

Preparation of Polyvalent Anti Snake Venom (ASV) By Caprylic Acid Method and Assessment of Neutralizing Capacity of ASV

Duraisamy Meena¹, Rajaram Megavannan²

^{1,2} Assistant Professor, Department of Biotechnology, AVS College of Arts & Science (Autonomous)- Salem-11.

ABSTRACT

In this study indicated that Polyvalent anti snake venom production was achieved in a Horse model. In vitro-In vivo results were also obtained for venom toxicity and ASVS efficacy data. Both the In vitro-In vivo neutralization capacity of the Polyvalent anti snake venom serum was effective. The toxic levels of the venom were determined by LD50 analysis and haemolytic activity. LD50 analysis of the venom was done by using Swiss albino mice. The LD50 of the CV, KV, RV and EV venom were calculated using Spearman Karber method and were found to be 12µl/ml, 4.0µg/ml, 16.8µg/ml and 9µg/ml. The horses were immunized and develop the ASVS in its blood. About 6 litres of blood was collected from the hyperimmunized horse. The plasma was separated from the collected blood. The plasma was purified by caprylic acid method. The immunodiffusion tests of Polyvalent ASVS against crude CV, KV, RV and EV venom showed clear and distinct precipitin bands. Thus the presence of Polyvalent antibodies in the purified plasma of the animal was confirmed. The aim of our current study is to raise Polyvalent anti snake venom against Cobra venom, Krait venom, Russell's viper venom and Echis viper venom by immunizing the horse and also to assess neutralizing the Capacity of polyvalent antisnake venom.

KEY WORDS: Polyvalent anti snake venom, Echis viper, Russell's viper, blood, caprylic acid

1. INTRODUCTION

India has rich assortment of snake fauna, of which only 242 species have been identified including 57 poisonous or harmful species. The four major species of venomous snakes ubiquitous in India know as "Big four" are considered responsible for life-threatening envenomation around the country. These include Indian cobra (*Naja naja*), the common krait (*Bungarus caeruleus*), the Russell's viper (*Daboia russelli*) and the sawscaled viper (*Echis carinatus*). Although many other species of venomous snakes, for example, Indian Banded Krait (*Bungarus fasciatus*), *Naja kaouthia*, *N. oxiana*, *N. sagittifera*, *Echis sochureki*, *Hypnale hypnale*, yet less attention has been paid in the supervision of snakebite wreaked by these neglected species of venomous snakes.

The species name of the Indian Krait is caeruleus. The Kraits are harmful variety of snakes. The snake has enlarged hexagonal vertebral scales and there are narrow white crossbars on the back more or less distinct in pairs. Its scales are shiny. Its body is long and cylindrical and its tail is one-sixth to one-eleventh of its total length. It is lustrous black or bluish black above with paired narrow white crossbars indistinct or absent anteriorly. In the young ones the cross bars are conspicuous. In old snakes the white is in the form of a series of connected spots.

Venom

The Indian krait's venom consists mostly of powerful neurotoxins which induce muscle paralysis. Clinically, its venom contains pre-synaptic and post-synaptic neurotoxins. These neurotoxins generally affect the nerve endings near the synaptic cleft of the brain (Anas Ahmed 2010 et.al).

The average length is 0.99 meters (3 ft 3 in) but they can grow to 1.75 meters (5 ft 9 in). Males are longer with proportionately longer tails. The head is flat, neck hardly evident. The body is cylindrical, tapering towards the tail. The tail is short and rounded. The eyes are rather small, with rounded pupils, indistinguishable in life. The head shields are normal, no loreals; four shields along the margin of the lower lip; 3rd and 4th supraoculars touching the eye. Scales are highly polished, in 15-17 rows; the vertebral row is distinctly enlarged and hexagonal. Ventrals 185-225 and Caudals 37-50, entire (Chellapandi P 2000, et.al).

The current study was carried out as a trial to produce potent polyvalent antivenom suitable for use in a specific area. Antivenom are antibodies which are blood serum proteins, especially called immunoglobulins. These are generated by the immune system in response to a foreign substance or any micro-organism (antigens). Antibodies are characterized by specific binding with their particular antigen and neutralizing by specific binding with their particular antigen and neutralizing them so that they are removed from the circulation. Several types of immunoglobulins (Ig) have been identified, such as IgG, IgM, IgD, IgA and IgE. Of these IgM is produced immediately after the exposure of antigen. IgG is produced slowly, say 2-4 hrs later and offers protection for a long period. Hence mostly IgG is isolated and purified for therapeutic purposes.

Several animals used for antivenom production are horses, sheep, goats, rabbits and chickens. In the last hundred years, the horse has been the preferred animal for antivenom production as it is easy to manage and large volume of plasma is obtained during bleeding and plasmapheresis (Anderson CG 1955 et.al). Internationally, antivenom must conform to the standards of pharmacopeia and the World Health Organization.

The aim of our current study is to raise Polyvalent anti snake venom against Cobra venom, Krait venom, Russell's viper venom and Echis viper venom by immunizing the horse and also to assess neutralizing the Capacity of polyvalent antisnake venom.

2. OBJECTIVES

- To characterize the Cobra venom, Krait venom, Russell's viper venom and Echis viper venom by haemolytic activity and LD50 analysis.

- To raise Polyvalent anti snake venom serum against Cobra venom, Krait venom, Russell's viper venom and Echis viper venom by immunizing the horse.
- To analyze the quality and quantity of antibodies by performing In vivo and Invitro test.
- To check the presence of Polyvalent antibodies in the animal and quantify them by using ELISA technique.

3. MATERIALS AND METHODS

Preparation of protein (PBS)

Standard solution:

The standard protein sample contains 2mg/ml BSA make dilutions of protein standards with concentration of 100,80,60,40 and 20µg/100µl by transferring respective amount of protein from the standard protein and adjusting it to a total volume of 100µl by adding PBS buffer.

Preparation of venom

1g of lyophilized dry form of venom was dissolved in a 50ml of normal saline. Centrifuge at 1500 rpm for 15 minutes. After centrifuge the unwanted mucus and tissues were discarded. The clear supernatant was filtered through membrane filter or cellulose filter (0.45µm-macromolecules were filtered, 0.22 µm-all microorganism were filtered). The aliquotes were stored in a sterile water bath and kept at 4°c hence the 1% of venom was prepared (Fry BG 2006 et.al).

Lethal dose (LD) 50 Analysis

Experiments were conducted in swiss albino mice (approved by the committee for the purpose of supervision and control of experiments on animals-CPCSEA guide lines).The CV, KV, RV and EV venom was diluted separately in a geometric series and the volume was made upto 5ml with the help of normal saline. Intravenous injection was administered to 6 batches of CV, 6 batches of KV, 6 batches of RV and 6 batches of EV (each containing 6 mice) of mice via the tail vein.

Haemolytic activity of venom in blood agar plates

Blood agar was prepared by adding 40ml of defibrinated blood with 450ml of nutrient agar.15ml of blood agar was poured into the three sterile petriplates and allowed to solidify A single well (5mm in diameter) was punched on each plate using a gel puncher.5µl of crude CV and RV venom was added to the first plate and labeled as CV and RV.5µl of crude KV and EV venom was added to the second plate and labeled as KV and EV. The final plate was labeled as a control, then the plates were incubated for 48 hours and zone of lysis were measured.

Separation of plasma from blood

Once the animal was hyper-immunized and the desired titer value was reached, it was bled. For this: At first weight of the horse was calculated so that desired amount of blood can be drawn from horse. Blood was drawn from the jugular vein of the Horse into the sterile citrated 10 liter bottles containing anticoagulant agent like sodium citrate.

Purification of plasma by caprylic acid method

To the sterile batch reactor 1.5 litre of hyper immunized plasma was taken and dissolved in a 3 litre of distilled water, mixed well. Adjust the P^H 3.4 by adding 5N of Hcl, 0.1% of pepsin was added to the digestion of the specific immunoglobulin protein (Gustaro Rojas Jose Manuel.1994., et.al). Adjust the P^H 3-3.4 by adding 50% of NaOH. After completion of digestion process 100ml of 5% Caprylic acid was added, protein precipitation was occurred.

Enzyme linked immunosorbent assay (ELISA)

U32 strips were coated with various concentrations of CV, KV, RV and EV venom (U16-RV and U16-EV). The plates were incubated overnight at 4°C. After incubation, the plates were washed 4 times with PBS-Tween solution. Then 250µl of blocking solutions was added and the plate was incubated for 2 hours at 37°C. Then the various dilutions of Polyvalent ASV were added to the wells and incubated for 1 hour at 37°C.

4. RESULTS

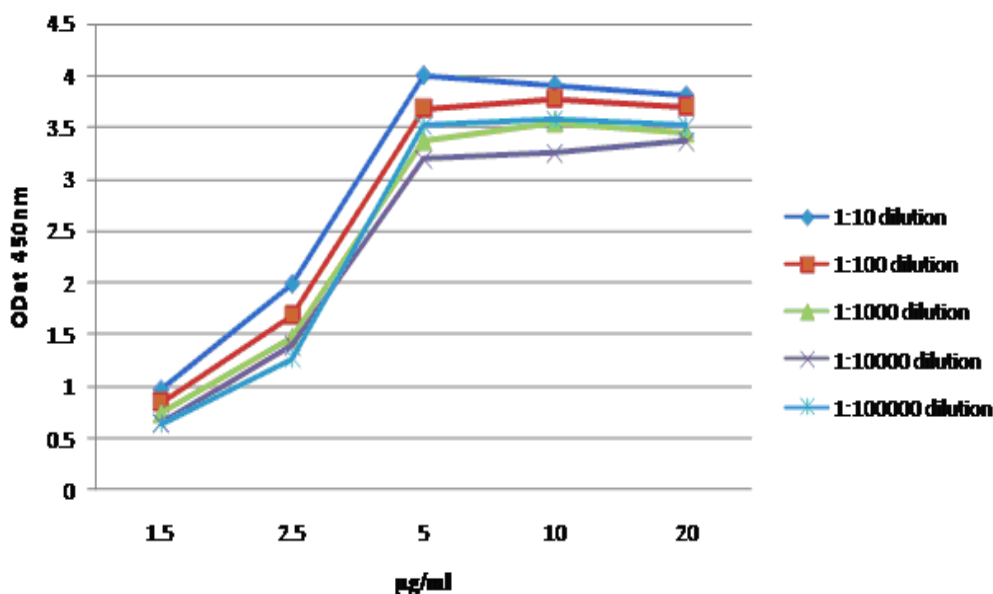
LD50 analysis

LD50 for four venom are classified by spearman karber method using Swiss albino mice of weight between 18-20 g. The Lethal dose for the mortality of 50% death, it prefers the toxicity in which the dose fixation for the immunization will be equalized accordingly. The LD50 values obtained for CV, KV, RV and EV were shown in the table 2, 3, 4 and 5.

The maximum quantity of venom used makes to the death of total number of mice is taken as X₁₀₀ with dilutions d=1.25. LD50 for CV, KV, RV and EV venom are classified by Spearman Karber method using Swiss albino mice. The lethality dose for the mortality of 50% prefers the toxicity. The dose fixation accordingly Table:1,

Table:1 LD50 analysis for CV venom

S. No	1	2	3	4	5
Venom in (µg)	7.81	9.26	12.2	15.3	19.1
Venom in (ml)	0.80	0.98	1.22	1.53	1.91
Normal saline	4.20	4.02	3.78	3.47	3.09
Death/live rate	0/6	4/2	6/0	6/0	6/0



ELISA for Cobra venom

Protein estimation (bradford method)

The unknown protein concentration of venom for CV, KV, RV and EV are estimated by Bradford Method. The quantities of total protein present in the four venoms are CV- 44µg/ml, KV - 59µg/ml, RV- 50µg/ml and EV - 37µg/ml were shown in the table 2.

Table 2: Protein estimation (bradford's method)

S. NO	Sample (µg)	Standard Protein (µl)	Venom (µl)				PBS buffer (µl)	Biuret reagent (µl)	Incubate 5 mts at room temperature	OD at 545nm			
			CV	KV	RV	EV				CV	KV	RV	EV
S1	100	50	-	-	-	-	50	500		90	90	90	90
S2	80	40	-	-	-	-	60	500		92	92	92	92
S3	60	30	-	-	-	-	70	500		72	72	72	72
S4	40	20	-	-	-	-	80	500		56	56	56	56
S5	20	10	-	-	-	-	90	500		24	24	24	24
Test (CV, KV, RV and EV)	-	-	10	10	10	10	90	500		52	70	59	44

Haemolytic activity of venom:

Venom of CV and RV, KV and EV was loaded in Blood agar plates. It was observed after 48 hrs of incubation at 37°C. The zone of lysis was observed were shown in the figure 10.

It shows the characterization of Cobra venom, Krait venom, Russell's viper and Echis viper venom possess high haemolytic activity. Hence it confirms that CV, KV, RV and EV are haemotoxins. The zone formed by CV is 1cm and zone formed by RV is 1.2cm. This shows that the hemolytic activity in CV is minimum than RV. It confirms that Russell's viper venom gives hemorrhages. i.e., haemotoxin and Cobra venom is neurotoxin.

The zone formed by KV is 0.9cm and zone formed by EV is 1.2cm. This shows that the hemolytic activity in KV is minimum than EV. It confirms that Echis viper venom gives hemorrhages. i.e., Krait venom is haemotoxin and neurotoxin. The loss percentage of haemotoxin may present in CV and KV. The difference of characterization of CV, KV, RV and EV will be defined by the above method.

Zone formed by CV and RV



Zone formed by KV and EV



Fig 1: Haemolytic activity of venom

Purification of plasma by caprylic acid method

Purification of plasma by caprylic acid method was done by following three process were shown in the figure 2.

1. Pepsin digestion,
2. Separated globulin,
3. Desalting by Ultrafiltration.



Separated globulin



Desalting by ultrafiltration

Total protein estimation

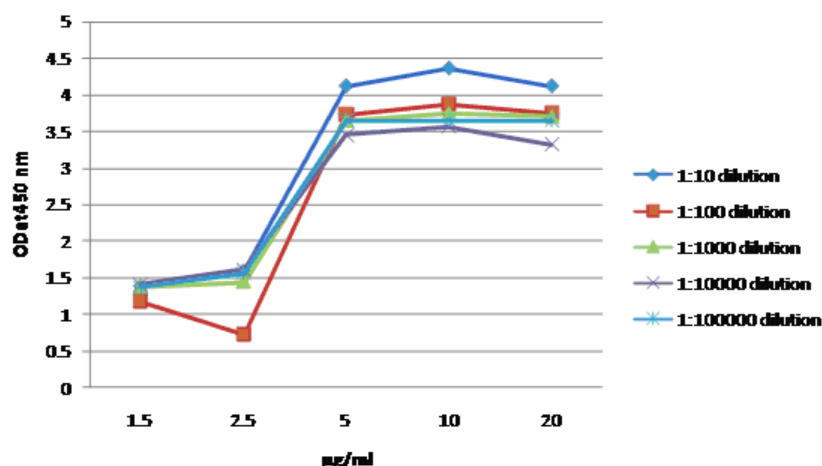
Total protein estimation in polyvalent anti snake venom was carried out by biuret method. 8.4g/dl of total protein is present in polyvalent ASV product.

5.9 In vivo - Potency test:

By using in vivo method, the potency of polyvalent anti snake venom serum produced by raising antibodies in horse neutralizes 6 LD₅₀ of (CV & RV) with 1 ml of ASV and 4 LD₅₀ of (KV & EV) with 1 ml of ASV. It shows the good yield and anti snake venom serum within the short period of immunization were shown in the table 3. From the result, it confirmed that 1ml of Anti snake venom serum (prepared by Caprylic method) neutralizes 0.6mg of Cobra venom.

- 1ml of Anti snake venom (prepared by Caprylic method) neutralizes 0.45mg of Krait venom.
- 1ml of Anti snake venom (prepared by Caprylic method) neutralizes 0.6mg of Russell's viper venom.
- 1ml of Anti snake venom (prepared by Caprylic method) neutralizes 0.45mg of Echis viper venom.

Sample	Venom (ml)	Final Product ASV(ml)	Saline (ml)	Number of mice	Result
1	Cobra Venom - 0.6	1	0.4	3	Alive
2	Russell's Viper - 0.6	1	0.4	3	Alive
3	Common Krait – 0.45	1	0.55	3	Alive
4	Echis Viper - 0.45	1	0.55	3	Alive



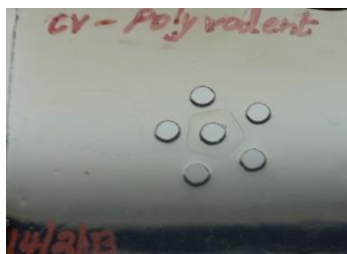
ELISA for Russell's viper Venom

Immunodiffusion

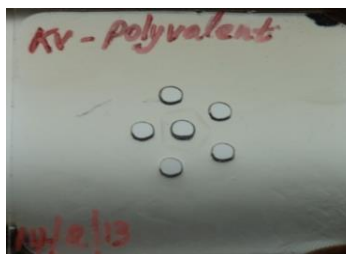
Antigen potency was determined against divalent ASV. Immunodiffusion plates were observed after 24 hrs of incubation. Precipitin line was observed. Antigen antibody neutralizes as they diffuse through the gel. The thick and the distinct band show the interaction between the antigen and antibody is very quick. It represents the presence of good yield of antibody in polyvalent anti snake venom serum.

Thick and distinct bands were formed by the four venom (CV, KV, RV, and EV) against the polyvalent anti snake venom serum and the Precipitin lines were obtained showed in the figure.3

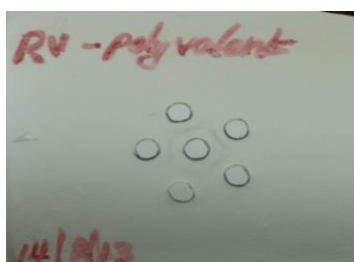
Indian cobra



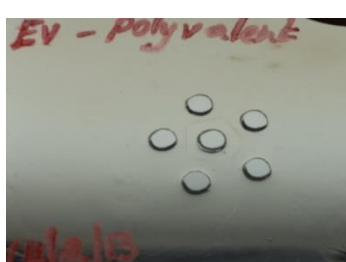
Common krait



Russell's viper



Echis viper



5. DISCUSSION

Snakebites represent a serious public health problem in many areas of the world. Traditional treatment is sought by most victims. Many survivors of snakebites are left permanently maimed. The Indian subcontinent, South East Asia, China, New Guinea and South America are the worst affected. Community based studies have shown that between 4 and 162 annual snakebites fatalities per 100,000 population. The present work revealed the possibility of simultaneous immunization of horses with different types of snake venom (CV, KV, RV and EV) for the production of polyspecific antivenom in horse with high titers of the respective specific antibodies. Polyvalent or polyspecific antivenoms have a broader spectrum of activity when compared to monospecific antivenom (Philadelphia Wahby et al., 2010).

Caprylic acid fractionation of animal sera or plasma constitutes a highly convenient methodology for immunoglobulin purification (Steinbuch and Audran, 1969). Our Present observation shows the preparation of polyvalent Anti snake venom by Caprylic acid, the hyper immunizes plasma, the yield and recovery of potency level is highly efficient. We suggested that the 5% Caprylic acid is giving good result in yield and potency level (Rojas G, et al. 2024), Rodriguez JP et al. 2023)

LD50 for CV, KV, RV and EV venom are classified by Spearman Karber method using Swiss albino mice. Ouchterlony immunodiffusion demonstrated immunoreactivity shows thick and distinct bands were formed by the four venom (CV, KV, RV, and EV) against the polyvalent anti snake venom serum. Both invivo and invitro tests are currently used to verify the neutralising efficiency of the antivenom. The principle of all these tests consists of measuring the decrease of venom toxic effects, lethality or particular biological activities, in relation to increasing concentrations of antivenom (Chippaux JP and Goyffon M, 1988).

The current study shows the maximum quantity of venom can be neutralizes the polyvalent anti snake venom (CV, KV, RV and EV) shows the efficient in potency titre value 5µg for CV, 10µg for RV, 5µg for KV, 5µg for EV and for all of these venom 1:10 ASV dilutions is found to be the minimum concentration to detect the potency of Ab-Ag reaction in ELISA test. Quantitative ELISA tests can also compete usefully with invivo and invitro assays (Theakston et al., 1977). Microtitration plates are coated by venoms used for the preparation of the antivenom. It can also be used in the same manner with the toxic fraction or the major toxin of venom. This type of test can be used only if significant correlation between invivo assays and ELISA are recorder (Theakston and Reid, 1979).

In view of this observation, if polyvalent antisnake venom production mechanisms are complimented to better facilitate, and also regulate the creation of venom production facilities, it would go a long way in promoting the production of high quality venoms (following WHO protocol) and thus overcome the snakebite deaths in India.

6. SUMMARY

In this study indicated that Polyvalent anti snake venom production was achieved in a Horse model. In vitro-In vivo results were also obtained for venom toxicity and ASVS efficacy data. Both the In vitro-In vivo neutralization capacity of the Polyvalent anti snake venom serum was effective. Polyvalent ASV made by immunizing the same animal using several different snake venoms producing effective treatment against the two or more different snake bites. Thus, finally concluded that Polyvalent ASV is more effective than Monovalent ASV can be used as snake bite treatment.

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