CHAPTER 4

RESULTS
4.1 Characterization of venoms

The four native venoms were assessed in reducing condition using SDS–PAGE (12%). The bands were visualized by silver staining of the gels (Fig.4.1). It was observed that RV has more number of prominent bands distributed below 97.4 kDa. The KV contains lower molecular weight proteins and a band near 66 kDa, probably of Bungarotoxin. During immunization, it was observed that RV was more immune responsive than the CV. The reason behind this may be high molecular weight proteins are more antigenic compared to the lower ones. The figure 4.1 demonstrates that each venom has the distinct banding pattern, nevertheless, some bands overlapped between venoms representing equivalent molecular weight proteins and may be the basis for the cross-reaction among venoms as established during further studies.

Fig. 4.1: SDS–PAGE (12%) of venoms. Lane 1 contains the standard molecular weight protein marker, whereas Lane 2: KV, Lane 3: CV, Lane 4: RV, and Lane 5: EV; the arrows indicate the standard molecular weight bands in kDa.

4.2 Protein content and LD50 of the venom

The amount of protein was estimated by Bradford’s method. The protein content of the venom varies from species to species. It observed that RV and CV has more protein content as compared to KV and EV (Table 4.1). Although the KV have less protein content, it was more toxic compared to other venoms. During LD50 determination, all deaths occurred
within 12 hrs of injection. The deaths due CV and KV are mainly due to neurological effects such as flaccid paralysis of hind limbs, disorientation, and respiratory failure. The deaths of mice inoculated with EV and RV are mainly due to hemorrhagic effects, bleeding gums, and blood in the urine.

<table>
<thead>
<tr>
<th>Sr. No</th>
<th>Venom</th>
<th>venom protein (g/g)</th>
<th>LD&lt;sub&gt;50&lt;/sub&gt; / mouse (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CV</td>
<td>0.91</td>
<td>7.52 (0.18)</td>
</tr>
<tr>
<td>2</td>
<td>RV</td>
<td>0.86</td>
<td>8.33 (0.39)</td>
</tr>
<tr>
<td>3</td>
<td>KV</td>
<td>0.72</td>
<td>2.90 (0.09)</td>
</tr>
<tr>
<td>4</td>
<td>EV</td>
<td>0.79</td>
<td>10.59 (0.25)</td>
</tr>
</tbody>
</table>

**Table 4.1**: Determination of LD<sub>50</sub> and protein content of the venoms. LD<sub>50</sub> values are average values (n=3) and SD values indicated in the bracket.

### 4.3 Immunization monitoring

For the beginning of immunization, the LD<sub>50</sub> of venoms taken into consideration and only sub-lethal doses of venoms injected subcutaneously. Immunization by means of detoxified venom permitted larger amount of antigen with no any harmful effects on physical conditions of the animal. During immunization monitoring, we observed that although the immunization carried out using detoxified venoms, generated antibodies have activity against the native venoms. The rate of seroconversion increased after 6 weeks of immunization, persisted till 18<sup>th</sup> week and maintained thereafter, which proved that the immunization with a combination of adjuvant gave better seroconversion. The change in antigen presentation led to yield more neutralizing antibodies. CV observed less immune responsive than RV, as it contains more number of lower MW proteins and this is the reason generally CV antibodies are used as a marker for immunization in manufacturing of polyvalent ASVA. Indirect ELISA used for monitoring of the immuno-conversion of the RV and CV, the red line represents CV and blue lines represent the RV and SD values shown by error bars. (Fig.4.2)
Fig. 4.2: Monitoring of immunization: anti-RV and anti-CV antibodies. The indicated values of OD are mean values (n=3), error bars indicates the SD.

4.4 Purity of antibodies

Fig. 4.3 demonstrates the protein profiles of monovalent plasma (rabbit), ARV- Abs and ACV-Abs resolved using SDS–PAGE (10%) in native conditions. The silver staining procedure carried out for visualization of the bands. The rabbit monovalent plasma shows multiple bands, mainly two distinct bands around 205 kDa and 66 kDa, which are suggestive of IgG and BSA respectively. In affinity purified samples, only a single band around 205 kDa, indicative of IgG. This confirms that, in affinity purification only antibodies separated from other proteins. The unbound section ascertained to have no activity against the venoms. The eluted IgG’s were further tested for their functionality against the venom. These antibodies remained functional in the process of affinity chromatography and showed activity against the venom.
Fig. 4.3: SDS–PAGE (10%) of purified antibodies and hyper-immunized plasma samples. Lane 1 contains standard molecular weight protein marker, whereas Lane 2: anti RV monovalent rabbit plasma, Lane 3: ARV-Abs, Lane 4: anti CV monovalent rabbit plasma, and Lane 5: ACV-Abs; the arrows specify the standard proteins. Monovalent plasma shows multiple bands whereas, purified antibody samples contains a band of IgG and confirms the purity of samples.

4.5 Indirect ELISA optimization for determination of ASVA

For the optimization of ELISA Standard ASVA and normal plasma were tested at different dilutions ranging from 1:1000 to 1:15000 were allowed to react with different concentration of coating venoms from one to five µg per ml. For secondary antibody dilutions viz. 1:5000, 1:10000, and 1:20000 were considered. All these dilutions were optimized using Checkerboard analysis. The criteria for selection of dilutions were that blank OD should be below 0.1 and positive samples having potency 0.1 mg/ml should have OD around 1.0. It was observed that two µg per ml of venom used for coating, primary antibody diluted to 1:10000 and secondary antibodies at dilution 1:20000 gave better results. The optimized Indirect ELISA protocol and followed during entire experimentation.
4.6 Standardization of ELISA for ASVA potency determination

To Standardize ELISA for ASVA potency determination specificity, linearity, and the range of the method was evaluated. The standard plasma diluted in such a manner that the potency was in the range from 0.025 to 0.2 mg/ml. The result interpretation carried out using Graphpad Prism 6 software by linear regression parameter. The specificity, accuracy, and linearity ascertained during standardization. The obtained R^2 values for CV and RV were 0.985 and 0.982 respectively. The SD values for CV and RV are 0.029 to 0.044 and 0.012 to 0.022, respectively (Fig. 4.4)

![Graph showing OD vs Titre](image)

**Fig. 4.4:** The estimation of ASVA using ELISA. Two µg per ml of venom used to coat the Micro ELISA plates using a carbonate buffer and added with dilutions of ASVA ranging 0.025 - 0.2 mg/ml. Normal equine plasma used as a blank. The OD values are the average values (n=3), error bars indicates the SD.

4.7 Sandwich ELISA optimization for the venom estimation

Optimization of indirect sandwich ELISA was performed by the Checkerboard analysis. It was observed that the ELISA selectively detects RV and CV venoms. The BSA used as blank, gives OD equivalent to blank at all stages of dilution. The sandwich ELISA was sensitive to detect the venom about 0.01 ng/ml and the graph observed linear from 0.1 to 1000 ng/ml for CV and RV venoms indicating the suitability of the method for
envenomation detection of snakebite patients. The SD values for anti-CV and anti-RV antibodies were inside 0.020 to 0.049 and 0.038 to 0.058, respectively. The R² values for anti-CV and anti-RV antibodies were 0.997 and 0.996, respectively (Fig. 4.5). The result interpretation was carried out using Graphpad Prism 6 software.

**Fig. 4.5**: Calibration plot of sandwich ELISA for estimation of the RV and CV. ELISA wells coated with horse ASVA using a carbonate buffer. The wells incubated with venom ranging from 0.01 ng/ml to 1000 ng/ml and BSA used as a blank. The OD indicates average values (n=3) error bar indicates the SD.

### 4.8 Assessment of cross-reactivity of affinity purified anti-RV and anti-CV antibodies.

Indirect sandwich ELISA, used to assess the cross reactivity monovalent affinity purified antibodies with heterospecific venom. To estimate the cross reactivity standard ASVA (equine) used as a capture antibody. The dilutions of conspecific and heterospecific venom ranging from 0.01 to 1000 ng/ml were analyzed during the study to evaluate the cross-reaction between ARV–Abs and ACV–Abs. The result showed that antibodies strongly responded to conspecific venom. Also, it was observed that snakes belonging to the same family have more cross reactivity, ACV-Abs shown maximum cross-reactivity to KV and ARV – Abs to EV, The cross reactivity with heterospecific venom is significant till 1 ng/ml dilution. The observed cross-reactivity of antibodies may give ambiguous results in
selective detection of snake venom without removal of cross-reacting antibodies by immuno-affinity chromatography. Therefore, the protein A purified antibodies were passed through conspecific and heterospecific venom bound columns to get the RV and CV SSAb’s.

**Fig. 4.6:** Cross-reactivity evaluation using sandwich ELISA. To evaluate the cross reactivity ASVA (equine) used as a capture. The wells incubated with venoms (CV, KV, RV, and EV), the quantity of venoms ranges from 0.01 to 1000 ng/ml. The figure (A) indicates cross-reactivity of ACV-Abs (rabbit) with conspecific and heterospecific venoms and fig (B) represent the cross reactivity of ARV-Abs (rabbit). The OD values are mean values (n=3) error bar indicates the SD.

**4.9 Specificity determination of CV and RV SSAb’s**

Indirect sandwich ELISA used to assess the specificity of SSAb’s. To estimate the specificity standard ASVA (equine) used as a capturing antibody. The dilution of conspecific and heterospecific venom ranging from 0.01 to 1000 ng/ml analyzed during the study to evaluate the specificity of RV and CV SSAb’s. The result showed that antibodies strongly responded to conspecific venom and SSAb’s shown no cross-reactivity with heterospecific venom for all the dilutions selected during the study indicates that SSAb’s
are specific for conspecific venom (Fig. 4.7). This result confirms the use of SSAb’s in snake envenomation detection tests.

**Fig. 4.7**: Specificity evaluation of SSAb’s using indirect sandwich ELISA. To evaluate the specificity of SSAb’s the equine ASVA used as a capture. The wells incubated with venoms (CV, KV, RV, and EV), the quantity of venoms ranges from 0.01 to 1000 ng/ml. Figure (A) indicates specificity of ACV-Abs and fig (B) represent the specificity of ARV-Abs. The OD indicates the average values (n=3) error bar indicates the SD.

4.10 **Determination of binding capacity of GNP solution.**

The minimum quantity of antibodies to stabilize the GNPs determined according to protocol mentioned by Slot and Geuze, (1984). The pH of GNPs and antibodies adjusted to 7.0. In each test tube added 2 ml of gold solution and 0.2 ml of antibody solution containing protein concentrations from 100 to 700 µg/ml and mixed well. After 1 min, 2 ml of 10% w/v NaCl added to each tube (Fig. 4.8). The stability of gold solution was observed by taking the absorbance at 580 nm wavelength. It was observed that 20 µg/ml proteins required to stabilize the GNPs as OD values stop decreasing from test tube, no. 4 and for conjugation experiment 10% excess antibodies taken.
**Fig. 4.8:** The estimation of the quantity of protein required to stabilize the dispersed GNPs. The first test tube contains the control GNP solution. The test tube 2 to 9 contains 2 ml GNP solution incubated with increasing concentration of proteins and consequently, 2 ml NaCl (10% w/v) added to observe the color change.

### 4.11 Performance of the SEDIA device

For the preparation of SEDIA strips, the NC membrane used about 10-µm size and gave excellent results. The pre-treated sample pad and the conjugate pads used; the concentration of GNPs optimized on the conjugate pad. For impregnation of antibodies on NC membrane concentration analyzed from 0.5 to 2 mg/ml by manual application and it was observed that 1 mg/ml was the optimum concentration. After optimization, one µl/cm solution was applied at the test and control lines using TLC spotter (Linomat IV, Camag, Berlin, Germany). The strips manufactured and assembled in cassettes. The devices packed, stored at ambient temperature, and protected from humidity and light.
4.11.1 SEDIA results interpretation

The result interpretation of SEDIA shown in (Fig. 4.9). The test region immobilized with SSAb’s of CV and RV. A positive result indicated by the observation of the red lines at corresponding test regions on the membrane, the lines produced due to the capture of gold conjugated antibody and venom complex. In the absence of venom, gold-conjugated antibodies cannot capture at the test regions. In the control region, immobilized anti-horse antibodies, which captures the gold conjugated antibodies and gave a red line with or without in the presence of venom indicating the validity of the device. If the control region does not produce red line, the test considered as invalid.

![Schematic representation for interpretation of SEDIA results](image)

**Fig. 4.9:** Schematic representation for interpretation of SEDIA results

4.11.2. Specificity

To determine the specificity of SEDIA device, all venoms (CV, RV, EV, and KV) spiked individually using PBS buffer such that the final concentration of venom in the solution was 1000 ng/ml. To ensure specificity and cross-reactivity of SEDIA strips, applied diluted
sample (100 µl). The results obtained by the visualization of red lines at control and the respective test region. The results of test obtained within five to ten min. All SEDIA devices demonstrated its validity by producing red lines at all control regions (Fig. 4.10). In the strip 1, where only PBS was added showing red line at control region only, representing the negative control. The device 2 was added with a mixture of RV and CV in PBS, a red line at control as well as both test regions was obtained indicating the device can identify a mixture of venoms, also displaying the positions of lines on a device. The device 3 and 4 inoculated with PBS containing CV and RV, respectively, and the red lines observed at their respective test zones. The SEDIA device did not show any cross-reactivity between CV and RV. In device 5 and 6 the red lines observed at the control region, representing the definite specificity of SEDIA device for the RV and CV.

![Image of SEDIA test strips](image-url)

**Fig. 4.10:** Specificity evaluation of the SEDIA device using different venom spiked (1000 ng /ml) samples. The control region developed red lines in all tests indicating the validity of the device. The device 2 contains a mixture of the RV and CV venom hence developed color in all regions. The device 3 and 4 applied with the CV and RV venom spiked sample and exclusively showing red lines at respective test zones. The device 5 and 6 applied with heterospecific venoms spiked samples of KV and EV and shown only control line indicating specificity of SEDIA device.
4.11.3. Sensitivity

Assessing sensitivity of SEDIA is the most crucial part in detection snake envenomation as the levels of venom in serum are very low. According to Hung et al. (2003), during snake envenomation detection of cobra bites in Taiwan, victims with severe systemic envenomation had a concentration of venom in the serum between 228–1270 ng/ml and for a mild systemic envenomation, concentration of venom in the serum between 0–24 ng/ml. Therefore, it becomes mandatory for snake envenomation assay, to detect venoms in nanograms. To assess the sensitivity of the SEDIA device, a logarithmic dilution (1000 to 0.01 ng/ml) of CV and RV venoms prepared in PBS. All the simulated samples were applied to the SEDIA strips (about 100 µl) to demonstrate the sensitivity of SEDIA. Device 1 was applied with PBS and device 2 to 7 with a dilution of venom respectively. As device 1 applied with PBS, hence no capture of the conjugate at test region resulted in development of the red lines only at control region. The intensity of the red line is dependent on the concentration of antigen in the sample and results are reproducible. Device 7 shown only the control line therefore sensitivity of SEDIA device is about 0.1 ng/ml (Fig. 4.11).
Fig. 4.11: Sensitivity determination of SEDIA device. A logarithmic dilution (1000 ng/ml to 0.01 ng/ml) of CV and RV venoms prepared in PBS and applied on SEDIA device. Fig. (A) and (B) representing the sensitivity CV and RV respectively. Device 1: negative control, the device 2 to 7 applied from left to right with decreasing logarithmic dilutions of spiked venom samples. In both figures device 7 shown only the control line, therefore sensitivity of SEDIA device is 0.1 ng/ml.
4.12. Evaluation of venoms from experimental envenomation

The RV and CV venoms injected s.c. in Swiss albino mice for systemic envenomation. The blood samples were collected after 30 and 60 min of duration (Fig 4.12). Plasma separated and diluted equally with PBS. The samples tested using SEDIA and sandwich ELISA for comparison of the results (Table 4.2). Device 2 and 3 applied with the CV samples and device 4 and 5 with the RV samples produced a prominent line at the respective test region. The results of SEDIA compared with sandwich ELISA using the same samples and there was 100% agreement between both results.

![Experimental envenomation determination by the SEDIA.](image)

**Fig. 4.12:** Experimental envenomation determination by the SEDIA. The device 2 and 3 representing results of CV and the device 4 and 5 for the RV envenomation produced the red lines at test as well as control zones, while device 1 produced a red line at control zone only; confirms the efficiency of the SEDIA device to selectively detect experimental envenomation.

<table>
<thead>
<tr>
<th>Time period</th>
<th>CV</th>
<th>RV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ELISA (µg/ml)</td>
<td>SEDIA</td>
</tr>
<tr>
<td>30 mins</td>
<td>0.022</td>
<td>+</td>
</tr>
<tr>
<td>60 mins</td>
<td>0.020</td>
<td>+</td>
</tr>
</tbody>
</table>

**Table 4.2:** Comparison of experimental envenomation detection. The values indicated in the table are mean ELISA values, the + symbol represents the red line at the test zones on the SEDIA device.