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DETECTION AND NEUTRALIZATION OF VENOM OF INDIAN COBRA BY RABBIT ANTISERUM IN EXPERIMENTAL ENVENOMATION USING MICE

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Abstract: A sandwich enzyme linked immunosorbent assay (ELISA) was developed to detect Indian cobra (*Naja naja naja*) venom in various organs (brain, heart, lungs, liver, spleen, blood, site of injection and kidneys) as well as tissue at the site of injection of mice, at various time intervals (0, 2, 4, 6, 8 and 12 h intervals up to 24 h) after venom injection. Antiserum significantly neutralized venom levels in serum and tissue samples. Whole venom antiserum or individual venom protein anti serum (14 kDa, 29 kDa, 65 kDa, 72 kDa and 99 kDa) of venom could recognize *Naja naja* venom by Western blotting and ELISA, and *Naja naja naja* venom presented antibody titer when assayed by ELISA. The assay could detect *Naja naja naja* venom levels up to 2.5 ng/ml of tissue homogenate and the venom was detected up to 24 h after venom injection. A highly sensitive and species-specifc microtitre ELISA was also developed to detect venoms of four medically important Indian snakes (*Naja naja naja*) in autopsy specimens of mice. Venoms were detected in brain, heart, lungs, liver, spleen, kidneys, tissue at the bite area and blood. As observed in mice, tissue at the site of bite area showed the highest concentration of venom and the brain showed the least. Moderate amounts of venoms were found in liver, spleen, kidneys, heart and lungs. Development of a simple, rapid and species-specifc diagnostic kit based on this ELISA technique useful to clinicians is discussed.

Keywords: ELISA; Antiserum; Naja *naja naja;* Cocktail Antiserum; Cobra.

INTRODUCTION

Snake bite is an occupational hazard, especially among the snake catchers, forest workers and agriculturists in rural India. It is estimated that about 200,000 persons are bitten in India annually and about 15,000 are fatal[1] but hospital statistics include only about 10% of the total cases in the population[2]. The incidence is more in rural areas during the rainy season, particularly at nights. About 216 species of snakes are found in India of which only 52 species are venomous. The four major venomous snakes chiefly responsible for the injuries and fatalities in India are Krait (*Bungarus caeruleus*), cobra (*Naja naja naja*), saw-scaled or carpet viper (*Echis carinatus*) and Russell's viper (*Daboia russelli russelli*)[3,4]. There is no rapid and dependable diagnostic test for the identification of species responsible for envenomation hence polyvalent antiserum raised against a mixture of venoms from four major Indian poisonous snakes is being used for treatment. Production of polyvalent serum is a long and complex process and is expensive[5].

Although it is effective, the recovery is slow and large volumes are needed for treatment of any particular snake bite. Further, the severe allergic reactions, serum sickness and other side effects which such a treatment brings about is of serious concern[6,7]. Establishment of identity of the species of snake invicting the bite would facilitate administration of monovalent (species-specific) antiserum for rapid and effective recovery with reduced side effects.

There were several reports on detection of snake venoms from various parts of the world and the techniques were extensively reviewed8-11. In several respects, enzyme-linked immunosorbent assay (ELISA) is of more practical use than any other tests and it can be readily modified into kit for field use. To begin with, a mouse model was adapted to develop ELISA for detection of cobra venom. This was owing to the low venom levels in autopsy specimens which made it essential that the ELISA should be more sensitive. Also it would be appropriate to decrease the assay time. Considerably in view of the fact that venom could be detected in the samples as fast as possible. In this study, we report the development of a sandwich ELISA for detection of Indian cobra venom in autopsy specimens of mice.

MATERIALS AND METHODS

Materials:

Adult male Swiss albino mice (18±22 g) and New Zealand white male rabbits (2±3 kg) maintained at the Abdul Hakeem College, Melvisharam, Tamil Tamil Nadu, India. The animal studies were conducted with the prior permission of the Institutional Animal Ethics Committee, C. Abdul Hakeem College, Melvisharam, Tamil Nadu, India (No.1011/c/06/CPCSEA, dt 19.12.2006), India, were used. Lyophilized whole venoms of four major Indian venomous snakes viz., *B. caeruleus, N. naja, E. carinatus* and *D. r. russelli* were purchased from the Irula snake catcher's industrial co-operative society, Tamilnadu, India. Venoms of each species were constituted from a pool of 35±80 adult donor snakes species (*B. caeruleus* 80, *N. naja* 50, *E. carinatus* 75 and *D. r. russelli* 35). Tween 20, bovine serum albumin (BSA) (Sigma, St. Louis, USA), ortho-phenylenediamine (OPD) (Aldrich, Milwaukee, USA), sephacryl S-200, protein A-sepharose CL- 4B (Pharmacia, Uppsala, Sweden), rabbit anti-goat IgG and goat anti-rabbit IgG±horse radish peroxidase (HRP) conjugates (Dako, Denmark), biotin±N- hydroxysuccinimide ester (Vega Biochemicals, Tuscon, USA), HRP±avidin conjugate (Vector Laboratories, Burlingame, USA), hydrogen peroxide (H_2O_2) (Glaxo Laboratories, Bombay, India), polystyrene microtiter plates, 96 wells, at bottom (Costar, Cambridge, USA) were obtained from the manufacturers. All other chemicals and reagents used were of analytical grade.

SDS-PAGE analysis of snake venom:

Snake venom protein is medicinally important and analyzed by 12% SDS-PAGE under reducing conditions[12,13]. Prior to electrophoresis, venom samples (10ug) were mixed 1:3 (v/v) with Laemmli sample buffer (10% SDS, 10% w/v β-mercaptoethanol, 50% sucrose, 0.02% bromophenol blue), boiled for 5 min, and electrophoresed at a constant current of 30 mA. After electrophoresis the gels were stained with Coomassie Brilliant Blue. Molecular weight standards were co-electrophoresed. Phosphorylase b (97.4kDa), Bovine serum albumin (66kDa), Ovalbumin (43kDa), Carbonic anhydrase (29kDa), soyabean trypson inhibitor (92kDa) and lysozyme (14.3kDa) were used as colored molecular weight markers.

Electro elution of different proteins of *Naja naja* venom:

The major venom proteins were electro-eluted on the basic method[14]. A preparative SDS-PAGE was run with proteins of cobra venom. After the run, the gel was soaked in prechilled KCl (0.4M). The prominent venom protein bands were excised and the gel slices were minced into small pieces (~1mm) using a sterile razor blade. The gel pieces were transferred into a dialysis bag with TE buffer (10mM Tris-HCl and 1 mM EDTA, pH 8.0) and the bag was kept in a horizontal electrophoretic tank filled with TE buffer. Constant power supply (50 mA) was set and run for 6 h. After elution the sample was dialyzed and concentrated by a Speed vac evaporator. The purified venom proteins were estimated and confirmed on SDS–PAGE.

Production of antiserum:

Male healthy New Zealand white rabbits (2 -3 kg body mass) were chosen for the production of polyclonal antibodies. Detoxified cobra venom (200 µg/kg of body mass) was emulsified with equal volume of Freund's complete adjuvant[15]and injected intramuscularly at multiple sites. First booster dose (200 µg/kg of body mass) was given along with Freund's incomplete adjuvant intramuscularly, after 4 weeks of first dose. After first booster dose, injections i.e. second and third booster doses (200 µg/kg of body mass) were administered intramuscularly along with Freund's incomplete adjuvant at an interval of 2 weeks[16]. The blood was collected from the marginal ear vein. After coagulation, the blood was centrifuged at 2,000-X g for 10 minutes and the serum was collected for determination by ELISA and Westernblot analysis[17,18]. The increase in antibody levels in both goat and rabbit were monitored by assaying sera samples, obtained one week after the booster dose by ELISA using the respective venom as coating antigen

ELISA for the determination of anti-snake venom antibodies:

Microtiter wells were coated with the four venoms (5 mg/ml) and incubated with serum samples diluted in PBS, pH 7.4±2% rabbit serum±Tween for 1 h at 37.8°C. Bound anti-snake venom antibodies were detected using goat anti-rabbit IgG±HRP conjugate and the enzymatic activity was measured with peroxide substrate solution.

Western blot analysis:

Cobra venom (20µg) was first fractionated by SDS-PAGE, as described above. The gel was placed in the electroblotting apparatus adjacent to nitrocellulose paper in buffer, as described by Towbin and his co-workers[19]. After transfer, the nitrocellulose paper (NCP) was blocked for 1 h with 3% skimmed milk in PBS (20nM sodium phosphate containing 0.9% NaCl, pH 7.2). The NCP was washed in PBS for 5 min and then incubated with 1:10000 dilution of rabbit anti cobra antiserum (or) rabbit anti individual venom proteins antiserum for 1 h. This membrane was washed three times in PBS containing 0.05% Tween-20 (PBS/T) followed by PBS was three times for 20 min each. The membrane was incubated with 1:30,000 dilutions of alkaline phosphatase conjugated goat anti-rabbit IgG for 2 h. The membrane was washed as described above

and developed with the substrate Nitroblue tetrazolium and 5-bromo-4-chloro indolyly phosphate in substrate buffer 10% 1 M Tris PH 9.5, 2.5% 4M NaCl and 0.5% 1 M $MgCl_2$. Same molecular weight markers were used in the gel electrophoresis.

Purification of monospecific antisera:

Hyperimmune sera raised against the venoms of Indian cobra (14 kDa, 29 kDa, 65 kDa, 72 kDa and 99 kDa proteins) were purified by affinity chromatography using a protein A-sepharose CL- 4B as described by[20]. In brief, protein A-sepharose (1.5 g) was swollen in BBS, pH 8.0, for 1 h at room temperature and the material when settled, was transferred to a column of approximately 6 - 1.5 cm. The monospecific antiserum was dialyzed against BBS and 2 ml of diluted antiserum was passed through the column. The column was washed thoroughly with BBS and bound IgG was then eluted with 4 M MgCl2. The absorbance of each fraction was monitored at 280 nm. Protein rich fractions were pooled, dialyzed against BBS and the lyophilized powder was stored at -70. 8°C.

Median Lethal Dose (LD_{50}) determination:

The lethal toxicity was determined in male Swiss strain mice. Groups of six animals were injected (i.p with 0.5ml of 0.85% NaCl containing increasing concentrations of cobra venom by the method[21]. The different concentrations of venom used for determine of LD_{50} value for the route was 6.25, 7.81, 9.76, 12.20, 15.25 and 19.90 µg per animal. The dose was killed 50% of animals within 24 h after determined by Spearman-Karber method[22].

Intraperitoneal administration of antiserum at 0, 1, 2, 4, 6, 8 and 10 h after injection of *Naja naja* naja venom:

Three sets of experiments were conducted to study the efficacy of whole venom antiserum, cocktail antiserum and commercial antiserum to neutralize cobra venom in mice. Each set consisted of eight groups of mice (six per group). In the first set, the mice of Group I was injected (i.p.) a lethal dose (25 µg per animal) of *Naja naja naja* venom and served as positive control. In Group II, the mice were administered *Naja naja naja* venom and whole venom antiserum (400 µl/mouse, i.p.) simultaneously through i.p. In Groups III, IV, V, VI, VII, VIII and IX the mice were injected *Naja naja naja* venom (25 µg/mouse, i.p.). The mice in Group III were administered whole venom antiserum (400 µl/mouse, i.p.) 1 h after venom injection, Group IV (2h), Group V (4h), Group VI (6h), Group VII (8h) and Group VIII (10h). The mice of Group IX were given PBS alone and served as a negative control. The number of surviving mice was recorded 24 h after injection of antiserum. The experiment was conducted in triplicate. The volume of cocktail antiserum and commercial antiserum used in this experiment were 300 µl and 400 µl per mouse.

Mouse autopsy specimens:

One LD50 of *Naja naja naja* venom (12.37 mg/kg) was administered s.c. to mice. Three experimental mice and two normal controls were used at each time interval. Different organs (brain, heart, lungs, liver, spleen and kidneys) as well as tissue at the site of injection were collected at various time intervals (0, 2, 4, 6, 8, 10, 12 and 24 h) after venom injection. The tissues were weighed and homogenized in PBS, pH 7.4 (100 mg/ml) with a Virtis homogenizer (Virtis, USA) for 10 min at 10,000 rpm. The homogenized materials were centrifuged at 30,000g for 10 min and the clear supernatant in each case was analyzed for the presence of venom by ELISA. Tissues from normal mice collected at similar time intervals after death and processed identically were used as control.

ELISA for detection of venom in envenomated mice:

An experiment was conducted to make use of antisera raised against different proteins of cobra venom or whole venom antiserum to detect the venom in different organs of envenomated mice at different time intervals using ELISA. The organs such as heart, liver, kidney, brain, lungs and spleen were dissected out from the envenomated mice at 0, 2, 4, 6, 8, 10, 12 and 24 h or moribund stage after injection of venom. Blood and exudates at the site of injection were also collected along with other samples. The organs were washed with PBS to eliminate blood contamination and then homogenized in PBS for 10 min. The ratio of tissue and buffer was 1: 5 (1 g in 5 ml of PBS). The homogenate was centrifuged at 10,000 rpm for 30 min at 4°C and the clear supernatant was subjected to venom detection test. Tissue from normal mice injected with physiological saline served as negative controls. The wells of microtiter plate were coated at 5 µg/well with blood and tissue samples of envenomated mice collected at different time intervals after estimating protein concentration. The venom was detected by ELISA using anti-14 kDa protein of cobra venom. The optical density was measured at 405 nm using an automated ELISA reader and the titers were determined[23].

Quantitation of venoms:

In order to quantitate the amount of venom present in tissue samples, a titration curve was constructed by plotting the log of venom concentration against absorbance value for each snake venom. Known amounts of venom spiked with tissue supernatants from normal controls were included with each test to obtain a titration curve. Quantitation of each venom was carried out by working out the difference in the absorbance values between the

experimental and control wells and by subsequent comparison to the titration curve. The venom concentration was calculated and expressed in terms of ng/ml of the tissue homogenate of mice.

Statistical analysis:

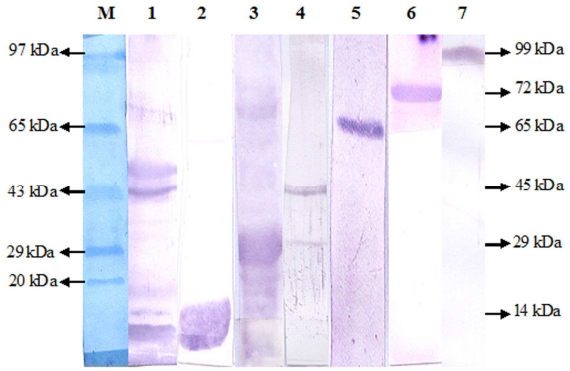
Data are expressed as mean ± SE. The Mann Whitney non-parametric tests and parametric student's t test were used for tests of significance of differences between groups. A probability of less than 0.05 was accepted as significant. Statistical calculations were performed using SPSS (version 9) software.

RESULTS

Protein concentration and SDS-PAGE analysis of cobra (*Naja naja naja*) venom:

The concentration of protein in the venom of Spectacled cobra was 62.56 ± 0.59, µg/µl. The protein profile of cobra venom was studied by SDS-PAGE. The Fig. 1 shows the protein pattern of cobra venom. Eight protein bands (six major bands and two minor bands) were observed on SDS-PAGE in reducing condition after staining with Coomassie Brilliant Blue. The approximate molecular weight of protein bands I, II, III, IV, V, VI, VII and VIII calculated based on the standard protein markers such as 14, 24, 29, 45, 48, 65, 72 kDa and 99 kDa respectively (Fig. 1).

Figure.1 : SDS – PAGE showing the protein pattern of Indian Spectacled cobra (*Naja naja*) venom.

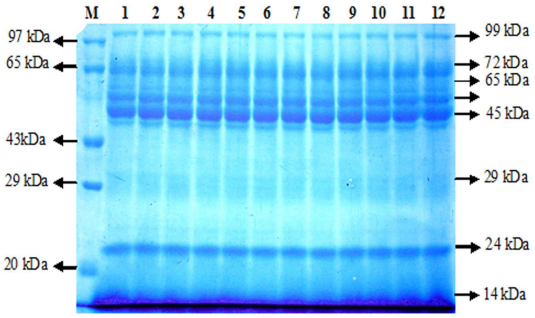


Lane M - Marker; Lanes 1 to12. *Naja naja* venom

Western blot analysis:

Antiserum was raised individually against different eluted proteins (band I, II, III, IV, V, VI, VII and VIII) of cobra venom and against whole venom of cobra. The concentration of protein in the samples eluted from the SDS-PAGE corresponding to Band I, II, III, IV, V, VI, VII and VIII were 23.57 ± 0.61, 32.26 ± 0.56, 21.23 ± 0.55, 23.24 ± 0.48, 16.68 ± 0.36, 18.24 ± 0.54, 35.35 ± 56 and 28.24 ± 0.61, respectively. The production of antisera was confirmed by Western blot analysis (Fig. 2). The results of Western blot analysis showed the appearance of six bands when the whole venom was treated with antiserum raised against whole venom. The results of Western blot confirm the production of antiserum raised against the venom protein bands I, III, IV, VI, VII and VIII (clear bands were not observed in the case of bands II and V).

Figure.2: Confirmation of production of antisera against whole venom and individual proteins of cobra venom in rabbit by Western blot analysis.



Lane M – Marker; Lane 1 – Whole venom; Lane 2 – 14 kDa; Lane 3 – 29 kDa; Lane 4 – 45 kDa; Lane 5 – 65 kDa: Lane 6 – 72 kDa: Lane 7 – 99 kDa

Immunization and Antibody response:

The whole cobra venom was assessed by ELISA for immunization. The antibody levels against whole venom were statistically significant in immunized rabbits when compared to negative controls to the PBS (Table 1).

Table.1: Antibody titers of whole venom antiserum and individual venom proteins antisera of cobra venom*

Groups	Antibody dilution
I	5,12,000
II	No reaction
III	2,56,000
IV	16,000
V	No reaction
VI	1,28,000
VII	1,28,000
VIII	1,28,000
Whole venom	1,28,000

*The antibody titers were accessed by ELISA using whole cobra venom or individual venom protein as antigen. Antibody titer corresponds to the maximal dilution of the serum resulting in OD 405 nm values higher than 0.100.

Determination of LD_{50}:

The LD_{50} value of cobra venom was determined in the mice model by different routes and the results are shown in Table 2. For intraperitoneal route the LD_{50} was found to be 13.73µg per mouse. The upper and lower limits were 2.03 and 15.55 µg per mouse.

Table.2: LD_{50} values of cobra venom for mice by different route of injection at 24 h post injection

Route of Injection	LD_{50} (µg/mouse) (Lower limit …… Upper limit)
Intraperitoneium (i.p)	13.73 (12.03 ……15.55)
Intravenous (i.v)	8.44 (7.35……9.53)
Intramuscular (i.m)	13.37 (12.03……15.55)
Sub-cutaneous (s.c)	13.73 (12.06……15.55)

Intraperitoneal administration of antiserum at 0, 1, 2, 4, 6, 8 and 10 h after injection of cobra venom:

100% survival was observed in envenomated mice treated with whole venom antiserum at the dose of 400 µl/mouse at 0, 1, 2 and 4 h after injection of cobra venom and 83.32% survival in mice administered the antiserum at 6 and 8 h after venom injection. Similar results were observed in the mice treated with commercial antiserum but the dose was 400µl/mouse and 100% survival was observed in the case of mice treated with cocktail antiserum (300 µl per mouse) at 0, 1, 2, 4 and 6 h after injection of venom (Table 3).

Table.3: Percentage survival of envenomated mice treated with different types of antisera at different time intervals (h) after injection of cobra venom. (n =18). The values are means ± SE.

Antiserum type	Concentration of venom (µg/mouse) used	Percentage of survival at 24 hrs after administration of the respective antiserum						
		0 h*	1 h*	2 h*	4 h*	6 h*	8 h*	10 h*
Whole venom antiserum (400 µl/animal)	25	100.00±0.00	100.00±0.00	100.00±0.00	100.00±0.00	83.32±5.57	83.32±5.57	0.00±0.00
Cocktail antiserum (300 µl/animal)	25	100.00±0.00	100.00±0.00	100.00±0.00	100.00±0.00	100.00±0.00	83.32±5.57	0.00±0.00
Commercial antiserum (400 µl/animal)	25	100.00±0.00	100.00±0.00	100.00±0.00	100.00±0.00	100.00±0.00	83.32±5.57	0.00±0.00
Positive control (PBS and venom)	25	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Negative control (PBS only)	0	100.00±0.00	100.00±0.00	100.00±0.00	100.00±0.00	100.00±0.00	100.00±0.00	00.00±0.00

Administration of antiserum at 0, 1, 2, 4, 6, 8 and 10 hr after injection of cobra venom

ELISA for detection of *Naja naja* venom in envenomated animals:

The ELISA technique was used to detect the venom in envenomated mice. The organs (heart, liver, kidney, brain, lungs and spleen) of envenomated mice were used for screening for venom. The organs were collected at different time intervals (1, 2, 4, 6, 8 hours post injection of venom and moribund stage) and used to detect the venom using ELISA. Blood and exudates at the site of injection were also collected along with other samples for screening purpose. The results are presented in Table 4. The venom was detected in all the organs of envenomated mice at 4 hours post injection of venom. The venom was detected in the blood of envenomated mice only up to 6 hours post injection and it showed negative at 8 hours of post injection and moribund stage. The venom was detected in all other organs up to moribund stage.

Table. 4: Detection of Indian cobra venom in different tissues obtained from the experimental envenomated mice injected with lethal dose of venom by ELISA at different time intervals

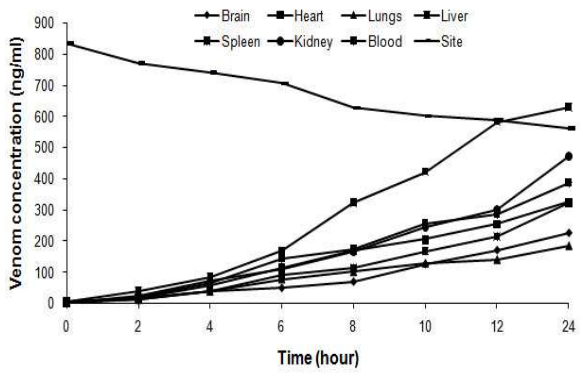
Groups (n = 6)	Venom injected	Number of positive/tested							
		Blood	Brain	Heart	Liver	Kidney	Lungs	Spleen	Site of Injection
I (1 hr)	*Naja naja*	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6
II (2 hrs)	*Naja naja*	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6
III (4 hrs)	*Naja naja*	5/6	6/6	6/6	6/6	6/6	6/6	6/6	5/6
IV (6 hrs)	*Naja naja*	5/6	5/6	6/6	6/6	6/6	6/6	6/6	6/6
V (8 hrs)	*Naja naja*	0/6	5/6	6/6	6/6	6/6	5/6	6/6	5/6
VI (Moribund stage)	*Naja naja*	0/6	4/6	6/6	6/6	6/6	5/6	5/6	5/6
Negative control	PBS	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6

Quantitation of *Naja naja* venom in mice:

The titration curve for the quantitation of known concentrations of *Naja naja* venom showed that the assay can detect venom levels up to 2.5 ng/ml of tissue homogenate (data not shown). The venom concentrations in various organs of mice as well as tissue at the site of injection at various time intervals following death are shown in Fig. 3. Tissue at the site of

injection showed the highest concentration of venom whereas brain showed the least. Heart, liver, kidneys, spleen and lungs showed moderate concentrations in the decreasing order. A correlation was found between the amount of venom detected and time elapse. A large quantity of venom was detected at 1 h after death in all tissues and the venom levels decreased gradually up to 24 h. Tissue at the site of injection showed a high level of venom (800ng ml) even moribund state, suggesting that the tissue at the site of envenomation may serve as a best source for venom detection in human cadavers too.

Figure.3: Naja naja venom levels in different tissues of mice at various time intervals following death. 200 µl of PBS, pH 7.4 containing 1 LD50 of venom was injected i.p into mice and the supernatants of tissue samples were analysed in duplicates. Venom concentrations (ng/ml) were interpolated from a titration curve of Naja naja venom diluted in tissue homogenates from control mice. Results are presented as mean ±S.D. (n=6).



DISCUSSION

The protein pattern of venoms of the medically important Indian snake (Spectacled cobra) was studied by SDS-PAGE. Eight protein bands with molecular weights of 14 kDa, 24 kDa, 29 kDa, 45 kDa, 48 kDa, 65 kDa, 72 kDa, and 99 kDa were observed on SDS-PAGE in the venom of *Naja naja*. Similar type of protein banding pattern was observed by many workers [24-27]. Mendoza and Bhatti (1992) observed seven protein bands in SDS-PAGE and twelve bands in non-SDS electrophoresis. SDS-PAGE analysis was carried out on Indian cobra venom obtained from three different geographical regions and the results revealed the presence of seven bands and significant variation in the protein constituents of the three regional venoms [25]. Cobra venom subjected to SDS-PAGE analysis indicated the presence of prominent protein components with molecular weights of 10, 20, 24, 55, 105 and 110 kDa[26]. Immunotherapy using polyvalent antivenom raised in higher animals is the only effective treatment against snake venom poisoning. Moreover, although the advantages of polyvalent antivenom are quite obvious, there is still a belief that polyvalent antivenoms are less effective and cause higher incidence of adverse reactions when compared to monovalent antivenoms[28,29]. Hence, an alternate technology should be explored to produce antivenom. In the present study, an attempt was made to make use of a cocktail antiserum prepared by mixing of antisera raised against individual venom proteins of cobra venom.

Highly potent antivenom against Thai cobra venom was produced in horse using different adjuvants and the results showed that the peak ELISA titer rose slowly and reached high levels after 18th week when bentonite was used whereas the ELISA titer rose very rapidly and reached high levels by 4th week when complete Freund's adjuvant (CFA) was used [30]. In the present study, the CFA was used to raise the antiserum against whole venom or individual venom proteins and the ELISA results showed high level antibody titer values for whole venom as well as some of the venom proteins. [31]Chotwiwatthanakun et al. produced potent polyvalent antivenom against Thai cobra and King cobra venom using low volume of venom with multiple-site immunization protocol as in the present study.

The cocktail antiserum was used in the present study to neutralize the cobra venom for developing an alternate technology for the production of antivenom using monoclonal antibodies for specific antigenic venom proteins. The use of cocktail antiserum to neutralize the cobra venom will form the basis for developing antivenom using monoclonal antibodies. Many workers have tried to develop alternate technology for production of antivenom against different snake venoms due to various reasons particularly the adverse side effects such as anaphylactoid reactions and serum sickness[32-36]. All these workers have tried to generate polyclonal antibodies in chicken egg against venoms of cobra, krait and vipers. Almeida et al. reported that polyclonal antibodies raised in chicken egg were found to be capable of recognizing, combining with and neutralizing the toxic and lethal components of *Bothrops* and *Crotalus* venoms[32]. Anti-viper venom antibodies were raised in Rhode Island Red inbred hens and these antibodies were isolated using a simple method. These antibodies showed good antigen binding and neutralization of venom in *in vitro* assays[33]. Thallay and Carroll have generated antivenoms against rattlesnake and scorpion venoms in chicken egg yolk and their neutralizing activity was demonstrated by in vivo experiments using mice[4]. Antivenom against Saw scaled viper was generated in the egg of white leghorn chickens as an alternative to conventional polyvalent equine antivenom[35]. Meenachisundaram and his co-workers have used chickens as an alternative source of

antibody production against cobra, krait, Russell's viper and Saw-scaled viper venoms[36].

The antiserum raised against venom protein of 14 kDa was used to detect the venom in different organs of envenomated mice by ELISA. The results showed that the antiserum could detect the venom in most of the organs tested at moribund stage of experimental animals. The ELISA test kit developed by Dong et al was used to detect the venom of four medically important snakes in Vietnam[37]. In the present study, the ELISA technique was used to detect the venom in different organs of envenomated mice using the antiserum raised against single protein of cobra venom. The venom in different organs was determined qualitatively not quantitatively. Selvanayagam et al., developed a double antibody sandwich ELISA to detect *Echis carinatus* venom in various organs of envenomated mice at different time intervals[38].

For the detection of snake venoms, there are several reports on clinical cases of envenomed victims but only a few studies done on autopsy specimens. In the present study, we developed a sandwich ELISA to measure Naja naja venom in various autopsy specimens of mice. The venom was detected in all the organs tested, indicating the distribution of *Naja naja* venom in all vital organs to be irrespective of any specific target organ (Fig. 3). However, concentrations of venom varied from one organ to another. The highest concentration observed at the site of injection suggested that the venom slowly entered into blood circulation from the site of injection. Moderate amounts of venom in kidneys indicated possible renal excretion of venom; similar results were obtained with *D. r. russelli*[39] and Vipera ammodytes venoms [40]. Low concentrations in the brain showed that the blood-brain barrier has some role in preventing the movement of venom into the brain. This may also prove logical in view of the fact that Naja naja venom contains toxic proteins which mainly affect the haemostatic mechanism rather than the central nervous system.

Several sensitive ELISAs have been reported for detection of snake venoms in human victims[41-47]. The increase in sensitivity may be attributed to the avidin/biotin amplification which enables the detection of extremely low levels of venom antigens in autopsy specimens. In venom detection, non-specific reactivity was observed[47, 48] and severity of the problem was elaborately discussed by Ho et al[41]. In the present investigation, this problem of non-specific binding was not encountered when normal mice autopsy samples were used. The AB-microELISA reported here is not designed as an emergency investigation in clinical medicine, since the complete procedure requires a minimum of 2 h and 10 min after blocking[49]. However a simple, rapid and species-specific immunodiagnostic kit was developed using the ELISA technique which will be discussed later.

CONCLUSION

In conclusion, these data suggest that the present investigation have thrown up the possibility of developing alternate technologies for production of antivenom against cobra venom and Antiserum produced in rabbits and an enzyme-linked immunosorbent assay (ELISA) to evaluate cobra venom levels or detection in biological samples. The results obtained from various studies on use of cocktail antiserum in the present investigation can form the basis for developing antivenom using monoclonal antibodies for snakebite treatment in future.

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