only Peak 2, 4 and 9 were found in the retained fraction (Figure 4.6A) depicting the presence of epitopes specific for structurally similar toxin. Non-retained fraction, however, contained 8 peaks of varying intensity, of which the proteins

eluted after 50 minutes were poorly recovered. Peak 1, 3 and 5 were not recognized by antivenom at all and are thus non-immunodepleted toxins. Further, Peak 2 and 4 were partially immunodepleted proteins due to their occurrence in both retained and non-retained fraction suggesting the presence of fewer epitopes for these peaks which gets saturated very soon (Figure 4.6A).

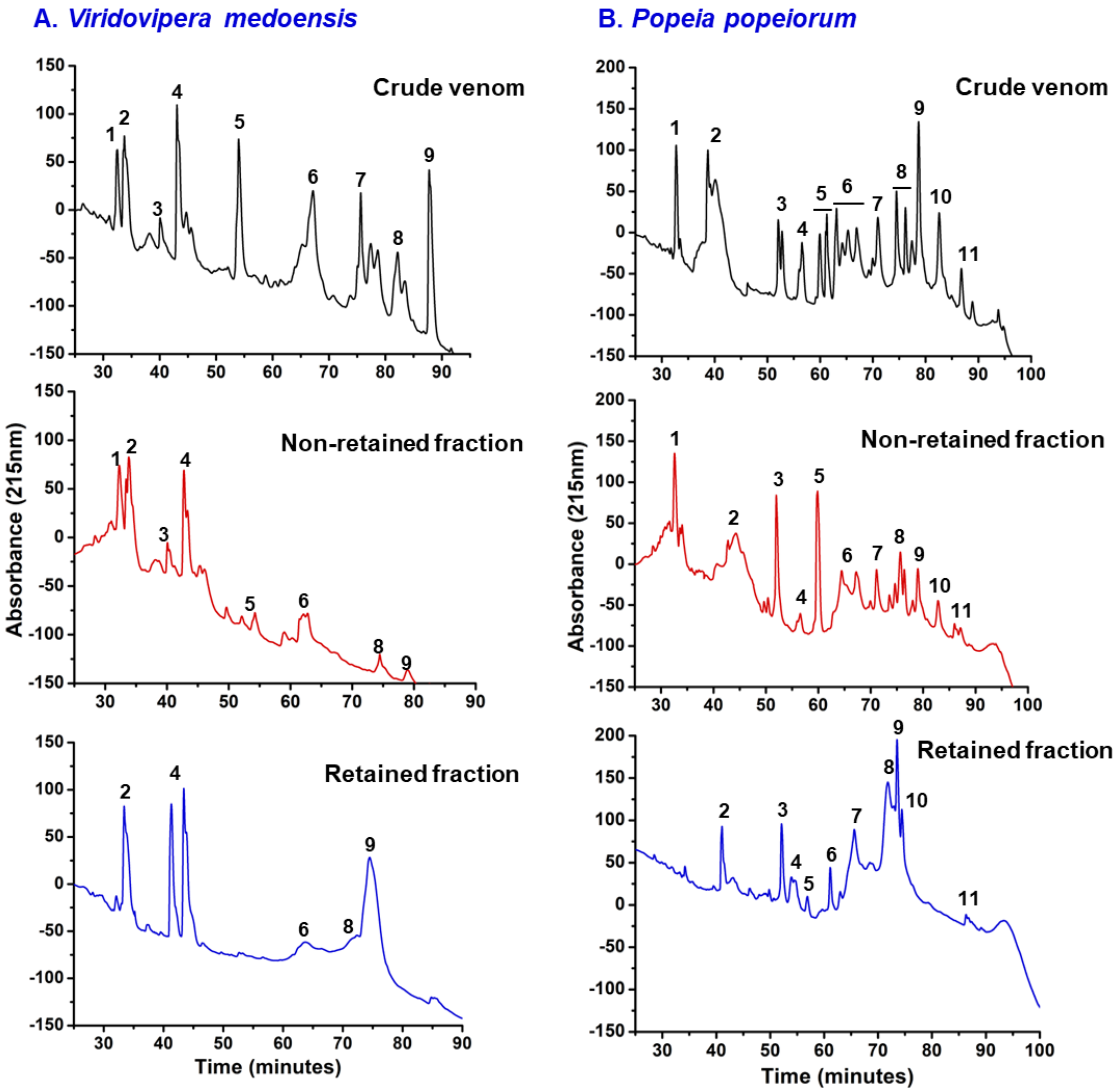



**Figure 4.6. Immuno-depletion study of crude venom of green pit vipers using Premium serum Polyvalent antivenom (PSPAV). (A) *Viridovipera medoensis*, (B) *Popeia popeiorum*.** Reverse phase- HPLC profiles of crude venom, non-retained and retained fractions retrieved from immunoaffinity columns incubated with 50 µg of venom.

All the 11 peaks of *P. popeiorum* venom was observed in the non-retained and retained fractions except Peak 1, however their intensity vary to a great extent (Figure 4.6B). Peak 1 represents the non-immunodepleted toxin as it was totally absent in

retained fraction. The peaks eluted after 60 minutes of run time were poorly recovered in non-retained fraction compared to a better recovery in retained fraction. Also, peak 4, 5 and 11 was observed as very small peaks in retained fraction inferring their partial recognition or the presence of few epitopes which could recognize very few proteins (Figure 4.6B).

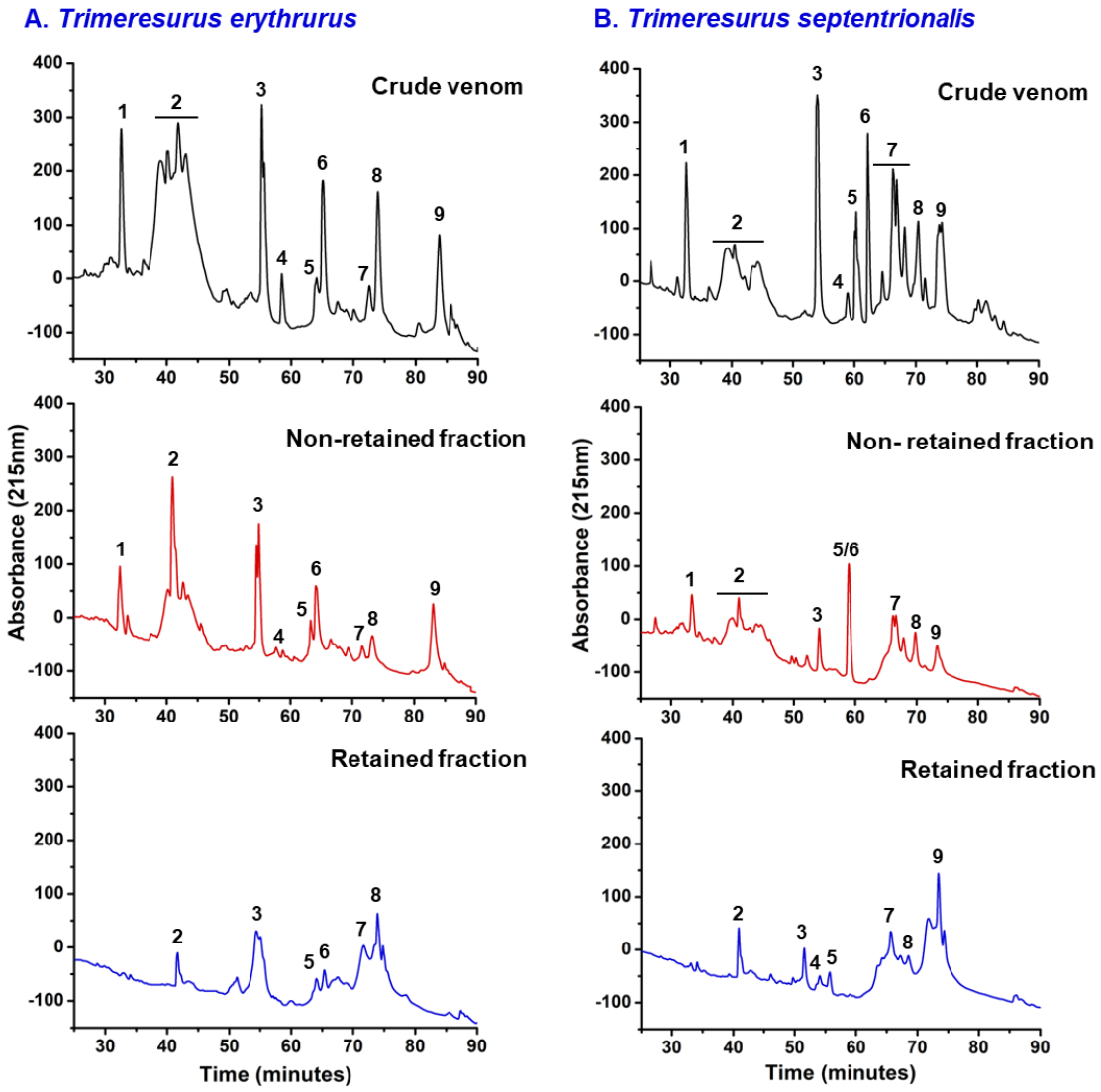



**Figure 4.7. Immuno-depletion study of crude venom of green pit vipers using Premium serums Polyvalent antivenom (PSPAV). (A) *Trimeresurus erythrurus*, (B) *Trimeresurus septentrionalis.*** Reverse phase- HPLC profiles of crude venom, non-retained and retained fractions retrieved from immunoaffinity columns incubated with 50 µg of venom.

The Rp-HPLC profile of both *T. erythrurus* and *T. septentrionalis* shows 9 peaks of variable heights, of which Peak 1 was present only in the non-retained fractions of both venoms, making it a non-immunodepleted protein (Figure 4.7A and 4.7B). Further,

Peak 9 contained completely non-immunocaptured proteins and was absent in retained fraction (Figure 4.7A), which, in contrast, was completely immuno-captured in *T. septentrionalis* venom (Figure 4.7B). For *T. erythrurus*, Peak 2, 3, 5 and 6 were partially immune-depleted peaks which although present in retained fraction, showed very low intensity (Figure 4.7A). However, Peak 7 and 8 were better recognized by antivenom, depicted as taller peaks in the retained chromatogram. Moreover, for *T. septentrionalis* venom, peak 2, 3, 7 and 8 were eluted in both retained and non-retained fraction suggesting their partial immuno-recognition by polyvalent antivenom (Figure 4.7B).

### 4.2.4. Clinical data on green pit viper bites

Data on green pit viper envenomated patients treated with Indian polyvalent antivenom was collected from Demow community health centre, Sivasagar, Assam. Clinical manifestations in 100 patients envenomed by green pit vipers were analysed. On admission, a thorough physical examination of patients was performed to observe fang marks, swelling, blisters, tenderness along with temperature and pulse rate. Blood tests included the standard WBCT20 test [209], prothrombin time (PT) and International normalized ratio (INR) on the day of admission and on $3^{rd,}$ $6^{th,}$ $8^{th}$, $10^{th}$ day after the bite. The symptoms of patients were monitored carefully throughout the hospital time and treatment was given accordingly.

All patients showed primary symptoms such as progressive pain, extensive swelling of bite site and developed prolonged coagulopathy (Figure 4.8). No blood clot was observed in the standard WBCT20 test and prothrombin time (PT) test. International normalized ratio (INR), assays that measure how long it takes for a clot to form in a blood sample, were unrecordable on admission and upto 10 days after the bite (Figure 4.8D). Before 2018, due to unrecordable INR, clinicians treating these bites assumed it to be typical viper bite and followed standard advisory, with immediate infusion of 10 vials polyvalent antivenom and referral to a higher centre where another 10 vials may have been given, along with infusion of blood products such as fresh frozen plasma. However, due to ineffectiveness of antivenom administration in reversing the coagulopathy for 10 days, the protocol was discontinued after 2018. Antivenom as well as blood products were no longer given and the patients were treated conservatively. Tramadol oral tablet was used for pain relief. Tetanus toxoid was administered after normalization of INR. Ofloxacin ornidazole, ranitidine, trypsin-chymotrypsin and magnesium sulphate

compression dressing 12 hours apart were prescribed for swelling. Patients were advised for absolute bed rest. After normalization of clotting confirmed by both bed side 20WBCT and normalisation of INR in 8-10 days, patients were discharged with follow-up to 30 days post discharge. No fatalities or kidney injury were seen in any patients.

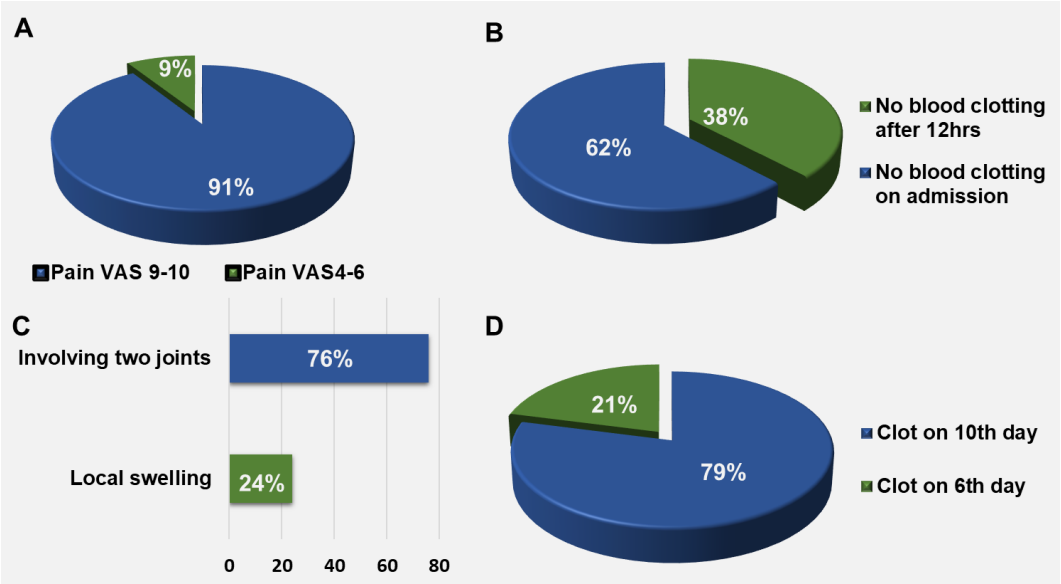



**Figure 4.8. Clinical features of 100 green pit viper bites recorded between 2010-2021 at Demow Community Health Centre, Sivasagar District, Assam.** Bites are mostly seen on limbs (62% on lower limb, 28% on upper limb). **(A)** Pain resulting from bites. VAS: Visual analogue score (10 indicating almost unbearable pain). Pain subsides within 26-50 hrs; **(B)** Swelling observed within 4-6 days of bite; **(C)** Coagulopathy observed in 100% of cases; **(D)** persistence of coagulopathy for an extended period.

### 4.3. Discussion

The clinical ineffectiveness of antivenoms due to venom variation is a serious concern in the field of snakebite management [47, 86, 210]. Inefficacy of antivenom is largely attributed to differences in immunogenic properties of various toxins [41] owing to the compositional variability of venom at taxonomic levels (both inter- and intra-specific), ontogenic and ecological factors [49]. The para-specific inefficacy of antivenom bestows restrictions in the use of polyspecific antivenom against heterologous and geographically distant homologous venoms. Thus, the improvement of antivenom quality as well as pre-clinical evaluation of antivenom efficacy has become the key to address this vital issue [51]. Various approaches have been prescribed for the assessment of cross-reactivity of antivenom with venom toxins such as immunoblotting, immunodiffusion, ELISA, *in vitro* and *in vivo* neutralization, and antivenomics [26, 41,

211]. Antivenomics is comparatively newer but most profound tool used now-a-days to quantify the extent of immuno-reactivity of an antivenom towards venom toxins of homologous and heterologous venoms [51]. In this context, a combination of various approaches, effectiveness of Indian polyvalent antivenom and Thai green pit viper monovalent antivenom against Indian green pit viper venoms were evaluated in the present study.

The crude venoms of Indian green pit vipers were subjected to immunoblotting using both Indian polyvalent antivenom and Thai green pit viper monovalent antivenom (GPVAV) as primary antibody. Analysis of blots to observe the pattern of immuno-recognition suggests differential antivenom efficacy of GPVAV in binding the venom toxins of the green pit viper species under study. The results were consistent with the generic revision of *Trimeresurus* sensu lato by Malhotra and Thorpe (2004) and the evolutionary relationships of $PLA_2$ toxins described by Malhotra et al. (2015) [99, 203]. Thai green pit viper antivenom is raised against the venom of *Trimeresurus albolabris,* and the venom of those snake species which are taxonomically closer to it viz. *T. erythrurus* and *T. septentrionalis* showed better recognition of bands in the blot than those which are more distantly related i.e., *Popeia popeiorum* and *Viridovipera medoensis*. Similarly, the differential antivenom efficacy of GPVAV in neutralizing coagulotoxicity of 13 species belonging to *Trimeresurus* sensu lato was demonstrated by Debono et al ; however, their results showed a prominent lack of phylogenetic pattern in venom variation and antivenom efficacy [212]. Further, the immunoblot of Indian polyvalent antivenom showed a consistent recognition of high molecular weight bands (55-72 kDa) and no recognition of mid-low molecular weight bands in the range of SVSPs and $PLA_2$s in all the tested crude venoms. The recognized high molecular weight bands representing SVMPs and LAAOs, possibly might be homologous to the venom toxins of viper venoms present in the immunization mixture used to prepare antivenom. Moreover, the non-recognition of certain proteins families such as SVSPs, $PLA_2$s etc. suggests poor or in-effectiveness of Indian polyvalent antivenom. Poor efficacy of Indian polyvalent antivenom in neutralization of *Craspidocephalus malabaricus* venom, belonging to *Trimeresurus* radiation has also been reported by Vanunjopadath and his team [82].

Green pit viper envenomated patients are characterized with painful swelling of bite site and incoagulable blood for several days. The clinical conditions of envenomated patients can be correlated with *in vitro* enzymatic activities shown by various venom protein families (discussed in chapter 3). Efficacy of monovalent and polyvalent antivenom in neutralizing *in vitro* enzymatic activity would provide insights on the enzymatic activity which cannot be neutralized even after administration of antivenom. Neutralization studies were performed by incubating crude venoms with antivenoms followed by *in vitro* enzyme assays. It was clearly demonstrated that GPVAV was better in neutralizing all the *in vitro* biochemical activities than Indian polyvalent antivenom. Previous studies also highlight better neutralization of green pit viper venoms with Thai green pit viper monovalent antivenom (GPVAV) compared to country-specific polyvalent antivenom in south-east Asian countries [121, 122, 124, 208]. However, the neutralization potency of GPVAV varied with respect to different biochemical activities as well as different species, which is again consistent with the previous reports of differential neutralization of coagulotoxicity of various green pit viper venoms by GPVAV [212]. On the other hand, Indian polyvalent antivenom has not been previously evaluated for cross-neutralization of *in vitro* biochemical activities in the crude venoms of *Trimeresurus* radiation. The present report demonstrates better neutralization of $PLA_2$ activity and procoagulant activity compared to partial neutralization of fibrinogenolytic activity and poor neutralization of thrombin-like activity of green pit viper species under study by the Indian polyvalent antivenom. Moreover, in addition to *in vitro* neutralization experiments, the incorporation of *in vivo* assays for preclinical evaluation of antivenom efficacy might provide substantiating confirmation regarding the immuno-reactivity of antivenom.

Although, antivenom raised as a result of immune response contains highly specific antibodies, tendency of antivenom to cross-react with venom toxins of heterologous snakes showing conformational similarities with immunogenic epitopes has been reported [31, 41]. Subsequently, the cross-reactivity of Indian polyvalent antivenom with toxins of heterologous venom of Indian green pit vipers were assessed by immuno-depletion studies using second generation antivenomics [63]. The toxins which could not be immuno-captured by antivenom was collected as non-retained fractions and the once that was captured was eluted as retained fractions. The collected non-retained and retained fractions were subjected to profiling by Rp-HPLC and compared with crude

venom profiles. The Rp-HPLC chromatograms revealed the occurrence of both non-immunodepleted and partially immunodepleted peaks in all the crude venoms. The non-immunodepleted peaks contains those proteins against which a specific antibody is absent in the antivenom, therefore, does not get immuno-captured by antivenom at all. These toxins might be the responsible components of the venom for the pathophysiological manifestations observed in the victims even after the antivenom administration. On the other hand, partially immuno-depleted proteins suggest presence of either an immunogenic epitope or a structurally similar epitope, which could generate an immune response during the production of antivenom, thereby immuno-captured by IgG present in the antivenom. However, the number of such immunogenic epitope appears to be relatively low leading to speedy saturation of epitope specific antibodies at a venom and antivenom ratio of 1:80. The toxins which remained non-immunocaptured as a result of epitope saturation, adds additional inefficacy to the antivenom treatment and demands for administration of an even higher dose to antivenom. The inefficacy of polyvalent antivenom in neutralizing para-specific venom is previously reported [46, 47, 86, 184, 213], the central cause being the diversity of species-specific toxins. Including venom toxins of region-specific snakes such as green pit vipers in the existing immunization mixture would lead to better neutralization of venom induced pathology, however, might increase the chances of additional non-specificity of polyvalent antivenom. Further, administration of higher doses of antivenom as well as non-specific antibodies might elicit an antigenic response and lead to side effects like anaphylactic shock, serum sickness, pyrogenic reactions etc [16, 39, 42, 214]. An alternative approach suggested by Ainsworth et al, 2018 demonstrates a preclinical basis of "pathology-specific" antivenom raised against mixture of toxins causing a particular pathophysiological effect (e.g., coagulopathy, haemorrhage) obtained from diverse taxa. (Ainsworth et al., 2018). The idea is theoretical at this stage, however, obtaining information regarding venom composition and pathophysiological effects of diverse snake families gives in the potential to recognize unexpected therapeutic benefits of existing antivenoms and lead to designing of a prototype for an effective pathology specific antivenom.

*In vitro* inefficacy of Indian polyvalent antivenom is reinforced by regional envenomation reports from Demow community health centre, Sivasagar, which confirms severe clinical conditions such as coagulopathy with incoagulable blood persisting for

many days post green pit viper bite even after administration of antivenom. The retrospective data obtained from the health centre establishes that Indian polyvalent antivenoms available in hospitals are inefficient to reverse the envenomation mediated pathology caused by green pit viper bites in northern and north-eastern India. The clinical study endorses the standard snakebite treatment protocol published in 2016 by the Directorate General of Health Services, Government of India (https://www.nhsrcindia.org/sites/default/files/2021-05/Snakebite_0.pdf). The standard treatment protocol advises against the use of antivenom for the treatment of envenomation featured by prolonged swelling accompanied by fang marks and incoagulable blood, which are the characteristics of a typical green pit viper bite. However, insufficient knowledge regarding snakebite management protocols among the health care workers as well as absence of a green pit viper specific monovalent antivenom in the region complicate the situation. The patient at any cost needs a prolonged stay in hospital till normalization of blood coagulation causing socio-economic burden to the victims and their families. Thus, there is an urgent need to develop an alternative approach for snakebite therapy specific for north and north-eastern India. Further studies should be aimed towards considering the feasibility and practical implications of the production and optimization of a regional/pathology-specific antivenom as well as exploration of alternative approaches such as APTMER based therapy for snakebite treatment [215, 216].