CHAPTER 3

MATERIALS AND METHODS
3.1 Materials

3.1.1 Venoms

The venoms (lyophilized) of snakes made available by Haffkine Biopharmaceutical Corp. Ltd. (HBPCL), Maharashtra, India. Venoms provided by HBPCL were a pool of venoms collected from various regions of Maharashtra. These venoms used for commercial manufacturing of antivenom. The venoms provided by HBPCL were reconstituted and lyophilized in aliquots, and maintained at -20\(^\circ\) C.

3.1.2 Animals

Swiss albino mice (male) weighing 18 - 20 g and rabbits (New Zealand white) weighing 2.0 - 3.0 kg maintained at HBPCL. The animals domiciled at room temperature, provision of food and water with dark/light cycle maintained during the experimentation.

3.1.3 Materials

I. CFA and IFA - DIFCO, USA.
II. BSA and Bentonite powder - HiMedia, Maharashtra, India.
III. Anti-horse (rabbit) IgG - horseradish peroxidase (HRP) and Anti-rabbit (goat) IgG - HRP - Merck, India.
IV. Tetramethyl benzidine/Hydrogen peroxide (TMB/H\(_2\)O\(_2\)) substrate- Merck, India.
V. Amicon centrifugal filters - Merck, India.
VI. Mabselect and CNBr-activated Sepharose 4B - GE Healthcare Bio-Sciences Corp. USA.
VII. MaxiSorp Nunc flat bottom 96 well plates - Nunc, Denmark.
VIII. Gold Chloride (AuCl\(_4\)) 49% - Sigma, Bangalore, India.
IX. NC membrane, conjugate pad, sample pad - mdi membranes, India, provided by Oscar Medicare Pvt. Ltd. Delhi, India.
X. All other reagents and chemicals used were of analytical grade.
3.2 Estimation of protein content

For determination of the sample protein, Bradford’s (1976) method was used. For the determination of antibody protein, immunoglobulin (equine) used as a standard. Venom protein content was determined using BSA as a standard. For preparation of standard graph 10 to 100 µg protein diluted in 0.1 ml with 0.15 M NaCl buffer as mentioned in (Table 3.1). The sample dilution adjusted according to standard graph range. Bradford’s reagent 5 ml, added to each tube including blank, mixed and incubated for five min. The absorbance taken at 595 nm wavelength. The sample protein content was evaluated from the standard graph.

3.2.1 Reagent preparation

I) Stock solution preparation

10 mg of standard protein dissolved in 10 ml of 0.15 M NaCl buffer.

<table>
<thead>
<tr>
<th>Sr.</th>
<th>Stock solution (µl)</th>
<th>Buffer (µl)</th>
<th>Volume (µl)</th>
<th>Final concentration (µg)</th>
<th>Bradford’s reagent (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-</td>
<td>100</td>
<td>100</td>
<td>Blank</td>
<td>5.0</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>90</td>
<td>100</td>
<td>10</td>
<td>5.0</td>
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<tr>
<td>3</td>
<td>20</td>
<td>80</td>
<td>100</td>
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<td>11</td>
<td>100</td>
<td>-</td>
<td>100</td>
<td>100</td>
<td>5.0</td>
</tr>
</tbody>
</table>

Table 3.1 Preparation of dilutions for standard calibration curve to estimate protein
3.3 Determination LD_{50} of snake venom

The LD_{50} of snake venoms determined by the method suggested by WHO (2010). For new venoms to avoid more usage of experimental animals as recommended by the guidelines LD_{50} range finding study was performed using single mouse (18-20 g) per dilution, 0.5 ml injected intravenously. The deaths of mice examined to find out the range of lethality from 0% to 100% and the same range used for determination of venom LD_{50}. To find out the LD_{50} of venom for statistically significant results requires minimum five mice per dilution, in our study; we used six mice group weighing in the range of 18-20 g per dilution. Each mouse was injected with 0.5 ml of diluted venom intravenously (i.v.), prepared in NS solution. The deaths of mice recorded up to 24 hrs and the minimum amount of venom, which kills 50% of animals in the group (LD_{50}), was determined. (Reed, L. and Muench, H., 1938; Sells, 2003).

3.4 SDS –PAGE profiles of venoms and hyperimmune plasma

SDS–PAGE of venoms, hyperimmune plasma, and affinity purified IgG’s were performed as procedure mentioned by the Laemmli (1970).

3.4.1 Preparation of reagents

I) 30% Acrylamide solution

Bis - acrylamide 0.8 g and Acrylamide 29.2 g dissolved in ultra pure water and diluted to 100 ml.

II) 1.5 M Tris HCl

Tris base (18.15 g) added in ultra pure water and pH adjusted to 8.8 with concentrated HCl and diluted with ultra pure water up to 100 ml.
III) 0.5 M Tris HCl

Tris base (6.05 g) added in ultra pure water and pH adjusted to 6.8 with concentrated HCl and diluted with ultrapure water up to 100 ml.

IV) 2X Laemmli sample buffer

The Laemmli sample buffer was prepared by taking 4.75 ml ultra pure water and adding 1.25 ml Tris HCl (0.5 M), 0.5 ml β-mercaptoethanol, 1.0 ml Glycerol, 2 ml SDS (10% w/v), and 0.5 ml Bromophenol blue (0.5%) produce 10 ml.

V) Running Buffer

Glycine 15.014 g, SDS 1.0 g, and Tris Base 3.02 g added to the ultra pure water dissolved completely, pH adjusted to 8.3 and diluted to produce 1000 ml.

VI) SDS- PAGE gel preparation

<table>
<thead>
<tr>
<th>Reagent Name</th>
<th>Resolving gel (12%)</th>
<th>Resolving gel (10%)</th>
<th>Stacking gel (4%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5 M Tris Buffer pH 8.8</td>
<td>2.5 ml</td>
<td>2.5 ml</td>
<td>-</td>
</tr>
<tr>
<td>1.5 M Tris Buffer pH 6.8</td>
<td>-</td>
<td>-</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>30% acrylamide</td>
<td>4.0 ml</td>
<td>3.35 ml</td>
<td>1.34</td>
</tr>
<tr>
<td>10% APS</td>
<td>0.05 ml</td>
<td>0.05 ml</td>
<td>0.05 ml</td>
</tr>
<tr>
<td>20% SDS</td>
<td>0.05 ml</td>
<td>0.05 ml</td>
<td>0.05 ml</td>
</tr>
<tr>
<td>Ultra pure water</td>
<td>3.4 ml</td>
<td>4.05 ml</td>
<td>6.05 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.007 ml</td>
<td>0.007 ml</td>
<td>0.01 ml</td>
</tr>
</tbody>
</table>

Table 3.2 SDS- PAGE gel preparation

VII) Fixing reagent:

10.0 ml Acetic acid (glacial) and 40 ml Ethanol dissolved in ultra pure water to produce 100 ml.
3.4.2 Reagents for silver staining

I) Sensitizing reagent

Sodium thiosulphate 0.2 g and Sodium acetate 6.8 g dissolved in ultra pure water. In the solution 30 ml absolute ethanol and added 0.5 ml Glutaraldehyde (25%) solution, the final volume produced to 100 ml with ultra pure water.

II) Silver nitrate reagent

0.25 g of Silver nitrate dissolved completely in ultra pure water and 40.0 µl Formaldehyde (37%) solution added, to make a final volume to 100 ml with ultra pure water.

III) Developing reagent

2.5 g Sodium carbonate dissolved in ultra pure water and 50.0-µl Formaldehyde (37%) solution added, to make a final volume to 100 ml with ultra pure water.

IV) Stopping reagent

1.5 g EDTA dissolved and the final volume made to 100 ml with ultra pure water.

3.4.3 Procedure

I. The gels casting unit was cleaned, 12 % resolving gel was prepared as mentioned in (Table 3.2) and added to casting gel unit with care to avoid air bubbles.

II. After 30 min, the stacking gel was prepared, poured carefully on resolving gel. Immediately comb was inserted avoiding air bubbles and allowed to solidify.

III. The comb removed carefully; the gel assembled in electrophoresis unit and added running buffer to equilibrate the gel.

IV. Samples treated with reducing Laemmli sample buffer at 95 °C for 2 min.

V. Five µg of protein sample and five-µl standard protein marker (broad range) was loaded carefully in the wells of the gel.
VI. Electrophoresis performed using an electrophoresis system (WEALTEC Corp., USA.) at ambient temperature. The current flow maintained at 20 mA till the dye front migrates to bottom.

VII. Washing given to the gel with ultra pure water, placed in fixing reagent on a shaker for 30 min, and again washed the gel with ultra pure water.

VIII. The gel was transferred to a sensitizing reagent on a shaker for 30 min and washed the gel 2 to 3 times with ultra pure water.

IX. Transferred the gel to a silver reagent for 20 min on the shaker and washed again 2 to 3 times.

X. Bands developed by adding developing reagent and the reaction terminated by adding stopping reagent.

XI. The profile of proteins captured on the Gel doc system (GeNei, India).

XII. Rabbit plasma (Monovalent) and antibodies purified by affinity chromatography treated with sample buffer (native) and proteins separated on 10% gel, for native sample buffer β mercaptoethanol not added, all the composition was same as mentioned in reducing sample buffer and the same procedure followed as mentioned above.

3.5 Detoxified venom preparation

All appropriate precaution taken at the time of venom handling, since lyophilized venom powder is highly antigenic in nature and may lead to cause severe side-effects including allergy and respiratory distress. The venom preparation area having separate air-handling unit and all the venom activities strictly performed in the biosafety cabinet. During handling of venoms, the appropriate gowning procedure followed with all necessary safety equipment. The detoxification method mentioned by Brunda et al. (2006), followed for detoxification of RV and CV. In brief, for the preparation of venom stock solution 1 mg/ml lyophilized venoms reconstituted with sterile normal saline. Venom stock solution heated at 60º C for 60 minutes and immediately cooled for 10 min, and the process repeated two times. Detoxified venoms prepared freshly for immunization.
3.6 Immunization

Immunization of rabbits carried out as mentioned by Brunda et al. (2006) with some modifications. New Zealand white (male) rabbits weighing about 2.0 to 3.0 kg were chosen for the generation of antibodies against RV and CV, respectively. The dose was prepared by considering LD$_{50}$ values of the venoms. To avoid contamination of microorganisms and reducing the microbiological load, dosages were prepared under strict aseptic environment.

During preparation and administering of dosages any direct contact to venoms and adjuvants was avoided, as some adjuvants contains inactivated *M. tuberculosis* and mineral oil, which can produce severe manifestations. The first immunization dose of rabbits was prepared using detoxified venom 100 µg per Kg body weight and CFA in equal amounts by following instructions. To obtain an emulsion of CFA repeated plunging was performed using sterile syringe (26½ gauge) untill a whitish emulsion produced and its consistency was checked by drop test. The immunization of rabbits was carried out by shaving area on each side of the abdomen. The area was thoroughly disinfected with 70% ethanol and injected 0.5 ml sub-cutaneously (s.c.) on each sides of abdomen. Subsequent two dosages of venom using IFA repeated with 3 weeks interval. The IFA emulsion was prepared similarly as described for CFA emulsion. Further, two subsequent venom dosages were prepared and injected by using native venoms as mentioned in Table 3.3 using 2% w/v solution of Bentonite in equal amounts. It was reported that absorption of antigen to Bentonite would enhances the immunogenicity of the antigen. The use of different adjuvant changes the presentation of the antigen resulting in better seroconversion. Additional, two booster venom doses of native venoms containing 100µg per Kg body weight were prepared in NS and administered. Blood samples from the ear vein of immunized rabbits were collected after 10 to12 days of immunization. The tubes for blood collection was prepared by adding 5% w/v Sodium citrate. The blood collected and centrifuged for 5 minutes at 5000 g to get clear plasma. The clear supernatant collected and filtered using 0.45 µ syringe filters and stored at 2-8 °C. Indirect ELISA was performed for monitoring of antibody levels in the plasma (Brunda et al., 2006; Pawade et al., 2016).
Table 3.3 Plan for immunization of rabbits

<table>
<thead>
<tr>
<th>Sr. No</th>
<th>Time (Weeks)</th>
<th>Venom(µg)</th>
<th>Adjuvant</th>
<th>Dose volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1&lt;sup&gt;st&lt;/sup&gt;</td>
<td>400 (Detoxified)</td>
<td>CFA</td>
<td>2 ml</td>
</tr>
<tr>
<td>2</td>
<td>3&lt;sup&gt;rd&lt;/sup&gt;</td>
<td>400 (Detoxified)</td>
<td>IFA</td>
<td>2 ml</td>
</tr>
<tr>
<td>3</td>
<td>6&lt;sup&gt;th&lt;/sup&gt;</td>
<td>400 (Detoxified)</td>
<td>IFA</td>
<td>2 ml</td>
</tr>
<tr>
<td>4</td>
<td>9&lt;sup&gt;th&lt;/sup&gt;</td>
<td>200</td>
<td>Bentonite</td>
<td>2 ml</td>
</tr>
<tr>
<td>5</td>
<td>12&lt;sup&gt;th&lt;/sup&gt;</td>
<td>400</td>
<td>Bentonite</td>
<td>2 ml</td>
</tr>
<tr>
<td>6</td>
<td>15&lt;sup&gt;th&lt;/sup&gt;</td>
<td>400</td>
<td>Nil</td>
<td>2 ml</td>
</tr>
<tr>
<td>7</td>
<td>18&lt;sup&gt;th&lt;/sup&gt;</td>
<td>400</td>
<td>Nil</td>
<td>2 ml</td>
</tr>
</tbody>
</table>

3.7 Plasma purification

The Mabselect is an affinity medium contains protein A (recombinant) produced in *E. coli*. The protein A particularly binds to the IgG at Fc region. The antibodies from the rabbit hyper-immunized plasma were separated using Mabselect™, as per the manufacturer instructions. To avoid cross-contamination of antibodies, the purification performed on separate columns manually for both monospecific plasma samples (Paul et al. 2007; Pla et al. 2012).

3.7.1 Reagent Preparation

I) Buffer A

0.283 g Sodium phosphate, 0.877 NaCl dissolved in 75 ml ultra pure water; the pH of the solution was adjusted to 7.2 and diluted to 100 ml.
II) Buffer B

2.941 g sodium citrate dissolved to produce 100 ml with ultra pure water and pH was monitored which should be between 3.0 - 3.6.

III) Tris HCl 1M

Tris base (12.1 g) added to dissolve in 70 ml ultra pure water, the pH of the solution was adjusted to 9.0 with concentrated Hydrochloric acid and diluted to 100 ml with ultra pure water.

3.7.2 Protocol for affinity purification

I. The buffer B was titrated with 1 M Tris-HCl to obtain neutral pH. Collection tubes prepared by taking 200 µl of 1 M Tris-HCl.

II. Rabbit plasma was diluted 1:1 with buffer A.

III. Column washed with eight column volumes with the buffer A to remove storage buffer and equilibrated with ten volumes of buffer A.

IV. The diluted plasma sample was applied to the column and kept for 10 min.

V. Washing given to the column until no traces of protein observed in the washing.

VI. Elution buffer added to the column, allowed to react and elution collected in collection tubes.

VII. The column regenerated with elution buffer and stored in buffer A.

VIII. The collected elution desalted and concentrated using Amicon® Ultra - 4 10K centrifugal filter devices.
3.8 Determination of ASVA by ELISA

The antibody titer of plasma was determined using the indirect ELISA method. Checkerboard analysis applied to optimize of Indirect ELISA (Pawade et al., 2016; Rial et al., 2006; Salvi et al., 2010, 2013; Wild, 2001).

3.8.1 Preparation of reagents

I) Carbonate buffer

2.930 g of NaHCO₃, 1.590 g of Na₂CO₃ allowed to dissolve in 750 ml of ultra pure water, the pH adjusted to 9.6 and diluted to 1000 ml.

II) Phosphate buffer saline, and Tween 20 (PBST)

1.500 g Na₂HPO₄, 8.0 g of NaCl, 0.200 g KCl, and 0.200 g of KH₂PO₄, dissolved completely in 750 ml of ultra pure water, the pH of the solution adjusted to 7.4, added slowly 0.5 ml of Tween 20, and diluted to 1000 ml with ultra pure water.
III) Blocking solution

1 g BSA dissolved in 100 ml PBST

IV) 1N H$_2$SO$_4$

0.54 ml of concentrated H$_2$SO$_4$ carefully added to ultra pure water to produce 20 ml.

3.8.2 Procedure

I. Venom diluted to two µg/ml using a carbonate buffer. The ELISA plate coated with venoms by adding 100 µl above diluted venom to the wells and incubated for 18 hrs at 2-8°C.

II. To remove traces of unbound venom, three washings given to the plate using PBST, on ELISA plate washer (BioTek, USA).

III. Two hundred µl of blocking buffer added to the wells and kept at RT for 60 min. The blocking buffer traces removed by washing (three times).

IV. The wells were applied with 100 µl of diluted (1:10000) equine ASVA samples and incubated for 30 mins at RT followed by three washing cycles using PBST.

V. The wells were added with a 100µl solution of anti-horse IgG-HRP conjugate (1:20000), incubated at RT for 30 minutes, and to remove traces of all unbound secondary antibodies the wells were washed five times with PBST.

VI. Afterwards, the wells were applied with 100 µl of TMB/H$_2$O$_2$ and incubated for 15 min in the dark.

VII. Finally, to terminate the enzymatic activity, the wells added with 100 µl of 1N H$_2$SO$_4$. The developed color in the wells was measured using an ELISA reader (BioTek, USA) at 450 nm. To determine rabbit ASVA, same protocol was followed using anti-rabbit IgG-HRP secondary antibodies. All the analysis performed in triplicate.
3.8.3 Standardization of ELISA for ASVA determination

To Standardize ELISA for determination ASVA potency linearity, specificity, and the range of the method was assessed. The standard ASVA provided by HBPCL, Pune. The plasma diluted in such a manner that the potency ranges from 0.025 to 0.2 mg/ml. Generally, the same range of potency considered for immunization and manufacturing. For the assessment, each dilution was prepared in triplicate and the same dilutions of normal plasma used as blank at each point. The optimized Indirect ELISA protocol followed during entire experimentation. The ELISA results analyzed by Graphpad Prism-6 software.

3.9 Detection of RV and CV by sandwich ELISA

The indirect sandwich ELISA carried out as stated by Selvanayagam et al., (1999) with minor changes and the Checkerboard method was used for optimization of ELISA (Gilliam et al., 2013; Pawade et al., 2016; Salvi et al., 2010, 2013).

I. Equine ASVA was diluted five µg per ml with coating buffer. The wells of ELISA plate was coated by adding 100 µl above diluted sample and incubated for 18 hrs at 2-8°C. Consecutively three washings were given using ELISA washer.

II. Two hundred µl of blocking solution was added to the wells and kept for 60 mins. The traces of blocking buffer removed by washing three times using PBST.

III. The wells applied with 100µl of diluted venom samples and incubation given for 30 min at RT, followed by three washing cycles using PBST.

IV. The wells allowed to react with 100 µl (1:1000) diluted purified ASVA (rabbit) for 30-min at RT, followed three washing cycles.

V. In the wells added with the 100 µl solution of anti-rabbit IgG-HRP conjugate (1:20000), incubated at RT for 30 minutes, and to remove traces of all unbound secondary antibodies the well were washed five times with PBST.

VIII. Afterwards, the wells were applied with 100 µl of TMB/H₂O₂ and incubated for 15 min in the dark.
IX. Finally, to terminate the enzymatic activity, the wells added with 100 µl of 1N H₂SO₄. The developed color in the wells was measured using an ELISA reader. All the analysis performed in triplicate and the values showed are blank corrected.

3.9.1 Standardization of sandwich ELISA for detection of RV and CV

To standardize indirect sandwich ELISA, for detection of RV and CV linearity, specificity, and the range of the test was evaluated. The plates coated with ASVA allowed to react with RV and CV in triplicate. The logarithmic dilutions (0.01 to 1000 ng/ml) of venoms applied separately using BSA as a blank at each point. All the procedure followed as mentioned in the indirect sandwich ELISA procedure for both venoms and checked for parameters like standard deviation, linearity, range, and R² values analyzed by Graphpad Prism-6 software (Pawade et al., 2016).

3.9.2 Assessment of cross-reactivity

For assessment of efficiency and cross-reaction between ARV-Abs and ACV-Abs, sandwich ELISA was performed. Conspecific and heterospecific venoms applied to pre-coated plates in triplicate with graded dilutions from 0.01 ng/ml to 1000 ng/ml. The ARV-Abs and ACV-Abs (1:1000) added in respective cross-reactivity experiments. The optimized sandwich ELISA protocol followed for cross-reactivity of affinity purified antibodies and SSAb’s of both venoms (Pawade et al. 2016; O’Leary et al. 2007).

3.10 SSAb’s preparation by immuno-affinity chromatography

3.10.1 Preparation of column

The method described by Dong L.V. (2003) and J. F. Gao et al., (2013) used to isolate the RV and CV SSAb’s (RV-SSAb’s and CV-SSAb’s). Briefly, the resin was swollen in 1.0 mM HCl (pH 3.0) medium for about 1 hr at RT with occasional gentle side to side stirring. The separated slurry was then equilibrated with coupling buffer (Sodium bicarbonate 0.1 M, with NaCl 0.5 M, pH adjusted 8.3). 20 mg of snake venom diluted in coupling buffer, applied to the slurry and incubated overnight at 2-8 °C on a horizontal rotator. The slurry extensively washed with coupling buffer. The unoccupied sites were blocked with the help of 0.1 M Tris HCl-pH 8.0 at RT for two hrs, followed by three alternate washings with
acetate buffer (0.1 M), NaCl (0.5 M)-pH 4.0 and Tris HCl (0.1 M), NaCl (0.5 M)-pH 8.0. The final wash given using Tris HCl (0.1 M) buffer and slurry filled into the column. The column equilibration performed using 20 mM PBS, pH 7.0 and stored at 2-8 °C until use.

3.10.2 Immuno-affinity purification of antibodies

ARV-Abs and ACV-Abs applied to the conspecific venom columns and permitted to react with the venom. The columns washed with PBS buffer pH 7.0 until no traces of protein observed in the washings. The venom-specific antibodies eluted by adding 0.1 M Glycine (pH 2.8), allowed to react, elution was collected in collection tubes and containing the previously determined amount of 1 M Tris HCl as described in protein-A affinity protocol.

**Fig 3.2** Schematic representation of immunoaffinity purification, Protein A purified rabbit IgG was applied on Sepharose 4B CNBr –activated conspecific venom-A column. Venom specific IgG was captured and unbound components are washed, venom specific IgG eluted by the use of low pH buffer (Dong L.V., 2003).
Fig. 3.3 Preparation of SSAb’s. For purification of SSAb’s, the conspecific venom immunoaffinity purified antibodies passed through heterospecific venom columns to remove cross-reacting antibodies by binding. The unbound part contains SSAb’s to the conspecific venom (Dong L.V., 2003).
3.10.3 Preparation species-specific antibodies (SSAb’s)

For the preparation of SSAb’s, the immunoaffinity-purified antibodies were loaded on heterospecific venom column to adsorb cross-reacting antibodies and collected unbound fraction. The collected fraction passed through next heterospecific venom columns as shown in the schematic diagram of SSAb’s preparation (Fig. 3.3) to yield SSAb’s. The antibodies subjected to desalting, concentration using Amicon® Ultra - 4 10K centrifugal filter devices as per manufacturer’s instruction and stored at 2-8°C.

3.11 Synthesis of GNPs

The 25 nm GNPs prepared by the method illustrated by Grabar et al. 1995. For the preparation of GNPs, care was taken to avoid contamination, all the glasswares used for synthesis were washed with 0.22 µm filtered ultra pure water. For preparation of HAuCl₄ solution and Sodium citrate solution, used 0.22 µm filtered ultra pure water. To prepare 0.01 % HAuCl₄ solution, 20 mg of HAuCl₄ dissolved in 200 ml ultra pure water. The solution heated and allowed to boil, rapidly added 2 ml Tri-sodium citrate (1%), the solution stirred continuously which initially formed blue color and then changed to a cherry red. The solution boiled additional for 10 min, and care taken during boiling that vapors to condensate and maintains the volume of the solution. Followed by stirring of the solution for 15 min; cooled to RT and stored at 2-8 °C in an amber glass bottle. The gold solution analyzed on an UV spectrophotometer (UV1800, Shimadzu Corporation, Kyoto, Japan) to find out the maxima (λ max) and the OD of the solution (Grabar et al., 1995; Pawade et al., 2016)
3.11.1 Determination of the amount of antibodies required to stabilize GNPs

The minimum quantity of antibodies required for stabilizing GNPs evaluated as stated by Slot and Geuze, (1984) with minor modifications. Briefly, GNP and antibody solution taken and pH maintained to 7.0 using carbonate buffer. In each tube, two ml of GNP solution was taken and 0.2 ml of antibody solution containing protein concentrations from 100 to 700µg/ml added, mixed well and after 1 min, 2 ml of 10% w/v NaCl added to each tube. The stability of GNP solution was observed by taking the absorbance using
spectrophotometer at 580 nm. The minimum quantity of protein required to stabilize the GNP was determined by the point from which there is no decrease in the OD of the solution. After addition of the NaCl, GNP start aggregating if not stabilized by proteins. For the final conjugation experiment, added 10% excess antibodies to the stabilization point (Slot and Geuze, 1984).

3.11.2 Preparation of GNP probes

Antibodies conjugated with GNP by the procedure illustrated by (Hung et al., 2014) with some changes.

I. The purified ASVA (equine) diluted to 1 mg/ml.
II. The pH of GNP was adjusted to 7.0 using carbonate buffer.
III. The determined quantity of antibodies added to the gold solution and stirred constantly for 1 hr.
IV. The available unbound sites on GNP blocked by the addition of BSA to make a final concentration in the solution to 1% and the solution centrifuged on the fixed rotor centrifuge at 30000 g for 30 min (Remi C-24BL, India).
V. To pellet dissolved in PBS, containing 1% BSA, 2% sucrose (pH 7.4) and kept at 2-8 °C.

3.12 LFA preparation

An LFA device contains of four key components; conjugate pad, NC membrane, sample pad, and absorbent pad. An NC membrane provide sites for capture antibodies. The LFA device prepared and optimized according to (Posthuma -Trumpie et al., 2008) with some changes.

I. The SSAb’s and anti-equine antibodies diluted to one mg per ml with carbonate buffer.
II. NC membrane having pore size 10 µm applied with different concentrations of antibodies at control and test lines, for optimum concentration determination, which subsequently finalized to one µl per cm.
III. TLC spotter (Linomat IV, Camag, Berlin, Germany) used to plot the control and test lines with anti-horse antibodies and SSAb’s of CV and RV with their respective positions as shown in the schematic diagram (Fig. 3.5). Care was taken to avoid any contact with NC membrane, as these membranes are delicate and may be damaged or contaminated, giving background noise, which may create ambiguity in the results.

IV. These membranes desiccated in an oven at 37 °C for one hr separately, to immobilize antibodies on the surface of the NC membrane.

V. The unoccupied sites on the membrane was blocked by applying solution 1% BSA in PBS for 30 min with gentle rotation. To remove the unbound BSA, the membranes washed two times with PBS. The membranes dried in the oven completely at 37 °C for two hrs.

VI. For the preparation of conjugate pad, the pads (PT-R7) immersed in the solution of GNPs conjugated with antibody, followed by complete drying in the oven at 37 °C for two hrs.

VII. The sample pad (GFB-R7) was pretreated and used without any further treatment.

VIII. The LFA device assembled by placing other components on plastic backed NC membranes, the adhesive protecting paper was removed and absorbent pad was placed in the direction of flow as shown in (Fig. 3.5) by overlapping five mm to the NC membrane.

IX. The treated conjugate pads kept towards the test line ending, by overlapping five mm to the NC membrane.

X. The sample pad kept at one end of the plastic backing in such a way that it overlaps five mm to the conjugate pad.

XI. On assembling all the components on plastic backing of the NC membrane, the strips prepared by cutting the membrane in three mm width vertically.

XII. These strips accommodated in a plastic cassette and the device kept at RT, protected from humidity and sunlight until use.
3.13 Assay performance of SEDIA device

3.13.1 Specificity

To determine the specificity of SEDIA device, all venoms (CV, RV, EV, and KV) were spiked individually using PBS buffer such that the final concentration of venom in the solution was 1000 ng/ml. To check specificity and cross-reactivity of the SEDIA device, applied diluted samples (about 100 µl). The results evaluated by the visualization of red lines at the respective test and control regions.
3.13.2 Sensitivity

To find out the sensitivity of the SEDIA device, a logarithmic dilution (1000 ng/ml to 0.01 ng/ml) of CV and RV venoms prepared in PBS. All the simulated samples applied to SEDIA device (about 100 µl) to demonstrate the sensitivity of SEDIA. Strip 1 was applied with PBS and device 2 to 7 with a serial dilution of venom respectively.

3.14 Experimental envenomation

Swiss albino mice used to simulate the experimental envenomation. In brief, concentration venom adjusted in NS such a way that it should contain two LD_{50} of venom per dose. The diluted RV and CV injected 250 µl subcutaneously to the group of Swiss albino mice weighing 20-22 g and concern given towards minimum pain to the animal during the entire experiment. The sample was collected from the heart of the mice by cardiac puncture method after 30 mins of envenomation (Group1, n=3), and after 60 mins (Group II, n=3). Anticoagulant 5% w/v solution sodium citrate added to the tubes to make a final volume to 5% of the total volume. These tubes centrifuged to separate the plasma on the fixed rotor centrifuge at 5000 g for 5 minutes. The plasma is stored at 2-8 °C until use. The samples were diluted 1:1 with PBS and 2-3 drops (100µl approx) applied to the SEDIA device with the help of dropper to assess its functionality. The sandwich ELISA of the samples was performed using one hundred µl of the sample to estimate the amount of venoms from the samples using the standard curve and OD obtained plotted on a graph to estimate the amount of venom (Brunda et al., 2006).