# CHAPTER 3

## Cross-reactivity of Indian polyvalent antivenom against *Bungarus fasciatus* venoms from different geographical locations of Eastern and North-East India

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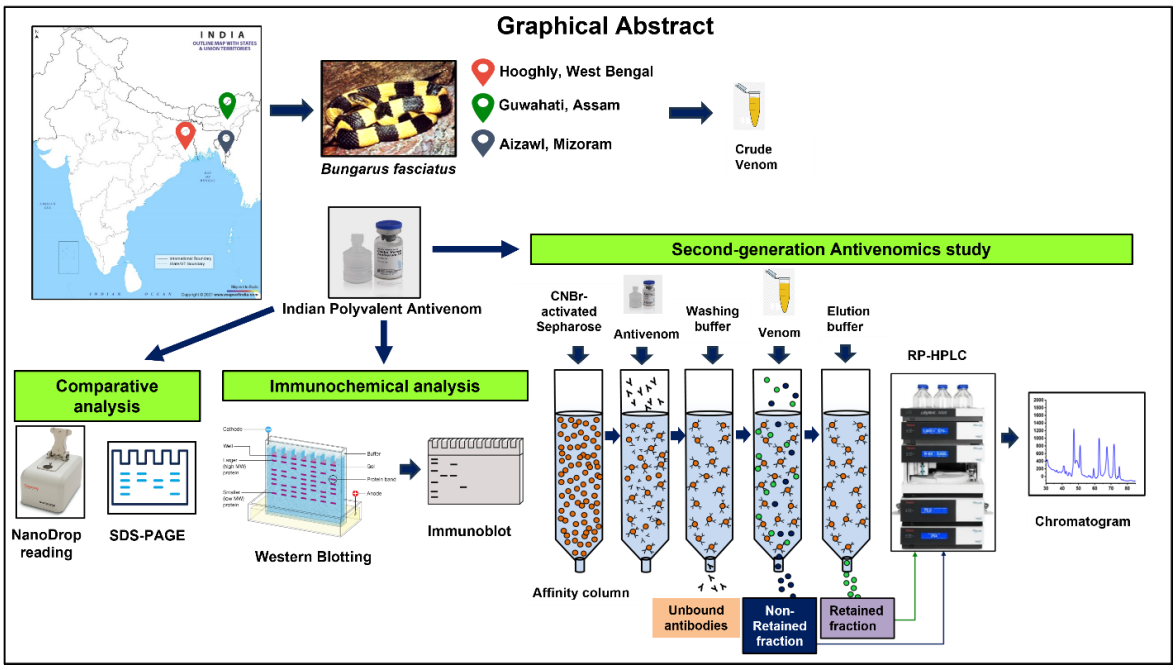


### 3.1 Introduction

Antivenom represent the sole efficacious therapy for snakebite envenoming. In India, polyvalent antivenom are administered for snakebite incidents including those from *B. fasciatus*. The polyvalent antivenom mainly contain purified immunoglobulin molecules such as whole IgG or fractionated fragments (F(ab) or $F(ab')_2$). Antivenom are stored at a low temperature (4 $^{o}$C) in lyophilized form, which needs to be reconstituted in purified water before intravenous injection in envenomated victims [174]. The major advantage of using polyvalent antivenom is its ability to protect and save lives against envenomations from numerous medically important snakes, including heterologous snakes, thus eliminating the need to identify the offending snake species. Moreover,

these antivenom are relatively less costly for production compared to monovalent antivenom [80].

In India, during the 1950s, the standards of manufactured antivenom were quite high, with minimum potencies against *N. naja* amd *D. russelii* venom being 2.0 mg/ml and 4.0 mg/ml respectively [175]. However, for unknown reasons, the standards were lowered in subsequent decades and currently, the polyvalent antivenom manufactured in India can neutralize a minimum of 0.6 mg/ml for *D. russelii* and *N. naja* venoms and 0.45 mg/ml for *E. carinatus* and *B. caeruleus* venoms [133]. Currently in India, polyvalent antivenom are mainly produced by four companies which are manufactured using the venom of "Big- Four" snakes of India, and serve as the primary treatment for majority of the snakebite cases. Along with the availability and accessibility, the quality of antivenom produced by these manufacturers should also be an important consideration for treating envenomated patients.

Using a variety of tools involving proteomics, toxicological and biochemical assays the variation in snake venom proteomes can be deciphered which is also known as "snake venomics". Moreover, in order to identify the venom proteins which bear epitopes for the antivenom, or the ones which can escape the immune system of the animal hyperimmunized for antivenom production, a proteomics based approach called "snake antivenomics" is used. By evaluating the immunoreactivity of snake venom proteins, they can be classified as C-toxins (completely immunodepleted), P-toxins (poorly or partly immunodepleted) and N-toxins (non-immunodepleted) [176]. The First generation (1G) antivenomics approach was designed to immunoprecipitate venom-antivenom complexes in solution followed by estimation of unbound venom proteins in supernatant using chromatographic approach. The poorly or non-immunorecognized proteins were quantified by comparison of chromatographic area with reference venom. However, the 1G antivenomics was designed for whole IgG antivenom and not fractionated $F(ab')_2$ or F(ab) antivenom [177]. In order to address this shortcoming the second-generation (2G) antivenomics approach was introduced to assess the $F(ab')_2$ and F(ab) antivenom [178]. This approach provided a better chromatographic profile of the unbound and bound venom proteins [177].

This chapter focused on the purity of three polyvalent antivenom manufactured in India viz. Bharat Serums, Premium Serums and VINS polyvalent antivenom, and their cross-

reactivity against *B. fasciatus* venoms from Eastern and North-East India which was investigated by following the 2G antivenomics approach.

## 3.2 Materials

### 3.2.1 Snake venoms

Snake venom samples from *B. fasciatus* were the same as that mentioned in chapter 2 (section 2.2.1).

### 3.2.2 Indian polyvalent antivenom

Lyophilized polyvalent antivenom were obtained from three Indian manufacturers, namely, A. VINS Bioproducts Ltd., Telangana, India (VPAV, Batch No. 01AS15007, Date of Expiry: Jan, 2019); B. Premium Serums and Vaccines Pvt. Ltd., Pune, India (PSPAV, Batch No. 212012, Date of Expiry: Aug, 2020); C. Bharat Serums and Vaccines Ltd. Navi Mumbai, Maharashtra, India (BSPAV, Batch No. A05317056, Date of Expiry: Mar, 2021) (Figure 3.1). The antivenom were stored at 4 $^{o}$C till use. Cresol (<0.25%) was present as a preservative in all the antivenom. VPAV also contained the stabilizer Glycine and excipient Sodium chloride. All the studies using these antivenom were performed within their shelf lives.


**Figure 3.1:** Indian polyvalent antivenom used for the study. **A.** VINS polyvalent antivenom (VPAV), **B.** Premium Serums polyvalent antivenom (PSPAV), **C.** Bharat Serums polyvalent antivenom (BSPAV).

### 3.2.3 Chemicals and reagents

Dialysis tubing (SnakeSkin™) having a molecular weight cutoff (MWCO) range of 3.5K was purchased from Thermo Fisher, USA. Sodium bicarbonate and Sodium chloride

were purchased from Merck (Sigma Aldrich, USA). Bromophenol blue, glycerol, tris base, glycine, sodium lauryl sulphate, methanol and glacial acetic acid, TEMED, APS, acrylamide, bisacrylamide, ethanol, 2-mercaptoethanol (BME), and Coomassie brilliant blue R-250 were procured from Sigma Aldrich, USA. PageRuler™ Plus protein ladder was purchased from Thermo Scientific, USA.

PVDF Membrane (Immun-Blot®, 26 cm x 3.3 m, 0.2 µm pore size) was purchased from Bio-Rad Laboratories, USA; Substrate solution (NBT-BCIP® solution premixed), and Secondary antibody (Alkaline phosphatase bound Anti-Horse IgG produced in rabbit) were purchased from Sigma-Aldrich, USA; Glacial acetic acid, Glycine, Methanol, Sodium lauryl sulfate, Sodium chloride, Tris-Base and Tween 20 were also purchased from Sigma-Aldrich, USA. Cyanogen bromide (CNBr)-activated Sepharose™ 4B was obtained from GE Healthcare, Germany. Gravity Flow Columns (9 cm height, 10 ml reservoir) were obtained from Bio-Rad Laboratories, USA. Pierce™ Centrifuge columns (2 ml) were procured from Thermo Fisher Scientific, USA. HPLC grade Trifluoroacetic acid (TFA) and acetonitrile (MeCN) were procured from Sigma Aldrich, USA.

### 3.2.4 Chromatographic Columns

Chromatographic analysis of retained and non-retained fractions and their corresponding crude venoms was performed in RP-HPLC. The C18 column Acclaim™ 300 (150 × 2.1 mm, 3 µm, 300 Å) was utilized for this purpose which was procured from Thermo Fisher Scientific, USA.

## 3.3 Methods

### 3.3.1. $F(ab')_2$ content and SDS-PAGE analysis of polyvalent antivenom

*Estimating the total $F(ab')_2$ content in antivenom:* Each of the lyophilized antivenom vials were dissolved in 10 ml of purified (Milli-Q) water which was followed by the estimation of total IgG content utilizing a NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific, USA). Milli-Q water without any dissolved antivenom was taken as the blank. Next, 2 µl of the dissolved antivenom sample was placed in the detector and measured utilizing the Protein A20 option and selecting the IgG extinction coefficient. Three reading were obtained for each of the antivenom.

*SDS-PAGE analysis of polyvalent antivenom*: In order to determine the purity of the molecules in the antivenom, they were subjected to 12.5% Tris-glycine gel electrophoresis [179]. Preparation of 12.5% resolving gel (final volume 6 ml) involved addition of 1.2 ml of distilled water, 1.55 ml of 1.5 M Tris-Cl (pH 8.8), glycerol (0.25 ml), 30% acrylamide (2.5 ml), 10% SDS (60 µl), 10% APS (60 µl) and TEMED (6 µl). The 5% stacking gel (final volume 3 ml) was prepared by adding distilled water (1.5 ml), glycerol (0.1 ml), 0.5 M Tris-Cl (0.75 ml, pH 6.8), 30% acrylamide (0.4 ml), 10% SDS (30 µl), 10% APS (30 µl) and TEMED (4.5 ml). The antivenom samples (50 µg) were mixed with loading dye containing 0.001% bromophenol blue. To induce reducing conditions, the samples were treated with 5% BME and then heated in water at 100 $^{o}$C for 3 min. For non-reducing conditions, the addition of BME and pre-heating of samples was avoided. The samples underwent electrophoresis at 80 V in the stacking gel and at 120 V in the resolving gel after which Coomassie staining was done for visualization of bands. The gel image was documented utilizing an imaging system (ChemiDoc XRS+, BioRad Laboratories, USA).

### 3.3.2 Dialysis of polyvalent antivenom

The dissolved antivenom was dialysed before use in all subsequent experiments. The dissolved antivenom was placed in a dialysis tubing membrane to allow desalting and removal of particles below 3.5 K MWCO. The dialysis tube containing the antivenom was then placed in a container containing dialysis buffer 1 (50 mM $NaHCO_3$, 100 mM NaCl) and a magnetic bead, and the container was then placed in a magnetic stirrer (SPINIT™, Tarsons, India) at 50% speed for 2 h. This was followed by a change in the buffer using dialysis buffer 2 (100 mM $NaHCO_3$, 200 mM NaCl) for 2 h, and 3 changes of dialysis buffer 3 (200 mM $NaHCO_3$, 500 mM NaCl) for 2 h each. The antivenom was then taken out of the dialysis membrane in a 1.5 ml centrifuge tube and stored at 4 $^{o}$C till further use.

### 3.3.3 Western blot analysis

The immune-crossreactivity of three polyvalent antivenom (Premium Serums, VINS polyvalent and Bharat Serums) manufactured in India towards the *B. fasciatus* venoms were studied using the western blotting technique [180]. The crude venoms (15 µg) were analysed on a reducing 10% Tris-Tricine gel at 120 V (resolving gel). Following the

completion of the run, transfer buffer was used to soak the gel for 10 min with PVDF membrane (pre-soaked in 100% methanol for 120 s). Similarly, the sponges and blotting papers were also soaked in the buffer for 4 min. The blotting arrangement was assembled and positioned in the transfer buffer. The electrophoresis transfer was carried out at 100 V, 300 mA for 90 min. After the transfer is completed, the gel was placed in blocking buffer (1% BSA in TBST) for 1 h at 25 $^{o}$C with mild shaking, after which it was washed with TBST thrice for 5 min each. Primary antibody (polyvalent antivenom) was added to the blocked membrane at 1:1000 dilutions (v/v) and incubated overnight at 4 $^{o}$C with mild shaking. This was followed by washing of the membrane thrice by TBST (5 min each wash). Next, secondary antibody was added in 1:2000 ratio (v/v) and incubated for 2 h at 25 $^{o}$C with gentle shaking. The immunoblot was rigorously rinsed thrice with TBST, each for 5 min. The immunoblot was then developed till the bands were visualized using a pre-mixed NBT-BCIP solution. The reaction was halted using a stop solution (1% acetic acid). The images of the immunoblot were recorded using an imaging system (ChemiDoc XRS+, BioRad Laboratories, USA).

### 3.3.4 Second-generation antivenomics

Second-generation antivenomics was employed to assess the capability of Bharat Serums polyvalent antivenom for depletion of venom poteins. The dialysed antivenom (10 mg/ml stock) was used to prepare immunoaffinity columns in accordance with the approach outlined by Pla et al. [178]. Gravity columns were used to prepare the affinity column (6 ml) using the matrix CNBr-activated Sepharose 4B. The matrix was washed with 1 mM hydrochloric acid (ice-cold) followed by another wash of two column volumes (CV) of coupling buffer (0.2 M $NaHCO_3$, 0.5 M NaCl, pH 8.3) and incubated at 25 $^{o}$C for 4 h. Next, 2 ml of dialysed antivenom was diluted in 12 ml of coupling buffer and added to the column followed by incubation at 25 $^{o}$C for 4 h. The unbound antivenom fraction was eluted and the amount of $(Fab')_2$ fractions present were measured in a NanoDrop spectrophotometer. The coupling yield, which was calculated by subtracting the eluted antivenom fraction from the total amount added to the column, was determined to be 44.85%. The remainder of the sites in the matrix were obstructed using a blocking buffer (100 mM Tris-Cl, pH 8.5) and incubated at 25 $^{o}$C for 4 h. Next, 500 µl of antivenom-bound matrix (containing 800 µg antivenom) was transferred to each of four Pierce centrifuge columns, followed by alternate washing with 1500 µl of

alkaline buffer (0.1 M acetate, 0.5 M NaCl, pH 8.5) and acidic buffer (0.1 M Tris-Cl, pH 8.5) for three times each. Next, 1500 µl of equilibration buffer (135 mM NaCl in 1X PBS, pH 7.4) was added.

The *B. fasciatus* venom samples (50 µg) were added to the matrix, after dissolving in PBS (250 µl), and kept in gentle shaking for 1 h at 25 $^{o}$C. The resulting venom-antivenom ratio was 1:16. A blank column, with activated matrix but without bound antivenom, was taken as the control and processed the same as the other columns to check any specificity of the venom towards the matrix. The non-retained venom proteins were obtained with five repeated washings of PBS (pH 7.4) and the retained venom proteins was collected using 5 washings of 0.1 M glycine buffer (pH 2.0) followed by the addition of half CV of 1.0 M Tris-Cl (pH 9.0) for neutralizing the solution. Lyophilization was performed on both the fractions utilizing a vacuum desiccator and subsequently dissolved in Milli-Q water followed by RP-HPLC profiling.

The binding efficacy of the antivenom for each peak was determined by calculating the percentage of non-retained fraction (*%NRi*) using the following equation:

$$\%NRi\ (peak)\ = 100 - \left(\frac{Ri}{Ri + NRi} \times 100\right)$$

The above equation depicts the areas of the retained (*Ri*), and non-retained (*NRi*) fraction in the chromatogram for the peak '*i*'. However, the binding efficacy for the venom peaks with limited elution in the non-retained fraction, owing to their strong interaction with the antivenom, was determined utilizing the following equation:

$$\%NRi\ (peak) = \frac{NRi}{Vi} \times 100$$

In the above equation, the term '*Vi*' depicts the chromatographic areas of the crude venom for the peak '*i*'. Moreover, the overall binding efficacy of the antivenom cumulative of all the peaks was determined using the following formula:

$$\%NRi\ (venom) = \frac{\Sigma NRi}{\Sigma Vi} \times 100$$

## 3.4 Results

### 3.4.1 Comparative analysis of Indian polyvalent antivenom

The quality of three Indian polyvalent antivenom used in this study was compared and the total $F(ab')_2$ content was determined using a NanoDrop spectrophotometer. The readings for each antivenom sample were taken in triplicates. The highest $\%F(ab')_2$ for the antivenom was exhibited by Premium Serums polyvalent antivenom (53.7%) followed by Bharat Serums (49.6%) and VINS (41.2%) (Table 3.1).

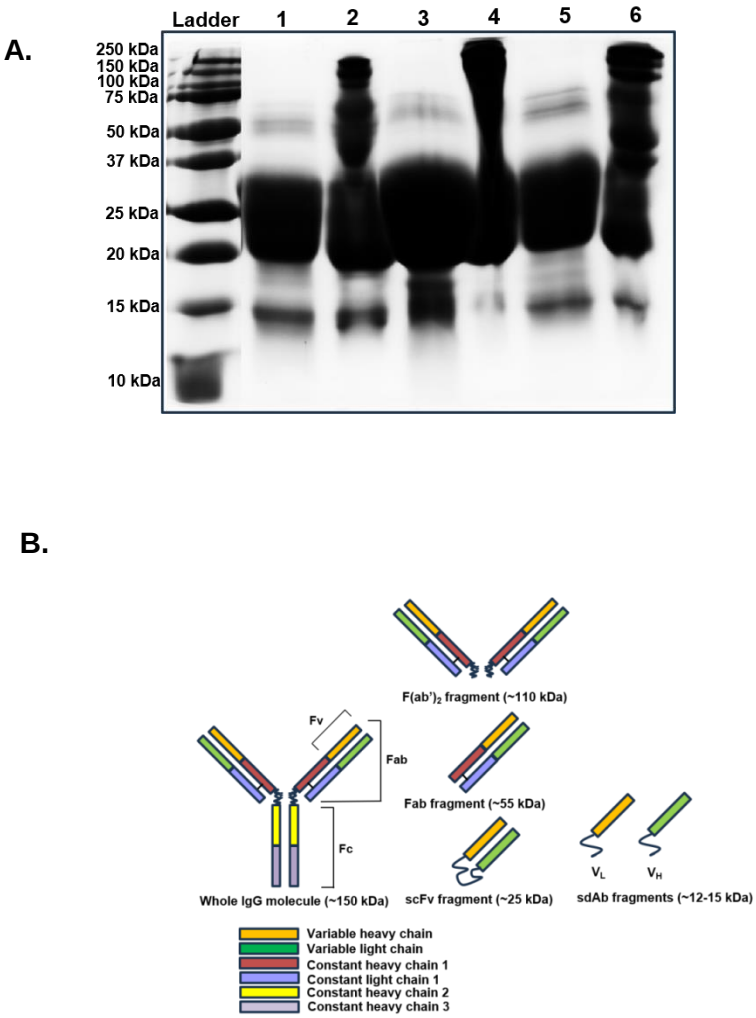


**Figure 3.2: A. Gel electrophoresis profile of Indian polyvalent antivenom (12.5% Tris-glycine gel) under reducing (R) and non-reducing (NR) conditions.** Lane 1: VINS (R), Lane 2: VINS (NR); Lane 3: Bharat Serums (R), Lane 4: Bharat Serums (NR); Lane 5: Premium Serums (R), Lane 6: Premium Serums (NR). Each sample (60 µg) was loaded in the wells of the 12.5% Tris-glycine gel under reducing and non-reducing conditions respectively. The bands were observed after Coomassie staining. Pre-stained protein marker (PageRuler plus, Thermo Scientific, USA) was utilized as the reference protein ladder. **B. Structure of whole IgG molecule and its fragments $F(ab')_2$, Fab, single chain variable fragment (scFv) and single-domain antibody fragment (sdAbs).**

Examination of the antivenom utilizing the 12.5% SDS-PAGE demonstrated the presence of thick bands in 20-25 kDa molecular weight (MW) range, and some minor bands in 55 kDa range under reducing conditions, whereas, in non-reducing conditions protein bands ranging from 20-150 kDa were detected. The protein bands in these regions indicate the presence of whole IgG molecule (~150 kDa MW), $F(ab')_2$ fragments (~110 kDa MW), Fab fragments (~55 kDa MW), single chain variable fragments (scFv) (~25 kDa MW) and single-domain antibody fragment (sdAbs) (~12-15 kDa) along with small quantities of other protein contaminants (Figure 3.2).

**Table 3.1:** Estimation of total $F(ab')_2$ content in commercially available Indian polyvalent antivenom.

| **Indian polyvalent antivenom** | **Dry weight (mg/vial)** | **$F(ab')_2$ content in each vial (mg/ml)** | **%$F(ab')_2$ in each vial** |
|---|---|---|---|
| VINS Bioproducts Ltd. | 715.70 | 30.91±0.62 | ~41.2% |
| Bharat Serums and Vaccines Ltd. | 534.84 | 26.52±1.23 | ~49.6% |
| Premium Serums and Vaccines Pvt. Ltd. | 701.32 | 37.66±0.98 | ~53.7% |

### 3.4.2 Immunochemical analysis of antivenom using Western blotting

The immune-crossreactivity of polyvalent antivenom manufactured in India was studied against *B. fasciatus* venoms from eastern and northeastern parts of India. The three immunoblots (Figure 3.3) revealed differences in binding capacity of the antivenom and incomplete recognition of different venom proteins when compared to the 10% SDS-PAGE profile (Figure. 2.2). For Premium Serum polyvalent antivenom, faint bands were observed between 17-37 kDa which implies poor and incomplete detection. However, most of the proteins of high molecular weights *i.e.*, in the 50-75 kDa range, were detected and prominent bands were observed. For VINS polyvalent antivenom, high molecular weight proteins were properly observed, and proteins in the 14-18 kDa range were also detected and prominent bands were observed. However, proteins ranging in low molecular weight (between 25-37 kDa and 18-25 kDa) were only partially detected and faint bands were observed, except for the sample from Guwahati. The detection of venom proteins by Bharat Serums polyvalent antivenom was mostly similar to that of

VINS, exhibiting a poor or partial detection capability of the proteins mostly in the 18-25 kDa range (Figure 3.3).

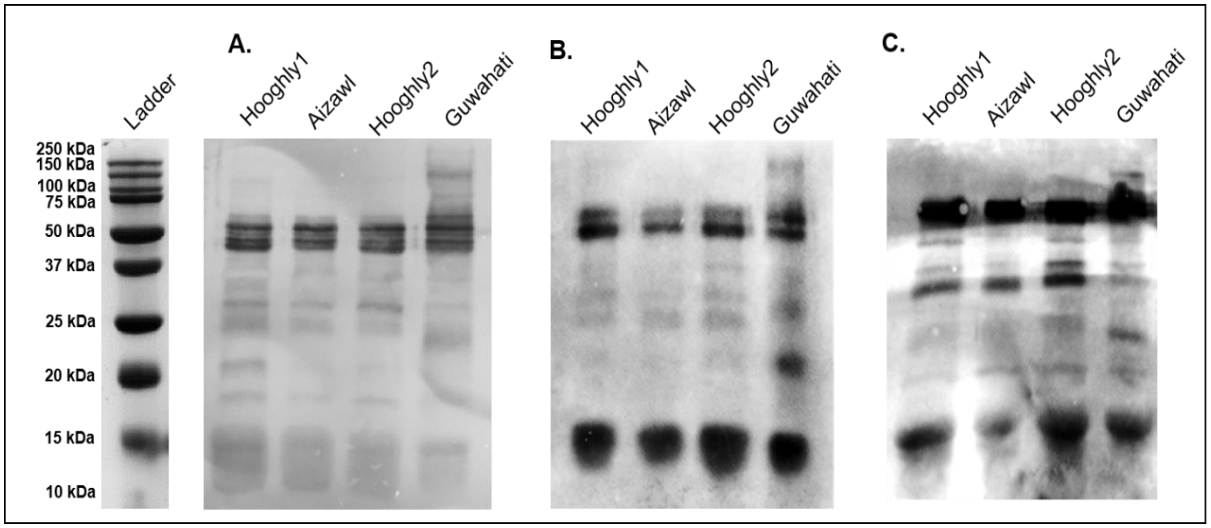


**Figure 3.3: Western blot of three polyvalent antivenom manufactured in India against the venoms of *Bungarus fasciatus*.** A. Premium Serums; B. VINS; C. Bharat Serums. Pre-stained protein marker (PageRuler plus, Thermo Scientific, USA) was utilized as the reference protein ladder.

### 3.4.3 Immunodepletion analysis using Second-generation antivenomics

From the 2G antivenomics approach, the capability of polyvalent antivenom (Bharat Serums) for immune-crossreactivity was determined for the *B. fasciatus* venoms from eastern and northeastern regions of India. The antivenom demonstrated moderate immunodepletion for some venom toxins, whereas, inadequate immunodepletion for others (Figure 3.4).

The areas of identified peaks from the crude, retained and non-retained fraction were calculated using the Chromeleon 6.8 software (Thermo Scientific, USA). From the total peak areas of respective samples, it was observed that the venom proteins in the non-retained fraction was higher for the samples from Hooghly2, Aizawl and Guwahati compared to their corresponding retained fractions utilizing a ratio of 1:16 for venom to antivenom. However, for the sample from Hooghly1, a greater quantitiy of the venom proteins were manifested in the retained fraction as opposed to the corresponding non-retained fraction. From the area of retained fractions (Ri), it was observed that the antivenom exhibited non-immunorecognition for Peak 5 from Hooghly2, and Peaks 1, 6 and 8 from Guwahati (Table 3.2).

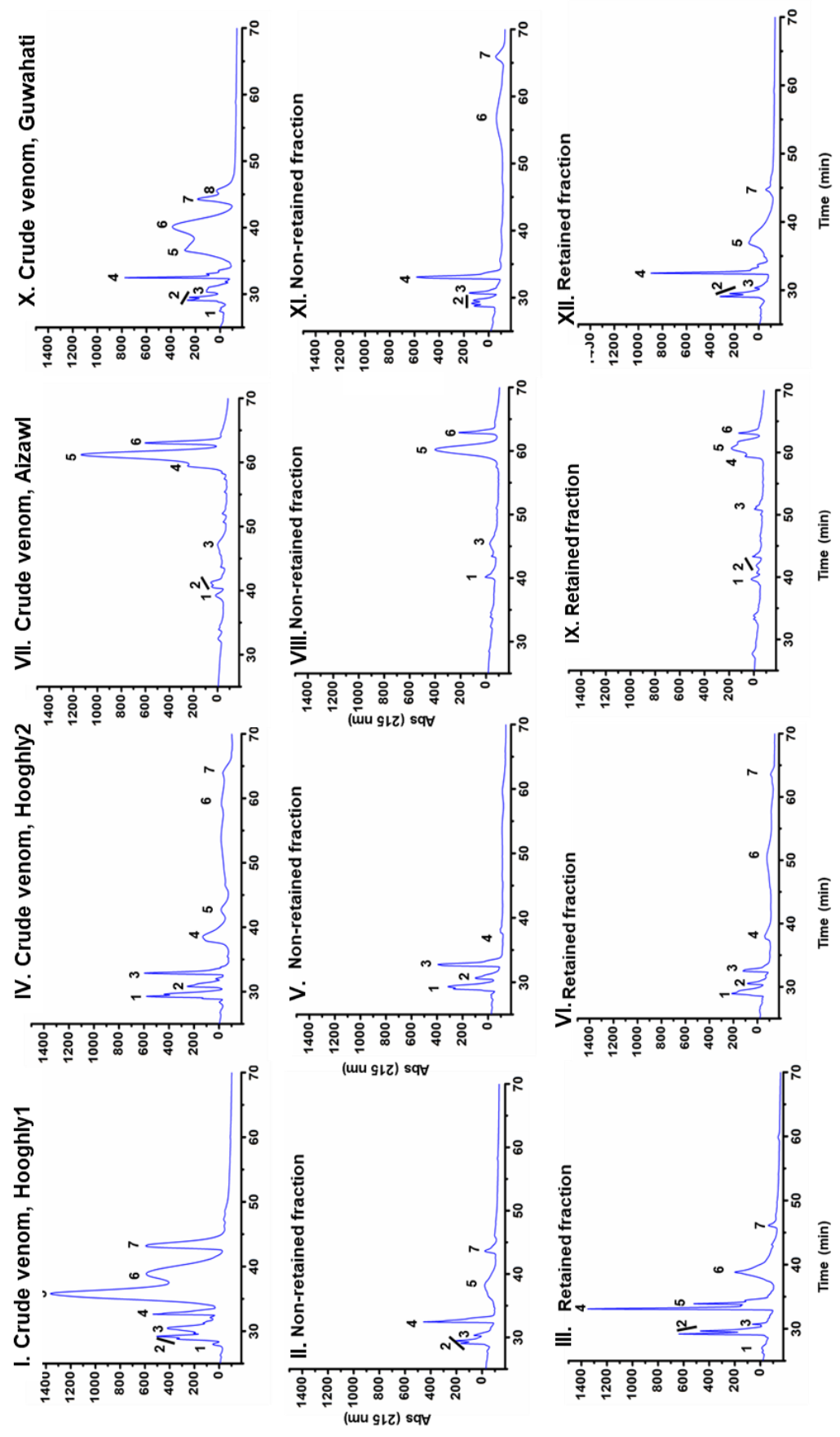


**Figure 3.4: Analysis of Bharat Serums polyvalent antivenom's immunodepletion potential against *Bungarus fasciatus* venoms utilizing second-generation antivenomics.**

**Table 3.2:** The chromatographic area of peaks from crude venom (Vi), non-retained (NRi) and retained (Ri) fractions are listed in this table. The percentage of non-retained fractions of every peak (%NRi (peak)) and the whole venom samples (%NRi (venom)) after seond-generation antivenomics using polyvalent antivenom (Bharat Serums) was obtained.

| Peak No. | NRi (mAU*min) | Ri (mAU*min) | Vi (mAU*min) | [Ri/(Ri + NRi)*100] | (NRi/Vi) *100 | %NRi (peak) | %NRi (venom) = [(ΣNRi / ΣVi) *100] |
|---|---|---|---|---|---|---|---|
| **(A) Hooghly1** | | | | | | | |
| **1** | 0.000 | 25.873 | 22.846 | 100.000 | - | 0.000 | |
| **2** | 148.390 | 358.149 | 223.465 | 70.705 | - | 29.295 | |
| **3** | 25.033 | 14.200 | 244.541 | 36.194 | - | 63.806 | |
| **4** | 259.827 | 387.933 | 232.448 | 59.888 | - | 40.112 | |
| **5** | 227.196 | 121.520 | 1790.258 | 34.848 | - | 65.152 | |
| **6** | 0.000 | 433.643 | 636.673 | 100.000 | - | 0.000 | |
| **7** | 69.489 | 35.616 | 709.302 | 33.886 | - | 66.114 | |
| **Total (Σ)** | **729.935** | **1376.934** | **3859.533** | | | | **18.913** |
| **(B) Hooghly2** | | | | | | | |
| **1** | 305.649 | 212.689 | 432.760 | 41.033 | - | 58.967 | |
| **2** | 102.286 | 56.788 | 201.477 | 35.699 | - | 64.301 | |
| **3** | 286.122 | 124.074 | 290.532 | 30.247 | - | 69.753 | |
| **4** | 13.135 | 62.140 | 387.266 | 82.551 | - | 17.449 | |
| **5** | 0.000 | 0.000 | 56.152 | - | 0.000 | 0.000 | |
| **6** | 0.000 | 146.740 | 121.646 | 100.000 | - | 0.000 | |
| **7** | 0.000 | 36.322 | 55.165 | 100.000 | - | 0.000 | |
| **Total (Σ)** | **707.192** | **638.753** | **1544.998** | | | | **45.773** |
| **(C) Aizawl** | | | | | | | |
| **1** | 61.230 | 51.478 | 54.028 | 45.674 | - | 54.326 | |
| **2** | 0.000 | 49.410 | 115.445 | 100.000 | - | 0.000 | |
| **3** | 123.820 | 54.455 | 196.794 | 30.546 | - | 69.454 | |
| **4** | 0.000 | 26.237 | 44.349 | 100.000 | - | 0.000 | |
| **5** | 752.207 | 255.108 | 1189.644 | 25.326 | - | 74.674 | |
| **6** | 126.134 | 69.670 | 301.120 | 35.581 | - | 64.419 | |
| **Total (Σ)** | **1063.391** | **506.358** | **1901.380** | | | | **55.927** |
| **(D) Guwahati** | | | | | | | |
| **1** | 0.000 | 0.000 | 26.982 | - | 0.000 | 0.000 | |
| **2** | 208.280 | 185.480 | 171.717 | 47.105 | - | 52.895 | |
| **3** | 96.448 | 13.684 | 120.396 | 12.425 | - | 87.575 | |
| **4** | 379.180 | 367.397 | 319.578 | 49.211 | - | 50.789 | |
| **5** | 0.000 | 341.560 | 345.056 | 100.000 | - | 0.000 | |
| **6** | 294.347 | 0.000 | 594.473 | - | 49.514 | 49.514 | |
| **7** | 83.815 | 36.167 | 158.646 | 30.144 | - | 69.856 | |
| **8** | 0.000 | 0.000 | 33.204 | - | 0.000 | 0.000 | |
| **Total (Σ)** | **1062.070** | **944.288** | **1770.052** | | | | **60.002** |

From the percent Non-retained fraction of peaks (%NRi (peak)), it was observed that the antivenom exhibited extremely poor recognition (%NRi>70%) for Peak 3 (87.57%) from Guwahati and Peak 5 (74.67%) from Aizawl. Further, from the percent Non-retained fraction of venom (%NRi (venom)), it was calculated that the overall percent of non-retained fractions for the samples followed the sequence: Guwahati (60.0%) > Aizawl (55.9%) > Hooghly2 (45.7%) > Hooghly1 (18.9%) (Table 3.2).

### 3.5 Discussion

There is little to no information available currently for the paraspecific neutralization capacity of polyvalent antivenom manufactured in India, against the medically relevant snake species *B. fasciatus* from eastern and northeastern India. The purity of polyvalent antivenom manufactured in India is compared in this chapter by their total $F(ab')_2$ content, along with their immunochemical cross-reactivity and immunodepletion capacity against *B. fasciatus* venom. From the protein estimation of the reconstituted antivenom, it was observed that the amount of total immunoglobulin content in the polyvalent antivenom was almost equal or less than half of their dry weights. The remaining portion may consist of impurities such as IgA, IgE, ceruloplasmin, and heterologous animal proteins such as alpha-2-macroglobulins and albumin [79,181]. Moreover, preservatives and protein stabilizers are also present in these antivenom which are mentioned in their product labels. The purity of antivenom should be considered an important factor and should be examined before assessment of its efficacy. The electrophoresis profile of the antivenom revealed that antivenom fragments ($F(ab')_2$, Fab, scFv and sdAbs) are present in these antivenom along with some amounts of IgG molecules since prominent bands were also visualized in the range of 150 kDa MW. IgG molecules which are inadequately digested during antivenom manufacturing may result in contamination of Fc fragments in the antivenom which may lead to adverse side effects if administered in victims [182]. Previous reports have indicated that up to 95% of the proteins in antivenom are immunologically active, however, less than 40% of these proteins specifically target snake venom proteins which is a cause of concern [79,181]. Moreover, recent studies have highlighted the co-relation of high antibody content in polyvalent antivenom with a relatively greater immune-crossreactivity and neutralization capacity [183]. Hence, there should be a focus on enhancing antivenom

purity by carefully modifying the immunization mixtures and recalibrating the antivenom manufacturing SOPs to have snake venom specific antibodies.

In the immunochemical study using western blotting, the antivenom exhibited better recognition of venom proteins belonging to high molecular weights (>25 kDa), which may belong to SVMP or CRiSP, compared to the low molecular weight proteins. However, majority of the protein bands in the 15-25 kDa MW were poorly recognized by all the venom proteins. Interestingly, although VINS and Bharat Serums polyvalent antivenom were able to prominently detect proteins ≤15 kDa, however, the polyvalent antivenom Premium Serums demonstrated low immunorecognition of these venom proteins which could belong to the phospholipase $A_2$ ($PLA_2$) or three-finger toxin (3FTx) superfamily [113]. Similar results were also obtained by Laxme et al. using polyvalent antivenom (Bharat Serums) targeting the venom of *B. fasciatus* from West Bengal [78]. Low MW venom proteins typically exhibit a poor immunogenic reaction when injected in equines for the production of toxin specific antibodies, due to which the resulting antivenom may contain insufficient antibodies against these venom proteins [184,185]. The venom proteins of *B. fasciatus* which are detected by the antivenom may be attributed to the presence of conserved antigenic determinants in the venom pool used to stimulate antibody production in equines. Leong et al. also studied the in vitro neutralizing potency of Bharat Serums and VINS polyvalent antivenom against cobras and kraits of Southeast Asia. Bharat Serums exhibited moderate efficacy against venoms of *Naja kaouthia* from Thailand but did not exhibit any efficacy against the venom from Malaysia. Similarly, the antivenom were weakly effective against a *N. naja* venom sample from Sri Lanka, but not against other samples from the country or from the neighbouring country India. Moreover, both the antivenom were ineffective against *B. fasciatus* venom samples from Southeast Asia. However, findings from our study suggest that the polyvalent antivenom manufactured in India exhibit limited efficacy in immunorecognizing venom proteins of *B. fasciatus* from diverse geographical regions of Eastern and North-East India [186].

Second-generation antivenomics study revealed the partial immunodepletion capability of Bharat Serums polyvalent antivenom utilizing a ratio of 1:16 for venom to antivenom. As a result, a higher antivenom dosage would be required to effectively deplete the venom proteins immune-recognized by the polyvalent antivenom. The recognition sites

present in the $F(ab')_2$ molecules must have reached saturation levels when 50 µg of crude *B. fasciatus* venom was added, as a result of which the remaining venom proteins eluted out in the non-retained fraction. It is known that a high dose of antivenom administration may lead to adverse side effects in the envenomated victim such as pyrogenic fever and severe anaphylaxis [59,187]. Interestingly, from this study it has also been observed that the venoms from northeastern India (Aizawl and Guwahati) contained more %NRi (55.9% and 60.0% respectively) compared to the ones from Eastern India (Hooghly1 and Hooghly2) *i.e.,* 18.9% and 45.7% respectively. Hence, for the convenience of study, we selected the *B. fasciatus* venom samples from Guwahati and Aizawl for further examination in subsequent chapters.

Using third-generation antivenomics approach with multiple venom-antivenom doses, the maximum binding capacity of the polyvalent antivenom may be determined. Such study may aid in determination of the appropriate antivenom dose in victims. However, in vitro binding of antivenom to venom proteins does not truly reflect its in vivo neutralization capability as sometimes non-neutralizing antibodies might also be present in the antivenom. Such non-neutralizing antibodies may compete with the neutralizing antibodies and bind to the immunodominant epitope, but fail to bind to the neutralization epitope leading to ineffective neutralization [75,76]. The WHO recommends neutralization studies in experimental animals along with antivenomics approach for determining the binding and neutralizing capacity of the antivenom [188]. The prior assessment of in vitro efficacy helps to minimize the number of experimental animals sacrificed during in vivo studies.

Snakes characteristically exhibit variations in their venom composition owing to several factors which may be phylogenetic, geographic origin, sex, food preference and availability, and life stage (juvenile or adult) [189]. Previously, intra-specific and intra-population diversity in venom composition have been documented for different snake species, some of which include *Bothrops jararaca*, *B. atrox*, *B. asper*, *Crotalus durissus*, *C. simus*, *C. viridis concolor*, *Naja naja*, *N. kaouthia*, *Ophiophagus hannah*, and *Vipera berus berus* [190–199]. In accordance with these studies, intra-population variation has been observed in this study for the venoms of *B. fasciatus* from Hooghly1 and Hooghly2. Although venoms collected from two snakes from Hooghly1 and Hooghly2 were males, however, the snake from Hooghly1 was a juvenile and the other was an adult. Thus, the

compositional differences in the venom may be attributed to the diversity among age groups. Moreover, the intra-specific differences among the *B. fasciatus* venom from Guwahati and Aizawl, with Hooghly1 and Hooghly2 correspond to geographical variations. Further, intra-species variation in krait venom has been reported previously by some authors. For instance, the venom of *B. fasciatus* exhibited differences in their composition from countries like China, Indonesia, Malaysia, Myanmar and Thailand [58]. Similarly, Oh et al. also reported venom compositional variations from *B. multicinctus* from Taiwan and China [200].

The potency of antivenom for neutralization of intra-specific venom from different geographies has previously been documented for the venom of *B. fasciatus* as well as other elapids from several Asian countries. In this context, the Thai monovalent antivenom raised to neutralize *B. fasciatus* venom, exhibited a variable neutralization potency (n-P) for the venom from Thailand (27.43 mg/g) > Myanmar (16.87 mg/g) > Indonesia (15.33 mg/g) > China (7.67 mg/g) > and Malaysia (3.24 mg/g) [58]. Also, the *B. multicinctus* monovalent antivenom (BMMAV) exhibited minor differences in half-maximum effective concentrations ($ED_{50}$) for binding the *B. multicinctus* venom from China (1.14 ± 0.09) and Taiwan (1.15 ± 0.04). Similarly, the Neuro bivalent antivenom (NBAV) was more effective against the *B. multicinctus* venom from its source country Taiwan (2.57 ± 0.07) than from China (2.96 ± 0.13) [200]. Moreover, Tan et al. described differences in the binding efficacy of few antivenom, namely, *Ophiophagus hannah* Monovalent antivenom (OHMAV) from Thailand, trivalent Serum Anti Bisa Ular (SABU) from Indonesia and *Naja atra* Monovalent antivenom (NAMAV) from China. The OHMAV also exhibited differences in binding the venom ($ED_{50}$) of *O. hannah* in the order Thailand (39.37 µl) > Malaysia (129.09 µl) > Indonesia (139.58 µl) > China (170.16 µl) [197].

This study highlights how antivenom efficacy can be strongly affected due to geographical differences in venom composition of serpents. Inclusion of non-immunodepleted or partly-immunodepleted venom toxins in adequate amounts in the venom pool is needed for production of antibodies in hyperimmunized healthy animals during antivenom manufacturing. The venom of *B. caeruleus* venom is utilized as one of the contributors in the venom mixture for manufacturing polyvalent antivenom. It may be anticipated that the antivenom would be able to detect paraspecific homologous

proteins from other snake venoms. Nonetheless, the polyvalent antivenom fail to detect particular toxins from *B. fasciatus* venoms which reflect the limitation of polyvalent antivenom manufactured in India in identifying all the venom proteins. The results are in line with other studies which reported inefficient detection and neutralization by Indian polyvalent antivenom against the paraspecific venom of Sri Lankan *B. ceylonicus* and Pakistani *B. sindanus* [157,201,202]. Similarly, *B. multicinctus* monovalent antivenin (produced by Shanghai Serum Biotechnology Co. Ltd.) raised against the venom of *B. multicinctus* from mainland China, also failed to neutralize the toxicity of *B. fasciatus* venom in Kunming mice in vivo, although the antivenom was effective in neutralizing *B. multicinctus* venom [203]. The non-retained venom proteins in this study may be further investigated to understand their pathophysiological role, if any, in the envenomated victims to determine whether the polyvalent antivenom could be used as an effective treatment. This study also highlights the need for the inclusion of non-retained or partly-retained venom fractions, having pathophysiological effects in victims, from different medically important species in the immunizing venom pool to develop more effective polyvalent antivenom.

## Conclusion

In this chapter, the comparative analysis of the quality of Indian polyvalent antivenom after protein estimation and SDS-PAGE analysis revealed minor differences in the $F(ab')_2$ content among the different polyvalent antivenom. Further, immunochemical analysis using western blotting technique has revealed that the antivenom were not efficient in immunorecognition of all the venom proteins, especially the lower molecular weight ones (15-25 kDa). Moreover, from the immunodepletion study using second-generation antivenomics, it was observed that Bharat Serums polyvalent antivenom exhibited incomplete immunodepletion of the *B. fasciatus* venom proteins utilizing a ratio of 1:16 for venom to antivenom. The maximum binding of venom proteins was observed for the samples from Guwahati followed by that from Aizawl, Hooghly2 and Hooghly1. Further studies using third-generation antivenomics approach with different venom concentrations might aid in identifying proper dosage of antivenom needed for neutralizing per unit amount of venom proteins. The study highlights how variations in venom composition due to age, geographical distance or other ecological factors may affect antivenom cross-reactivity and hence its efficacy.