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Cobra

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Cover – Spectacled Cobra (*Naja naja*)

Most celebrated snakes of India
Photo: Dr.C. Arivazhagan

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Cobra, the bi-annual journal of the Chennai Snake Park Trust, invites articles and notes on reptiles and amphibians, their ecology, biology, natural history, conservation or other aspects. These may be of scientific or popular interest. Black and white photographs are also welcome.

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Identification of Common Cobra (*Naja naja*) venom through Ag-Ab complex interaction

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Introduction

Snakebite is one of the most important public health problems of tropical countries including India. Almost 30000 to 40000 people getting killed by snakes across the country. According to Suraweera *et al.,* (2020), estimated that India had 1.2 million snakebite death (average 58,000/year) from 2000 to 2019. Nearly half occurred at ages of 30–69 years and over a quarter in children < 15 years. Most occurred at home in the rural areas. The risk of an Indian dying from snakebite before age 70 is about 1 in 250, but notably higher in some areas. More crudely, we estimate 1.11–1.77 million bites in 2015, of which 70% showed symptoms of envenomation. Prevention and treatment strategies might substantially reduce snakebite mortality in India. The actual number of bites may be much more because many events were not reported. Snake bite is an occupational hazard, especially among the farm workers and forest workers and in rural India.

Snake venoms are complex in nature. Most of the constituents are proteins, but low molecular weight compounds such as peptides, nucleosides and metal ions are also present. The death of the prey is due to respiratory or circulatory failure caused by various neurotoxins, cardiotoxins, coagulation factors and other substances acting alone or synergistically. The four major venomous snakes chiefly responsible for the fatalities in India are Cobra (*Naja naja*), Krait (*Bungarus caeruleus*), Russell's viper (*Daboia russelii*) and Saw-scaled viper (*Echis carinatus*) Their toxins are composed of neurotoxins, cardiotoxins, enzymes and proteins (Sivamogsthem and Tejasen, 1973). In addition to the respiratory crisis, local reaction of the bitten site is also a serious problem. Though not life threatening, the local reaction may prolong the duration of hospitalization and it may increase morbidity in some cases (Pongprasit *et al*., 1988).

Polyvalent Anti Snake Venom Serum (ASVS) is a therapeutic agent available throughout the world for snake bite. ASVS sometimes do not provide enough protection against venom induced haemorrahge, necrosis, and nephrotoxicity and often produce hyper sensitive reactions. (Sutherland, 1977 and Corrigan *et al.*, 1987).

Hence, it is preferable to use monovalent antivenom for treatment of envenomation (Chippaux and Goyffon, 1998). As 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. 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. Establishment of identity of the species of snake indict the bite would facilitate administration of monovalent (species-specific) antiserum for rapid and effective recovery with reduced side effects.

In this approach, establishment of identity of the species of snake inflicting the bite would assist in the correct administration of monovalent antivenom for rapid and more effective recovery of the victim with minimal side effects.

From the medicolegal point of view, detection of snake venom in autopsy specimens of human victim plays a major role in ascertaining the cause of death. In view of monetary benefits given by the Tamil Nadu Government in India to the dependants of those who die due to snake bites, several false cases of snake envenomation have been reported. It is of immense importance for the forensic expert to detect and quantitate snake venom in autopsy specimens of human victims.

There were several reports on detection of snake venoms from various parts of the world and the techniques were extensively reviewed (Theakston, 1983). 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 *E. carinatus* venom. However, the same ELISA when applied to detect *E. carinatus* venom levels in autopsy specimens of human victim of snake bite was proved to be ineffective. 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.

Establishment of identity of the species of snake indict the bite would facilitate administration of monovalent (species-specific) antiserum for rapid and effective recovery with reduced side effects for Common Cobra (*Naja naja*) venom through Ag-Ab complex interaction

Cobra is a common snake throughout Africa and Asia. Africa species are generally larger than those from Asia and have narrow hoods. Furthermore, each species is fairly distinct and there is little controversy regarding their classification. The cobras of Southeast Asia are generally smaller than the African counterparts and can spread their hood to a greater width. Southeast Asian cobras, however, have few distinct external characteristics. There is considerable variation in both colour and pattern, even among individual specimens from the same geographic area. Indian members of Genus *Naja* range from one to two meters in average length. They eat a wide variety of good including other snakes, frogs, lizards, birds, and small mammals. Indian members of Genus *Naja* appear to categorize within three groups. They are *Naja naja, Naja kaouthia, and Naja oxiana*. Naja naja is abundant in India. It is found near human habitation where there is abundant supply of rodents, ducklings, and chicks. It is the most dangerous snake and important cause of death from snakebite (Buranasin, 1993, Looareesuwan *et al*., 1988).

Cobra venom Composition

Cobra venom is a mixture of many different proteins. The most toxic components are neurotoxins, which are single chain proteins of about 60 or 70 residues. The neurotoxins are low molecular weight and diffused rapidly into the blood stream. These toxins bind to nicotinc acetylecholine receptors, and they are the cause of death by paralyzing respiratory muscle. Neurotoxins are the principle lethal toxins in cobra venom; therefore, the crude venoms produce the same neurotoxic effect as pure neurotoxins. The crude venoms are a mixture of various proteins and contain other lethal toxins. Cardiotoxins are the next most toxic components, and hence the crude venoms provoke cardiotoxic as well as neurotoxic effect (Tu.1977a). Neurotoxins and cardiotoxins of cobra venoms are structurally similar, but they have different pharmacologic effects. Cardiotoxins contain 60 to 62-amino acid residues that are similar to neurotoxins. They do not bind to choline receptors. Cardiotoxins cause the dropping in systemic blood pressure followed by bradycardia resulting in cardiac arrest. The lethality of cardiotoxins is greatly increases in the presence of phospholipse A_2, which display a synergistic action (Tu, 1977b) however the effects of cobra venoms are not restricted to neurotoxins and cardiotoxins. The venoms also cause local myonecrosis, although they do not produce local hemorrhage. Cobra venom

contains a variety of enzymes and hemolytic components, but the enzymes found are lesser than that in venoms of Viperidae and Crotaridae. They possess Caseinolytic, Plasma protease, Phospholipase A_2, Adenosine monophosphatase and acetylcholineesterase activities, with significant quantitative e differences (Mukherjee and Maity, 2002). Unlike, Krait venom, which contains both pre and postsynaptic toxins, the neurotoxins of cobra venoms are postsynaptic acting (Tu, 1977a).

Symptoms and Pathology of Cobra bite

Before going to the stage of paralytic, preparalytic symptoms, apart from the local symptoms of severe pain and swelling may be occurred, of these including; headache, vomiting, drowsiness, loss of consciousness, vasomotor sign such as pallor, sweating, weak to absent pulse and hypo tension. The recognition of the preparalytic symptoms and signs of envenomation assumes to be a great importance because, if the antivenom is given in time at this stage, it may effectively prevent paralysis developing or may limit its extent. These symptoms may appear within minute of the bite, so they may occur at the same time of muscle paralysis (Reid, 1964).

Local poisoning

Local poisoning of cobra bite is a serious problem. Cobra venom also contains potent cytotoxins, which is responsible for pain and rapidly spreading of swelling and tissue necrosis. The severity varies from mild to severe necrosis of skin and subcutaneous tissue. Local symptoms are pain, swelling with or without blistering and necrosis (Pongprasit *et al.,* 1988; Homma and Tu.1971). Pain then varying degrees of swelling and later necrosis, are outstanding features of local poisoning. Pain may be started immediately after the bite and remained for 10 days depending on the extent of necrosis. Swelling usually started two to three hours after bite and reached a maximum in 24 to 48 hours and may persists for up to 18 days (Reid, 1964; Pongprasit *et al*, 1988; Homma and Tu, 1971).

Local necrosis is now accepted at the most common squeal to and an effective cobra bite and once was classified as peculiar to cobra bite. Approximately fifty percent of the victims bitten by the cobra face the problem of local tissue necrosis, which is difficult to treat and antivenom cannot prevent, unless it was administered intravenously with 30 minutes after envenomation (Pongprasit *et al.,* 1988). After cobra bite is a dusky discoloration around the bite marks, extending in area and deepening in colour each day. About the third or fourth day the grey-black area becomes encircled by a red raised rim and

sometimes blisters developed on the middle of the dusky area. Then was often evident; incision released red yellow material and revealed necrosis of subcutaneous tissue. (Warrell *et al*, 1976). The area of skin necrosis may vary from a few cm^2 up to 600 cm^2 (Reid, 1964). The Most of the patients developed local necrosis and the bite marks, usually 4 days after the bite. While patients were discharged one to three days after the bite, so that local necrosis may well have been overlooked and often patient's comeback to hospital with severe local necrosis. The healing process needs long time in hospital may be 1 to 2 months, some cases required skin graft and often occurred of permanent tissue loss, morbidity or amputation (Pongprasit *et al*, 1988).

Systemic symptoms

The earliest symptoms of systemic poisoning are drowsiness, starting one to five hours after the bite. Difficulty in opening the eyes, speaking, opening the mouth, moving the lips, and swallowing followed three to four hour later. The susceptibility of various muscles to neurotoxins varies considerably; the most susceptible are extrinsic eye muscles and elevator of eyelids while superficial facial muscle and diaphragm are resistant. The general weakness was usually the last symptoms to develop, followed by paralysis of the muscle in severe cases (Reid, 1964) The outstanding feature of systemic poisoning is paralysis of the muscles due to rapid action of neurotoxin at the myoneural junction. Respiratory paralysis may occur within 3-4 hours in severe cases and is the important cause of death with or without complicating, shock, septicaemia and renal failure (Looareesuwan *et al,* 1988). Restlessness, irregular breathing, and mental confusion usually develop before respiratory paralysis, indicating the early significant clinical signs of impending respiratory failure. It is important for clinicians to recognize the early signs of systemic poisoning and the warming signs of respiratory failure.

Quantity of venom injected at a bite

This is a very variable, depending on the species and size of snakes, the mechanical efficiency of the bite, whether one or two fangs penetrated the skin and whether there are any repeated strikes. The snake may be able to control whether or not venom is injected. For whatever reason, a proportion of bites by venomous snakes do not result in the injection of sufficient venom to cause clinical effects. About 50% of bites by Malayan pit vipers and Russell's viper, 30% of bites by cobras and 5-10% of bites by saw scaled vipers do not result in any symptoms or signs of envenoming. Snakes do not exhaust their store of venom, even after several strikes and they are no less venomous after eating their prey.

Although large snakes tend to inject more venom than the smaller of the same species, the venom of smaller, younger viper may be richer in some dangerous components, such as those affecting haemostatic (Warrell, 1989). The severity of snake venom poisoning not only depend on the venom amount injected but also on a number of variables such as bite site, venom quality, the age, weight, and health of the victim bitten and the medical treatment.

Treatment of Snake Envenomation

First aid treatment

First aid treatment is carried out immediately or very soon after the bite, before the patient reaches a dispensary or hospital. Most of the traditional, popular, available and affordable first aid methods have proved to be useless or even frankly dangerous. These methods include making local incision or pricks\punctures ("tattooing') at the site of the bitten limb, attempts to suck the venom out of the wound, use of (black) snake stones, tying tight bands (tourniquets) around the limb, cryotherapy, electric shock, topical instillation or application of chemical. Most of studies have shown tourniquets did not prevent or adequately delay the spread of venom from the bite site (Warrell, 1999; Pe *et al.*, 1987). The two most important principle of first aid for snakebite are immobilization of the bitten limb and rapid transport of the patient to medical care. Bites by cobras, king cobras, kraits or sea snakes may delay absorption of venom from the site of the bite by pressure immobilization which bundle of crepe bandage around the entire bitten lib, starting distally around the fingers or toes and moving proximally, to conclude a rigid splint. Compression bandage or a tight tourniquet should not be released until the patient is under medical care in hospital because of it may result in the dramatic development of severe systemic envenomation. This method was extremely painful and very dangerous if the tourniquet was left on for too long (more than 40 minutes) as the limb might be damaged by ischaemia. Many gangrenous limbs resulted. Pressure immobilization is recommended for bites by neurotoxin elapid snakes, including sea snakes, but should not be used for viper bites due to its danger by increasing local effects of necrotic venom. However, it is not recommended for bite by cobra whose venom cause local necrosis.

Conventional treatment

The antivenom is the most recommended and the mainstay of treatment for snake envenomation. Monovalent or nonspecific antivenom neutralizes the venom of only one species of snake. Polyvalent or polyp specific antivenom neutralizes

the venom of several different species of snakes. However, antivenom treatment carries a risk of severe adverse reactions and in most countries, it is costly and may be in limited supply. It should therefore be used only in patients in whom the benefits of antivenom treatment are considered to exceed the risks. Indications for antivenom vary in different countries. Antivenom treatment is recommended if and when a patient with proven or suspected snakebite develops one or more of the following signs:

Systemic envenoming

- Haemostatic abnormalities: spontaneous systemic bleeding (clinical), coagulopathy (20WBCT or other laboratory) or thrombocytopenia (<100x10power3\liter) (laboratory).
- Neurotoxic signs: ptosis, external ophthalmologic, paralysis etc., (clinical)
- Cardiovascular abnormalities; Hypotension, shock, cardiac arrhythmia (clinical), abnormal EGC
- Acute renal failure: oliguria/anuria, dark brown urine, rising blood creatinine/urea (clinical), urine dipsticks, other evidence of intravascular haemolysis or generalized rhabdomyolysis (muscle aches and pains, hyperkalaemia) (clinical, laboratory).

Local envenoming

- Local swelling involving more than half of the bitten limbs (in the absence of a tourniquet)
- Swelling after bites on the digits (toes and especially fingers).
- Rapid extension of swelling (for example beyond the wrist or ankle within a few hours of bites on the hands or feet.
- Development of an enlarged tender lymph node, draining the bitten limb. (Warrell, 1999)

Antivenom treatment should be given as soon as it is indicated. It may reverse systemic envenoming even when this has persisted for several days or, in the case of haemostatic abnormalities, for two or more weeks. If there is only local envenoming, antivenom may be effective when it is given within the first few hours after the bite.

Problem of Polyvalent Antivenom treatment

Antivenom remains the only agent widely used in snakebite treatment. However, it leads to many problems.

1. The most effective antivenom is the monospecific. A species diagnosis must be made before the right treatment can be chosen. Frequently, patients cannot recognize or bring the snakes to hospital, so it is difficult to make right diagnosis. From data of the Indian ministry of Public Health, culprit snakes cannot be identified in about 80% of envenomation. In addition, clinical signs of snake envenomation are complicated; sometimes the medical staff misinterpreted and gave inappropriate antivenom (Looareesuwan *et al*., 1988).

2. Antivenom reactions occur usually more than 20% by developing react either early (within a few hours) or late reactions (5 days or more) after being given antivenom (Warrell, 1999).

Early anaphylactic reactions:

Usually develop within 10-180 minutes after starting antivenom. The patient begins with itching (often over the scalp) and develops urticaria, dry cough, fever, nausea, vomiting, abdominal colic, diarrhea and tachycardia. A minority of these patients may develop severe life-threatening anaphylaxis such as hypotension, bronchospasm and angio-oedema. Although, severe anaphylactic shock is rare, it is the cause of death at high risk (Gilon *et al*., 1989). Skin testing is necessary before antivenom is administered.

Late (serum sickness type) reactions:

1. Develop 1-12 (mean 7) days after treatment. Serum sickness reactions are less dangerous and less frequently reported. Clinical features include fever, nausea, vomiting, diarrhea, itching, recurrent urticaria, arthralgia, myalgia, lymphadenopathy, periarticular swellings, mononeuritis multiplex, proteinuria with immune complex nephritis and rarely encephalopathy. Patients who suffer early reaction and are treated with adrenaline, antihistamines and corticosteroid are less likely to develop late reactions. Pyrogenic (endotoxin) reactions: usually develop 1-2 hours after treatment. Symptoms include shaking chills (rigor, fever, vasodilation and a fall in blood pressure. (Warrell, 1989; Gilon *et al.,* 1989; Chippaux and Goyffon, 1998)

2. Antivenom development in animals such as horse or sheep, is time consuming, expensive and requires special storage condition (Chippauz and Goyffon, 1998)

3. Antivenom sometimes does not provide enough protection against venom induced hemorrhage and nephrotoxicity, which it is the important cause of death (Sutherland, 1977; Corrigan et al., 1987; Gilon *et al*., 1989; Warrell, 1989) and local tissue damage (Leon *et al*., 2000).

4. Antivenom is administered via intravenous: with the help para medical personnel (Chippaux and Goyffon, 1998).

Hence, it is preferable to use monovalent antivenom for treatment of envenomation (Chippaux and Goyffon, 1998). 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. 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. Establishment of identity of the species of snake indict the bite would facilitate administration of monovalent (species-specific) antiserum for rapid and effective recovery with reduced side effects.

Materials and Methods:

Animals

New Zealand rabbit and Adult Swiss mice were obtained from the Institute of Veterinary Preventive Medicine (IVPM), Ranipet, Tamil Nadu, and India were used for the study. They were kept in new plastic cages with sawdust as bedding under conditions of 12:12 h light and dark cycle and fed with standard diet. Equal numbers of male and female mice were used in each experimental group, keeping their mean weight as near as possible. The study was conducted as per the guide lines given by Institutional Animal Ethic Committee.

Commercial Polyvalent Antivenom

Hyper immune serum prepared by immunization of horses with a pool of venom from Common cobra, Common krait, Russell's viper, saw scaled viper and supplied by King Institute of Preventive Medicine, Chennai, India was used. The

potency assay was determined by the mouse protection test, in which *Naja* venom (10 LD_{50}) was pre-incubated with serial dilutions of antivenom, at 37ºC for 60 minutes, before i.p. injection. Antivenom potency was performed by probit analysis. One millilitre of this antiserum was able to neutralize the lethal activity of 1.mg of *Naja naja* venom

Cobra Snake venom

The lyophilized snake venoms cobra (*Naja naja*), have been obtained from Irula Snake-Catcher's Industrial Co-operative Society, Chennai and preserved in desiccators at 4°C for future use with necessary permission has been obtained from Tamil Nadu Forest Department (WL1/7/06 dated 22.11.2006). Venoms are dissolved in 0.9% saline and centrifuged at 2000 rpm for 10 min. The supernatant was stored at 4°C for further experiments. Venom concentration was expressed in terms of dry weight.

Phospholipase A2 activity

Phospholipase A2 activity was determined using egg phosphatidyl choline (PC) as substrate. The reaction mixture (1 ml) contained 1 μmole PC in 0.05 M Tris-HCl buffer pH 7.5, 0.2 ml diethyl ether, 40 μmoles Ca2+ and 10 μg of each regional venom was separately incubated for 1 h at 37°C. After inhibiting the reaction, the free fatty acids released were extracted in the upper organic phase. The cobalt soap of fatty acid formed gives a reddish yellow coloured product with α-nitroso-β-naphthol. The absorbance was measured at 500 nm. Phospholipase A2 activity was expressed as μmoles of free fatty acid released/ min/mg protein. Linoleic acid (up to 1μmole) served as standard.

Edema inducing activity

The procedure of Vishwanath *et al.,* 1987 was followed. Groups of six mice were injected separately in the right footpads with different doses (15 μg) of venom samples in 200 μl saline. The left footpads received 200 μl saline, which served as controls. Mice were sacrificed after 45 min by cervical dislocation and both the hind limbs were cut off at the ankle joints and weighed individually. The increase in weight due to edema was calculated as the edema ratio, which equals the weight of edematous leg × 100/weight of normal leg. Minimum edema dose (MED) is defined as the amount of protein concentration required to cause an edema ratio of 120%.

Determination of median lethal dose (LD) of Snake venom

Median lethal dose (LD_{50}) is defined as the least amount of venom (µg dry weight) injected intramuscularly to animals and resulted in the 50% death of animals within 24 hr. LD_{50} will be determined by conducting the lethal experiment. Mice weighing 18-20g, either male or female were used in this experiment. The venom solution having doses 3-12 µg/mouse were prepared in 0.9% saline. The 0.1 ml venom solution was injected intramuscularly (i.m.) to mice. Eight mice were used for each test dose. Control animals were injected with 0.9% saline only. The percent death of animals was recorded at 24 hr after the injection. The LD_{50} was calculated

Production of polyclonal antibodies against Indian cobra venom toxic proteins in rabbits

Polyclonal antiserum has been raised against whole venom of cobra and individual venom proteins of cobra in rabbit using standard immunization protocols. A modified method from Shashidharamurthy *et al*., (2002) was used to immunize mice. The whole venom of the snake and the individual proteins of venom (200 µg in 0.3 ml of phosphate buffered saline; 0.05 M, pH 7.5) of Indian *Naja naja* was thoroughly mixed with equal volume of Freund's complete adjuvant and injected into male rabbit intradermally at several spots. Three booster doses were administered at weekly intervals at the same concentration but with equal volume of Freund's incomplete adjuvant. After resting the animal for 9 days, blood was drawn from the marginal ear vein and antisera was separated after allowing the blood to coagulate at 8–10°C for 24 h.

Western blotting

Western blot analysis was done to confirm the production antibodies against whole venom and individual venom proteins. After separation on SDS–PAGE, the whole venom or individual venom proteins were transferred to nitrocellulose paper (NCP) at 300 mA for 3 h at 4^{o}C. The membrane was stained with Ponceau S to localize the marker and cut out the marker lane. Membrane was then washed thrice with Phosphate buffered saline (PBS) and blocking reagent (3% skimmed milk) was added and blocked overnight at 4ºC. After blocking, the blot was washed with PBS (NaCl 80.0g/l, KCl 2.0g/l, KH2Po4 4.4g/l, pH7.20) twice for 5 minutes each and primary antibody raised against *Naja naja* whole venom and individual venom proteins (1:1000) was added along with 1% BSA in PBS, 0.1% Tween 20, and rocked gently at room temperature for 3 hour. The blot was washed thrice with PBS

for 5 minutes each and was incubated ALP-conjugated goat anti-mouse IgG (1:10000, Sigma) for 1hr at room temperature.

After extensive washing, the blot was incubated in detection buffer (10mM Tris, pH 9.5, 100mM NaCl. 5mM MgCl20) for 10 minutes. The colour development was carried out using 33 ul of 5 – bromo-4-chloro-3-indolyl phosphate (50 mg/ml in 70% diethyl formamide) and 66ul of Nitro blue tetrazolium (50mg/ml in 70% diethyl formamide) in 10ml of detection buffer. The reaction was stopped after 15 minutes by addition of 10 mM EDTA.

Analysis of antibody response using ELISA

Eighteen mice from each treated group were bled on and assayed for antibody response against *Naja naja* whole venom and individual venom proteins by Enzyme-linked immunosorbent assay (ELISA). Mice were anaesthetized (MS-222, 50ug/ml) and the blood was collected from retino orbital vein in the eye region. After coagulation, the blood was centrifuged and the serum was collected and stored at -80C. ELISA plate (96 – wells) was coated overnight at 4C with *Naja naja* venom (10ug/ml) in 100 ul aliquots in carbonate buffer (15mM Na2 CO3 and 35 mM NaHCO3 at pH 9.6). The plate was washed with PBS containing 0.05% Tween 20 (PBS – Tween) and the blocked with 3% bovine serum albumin (BSA) in PBS for 1 h at 28C. After washing with PBS-Tween, serial dilutions of the sera (1:50 and 1:500 were added to triplicate wells of the plate and incubated for 90 min at 37C. The plate was then washed twice and rabbit anti-mouse immunoglobulin (1:1000) was added to the plate and incubated for 2 h at 37C. After washing thrice, Horse radish peroxidase (Sigma Chemicals Co., USA) was added to the wells and incubated for 2 h at 37C. After washing, the reaction was developed by the addition of DAB (Di amino benzidine) with TRIS and 6 ul of 30% Hydrogen peroxide, ph.7.6. After 30 minutes incubation in darkness at room temperature (the reaction was stopped by the addition of 3N NaoH) the plate was read with a micro plate reader (Thermo Lab Systems) at 405 nm. After 30 min incubation in darkness at room.

Myotoxic activity

Mice were injected intramuscularly (i.m.) in the right gastrocnemius with 2.5 mg of *Naja naja* venom in 50 ml of sterile saline and were bled 1, 3, 6, 12 and 24 h later through the ophthalmic plexus under light ether anaesthesia. Sera were collected after centrifugation and immediately assayed for creatine kinase activity (CKnac 2.5, Merck, USA). One unit corresponds to the amount of enzyme that hydrolyses 1 mm of creatine per min at 25°C. Enzyme activity was expressed in U/l.

Serum neutralization studies

Neutralization of myotoxic, hemolytic and lethal activities

The ability of anti- serum from rabbits to neutralize the myotoxic activity was estimated by incubating 100 µg of *Naja naja* venom with 50 ml of antiserum, undiluted and diluted 1:2, 1:4, 1:8, 1:32, 1:128 for 30 min at 37°C. Following incubation, the mixture was injected i.m. into mice. Blood was collected after 6 h by the ophthalmic plexus under light ether anaesthesia, and immediately assayed as described above for creatine kinase activity. Control groups were injected with either venom or antivenom (1/1). Experiments for the neutralization of hemolytic activity were performed by incubating venom (50 mg) with serial dilutions of antivenom, and then the remaining hemolytic activity was assayed as described above.

Neutralization of Antiserum for protective activity against cobra venom

Administration of both *Naja naja* venom (80µg/Mouse) and Antiserum i.p. after preincubation of the mixture for 30 min 12 groups of mice consisting of six mice per group were used in the experiment. The Positive control group was injected with commercial antiserum (8.5 mg/kg, i.p.) only. The negative control group was injected with *Naja naja* venom (i.p.) only. The other groups were each given a mixture of *Naja naja* venom (8.5 mg/kg) and different combination of antiserum produced against *Naja naja* venom fractions through i.p. The mixture of venom and antiserum was pre-incubated for 30 min at 37 ºC before administration. The times taken by the mice to die and the mean death times were recorded and analyses as described previously.

Intraperitoneal administration of the Antiserum, 2, 4, 6 and 8 hrs after injection of *Naja naja* venom (80 µg/mouse, i.m.)

10 groups of mice with six mice per group were used for the experiment. Group 1 was administered a lethal dose (10 mg/kg, i.m.) of *Naja naja* venom only and this served as the control. Groups 2,3,4,5,6,7 were given Antiserum at 2ml/kg i.p., 2,4,6,8,10,12hrs after the administration of *Naja naja* venom (80 µg/mouse, i.p.) respectively. Group 8 was given commercial antiserum at 2 ml/kg i.p. All the mice were observed for the number that died over the period of the experiment and the mean time of death was analysed using ANOVA.

Results

Median lethal dose (LD_{50}) of *Naja naja* venom

Median lethal dose (LD_{50}) of *Naja naja* venom was assayed by injecting different doses of venom in 0.1 ml physiological saline intramuscularly to left thigh of Swiss albino mice of both sexes weighed between 18-20g. Lethality data of *Naja naja* venom was shown in Table 1. LD_{50} was calculated. The LD_{50} of Naja naja venom obtained from this study was 13µg/mouse.

Table. 1. % Death of mice receiving various doses of *Naja naja* venom (LD_{50})

Dose (µg/mouse)	Dead animal/total	% Death
00	0/6	00
3.0	1/6	16
6.0	2/6	33
9.0	2/6	33
12.0	2/6	33
13.0	3/6	50
15.0	5/6	83.5
18.0	6/6	100

Edematogenic, Phospholipase A2 and Myotoxic activities

This venom presented high edematogenic activity, as demonstrated by its MED of 0.27 mg/ml. Edema started immediately after venom injection, reaching its peak at 30 min, decreased after 2 h, and disappeared completely at 24 h. (Table2) It shows that the myotoxic activity had its peak at 6 h after venom injection and decreased after 12 h.

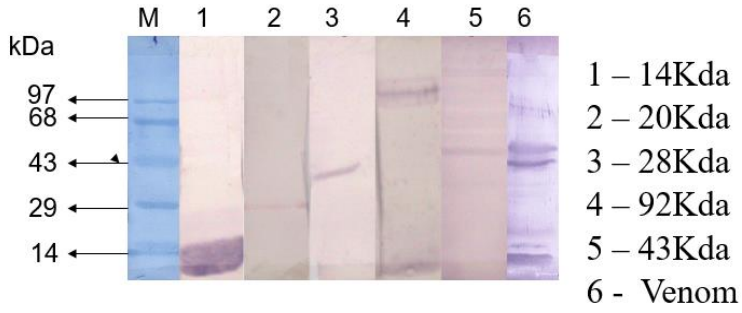
Table.2. PhospholipaseA2, Myotoxicity and Edematogenic activity

Protein Content (µg/ µl)	Phospholipase A2 (U/ml)	Myotoxicity (CK,U/ml)	Edematogenic (MED, µg)
66.25	114	2201.61	0.27

Production of polyclonal antibodies against Indian cobra venom toxic proteins in rabbits

When the electrophoretic profiles (Figure - 1) were considered together with the Western blotting profiles, we could observe that the anti-Naja naja serum recognized several venom components, mainly those lying between 84 and 16kDa were in Naja naja venom. And also, the antiserum against individual proteins recognized the 65,45,29,20,16 kDa were clearly detected.

Figure 1: Protein profiles as confirmed by Western Blot analysis



ELISA

The specificity of antibodies present in anti-elapidic serum was estimated by ELISA, using *Naja naja* venom as antigens. There was difference between commercial polyvalent serum antibody titers and monovalent antiserums using *Naja naja* as antigen. (Table - 3).

Table 3. Antibody titres of *Naja naja* antivenoms (Antibody titres were accessed by ELISA using *Naja naja* venom as antigens)

Groups	Ab titres dilution
Mice *Naja naja* whole venom antivenom	2,25,800
Mice 14kDa protein antiserum	2,00,800
Mice 20kDa protein antiserum	50,200
Mice 28kDa protein antiserum	30,100
Mice 43kDa protein antiserum	10,3000
Mice 93kDa protein antiserum	1,50,300

Cross-reactivity

Cross-reactions of *Naja naja* venom antiserums along with the three venoms (Russell's viper, Common krait and Saw-scaled viper) were performed. And the antisera of 14 kD Naja naja venom protein show negative cross-reaction with three venoms. That the antisera of 14Kda *Naja naja* venom protein been selected for the detection of *Naja naja* venom in envenomated mice.

Venom detection in envenomated animals by ELISA

All animals in the lethal dose injection groups showed systematic envenomation. Mice injected with *Naja naja* venom died within 1- 12 hr. after the injection with overall symptoms of blood loss and swelling at the site of injection. As soon as injected, 15 mins, the local symptoms developed. Under skin hemorrhage was seen and the symptom soon became severe after 2 h with continuous bleeding from the wound. The injected legs swelled and increased in sizes. Exudation from wound contained red blood cells as well as hemoglobin as results of hemolysis. The first mice injected with *Naja naja* venom died at 1 h and the last one died at 10 h after injection.

Mice injected with *Naja naja* venom quickly developed systemic neurotoxic symptoms. Paralysis started at the injected leg then the whole body became paralyzed after 45-60 mins. The animal could not stand, breathing became difficult and air way exudation was observed. Venoms were detected in blood and tissues taken from animals undergoing envenomation by performing ELISA in which antisera of 14KDa *Naja naja* venom protein used as primary antibody and they were tabulated below (Tables 4, 5, 6, 7, 8).

Table.4. The percentage of survival of mice injected with pre-incubated antiserum with Naja *naja* venom

Groups	Survival Animal/Total	% Survival
Control	0/6	00
Normal	6/6	100
Whole antiserum+Naja naja venom	5/6	83
Cocktail antiserum+Naja naja venom	6/6	100
Commercial antiserum+Naja naja venom	6/6	100

Note: Data of survival percentage was calculated from all mice (died and survival mice)

Table.5. The percent survival of mice injected with antiserums after *Naja naja* venom injection with different hours

Groups	Survival Animal/ Total After 24hrs	% Survival
Control	0/0	0
Normal	6/6	100
Whole antiserum	6/6	100
Cocktail antiserum	6/6	100
Commercial antiserum	6/6	100

Note: Data of survival time was calculated from all mice (died and survival mice)

Table 6. Inhibition of myotoxicity

Groups	CPK (Units/L)	% Inhibition
Normal mice	102+2.63	-
Control mice	2632+498.64	0
Whole venom antiserum+*Naja naja* venom	425+102.25	85.31
Cocktail antisrum+Naja naja venom	385+109.88	90.77
Commercial antiserum+*Naja naja* venom	485+105.25	82.25

Table 7. Snake venom detection in blood and tissues samples taken from experimental envenomation mice with different venoms

Groups N=6	Venom Injected	Venom detection	Serum before death	Brain	Heart	Liver	Kidney	Spleen	Lungs	Muscle
1	Spectacled cobra	+	+	+	+	+	+	+	+	+
2	Common Krait	-	-	-	-	-	-	-	-	-
3	Russell's Viper	-	-	-	-	-	-	-	-	-
4	Saw Scaled Viper	-	-	-	-	-	-	-	-	-

Table 8. Spectacled cobra (*Naja naja*) venom detection in blood and tissues sample taken in different hours from experimental envenomation mice

Concentration	Venom injected	Species identification	0	1	2	3	4	5	6	7	8	9	10	11	12
13ug/kg	Naja naja	Blood	0	+	+	+	-	-	-	-	-	-	-	-	-
13ug/ kg	Naja naja	Heart	0	+	+	+	-	-	-	-	-	-	-	-	-
13ug/kg	Naja naja	Liver	0	+	+	+	-	-	-	-	-	-	-	-	
13ug/kg	Naja naja	Brain	0	+	+	+	-	-	-	-	-	-	-	-	-
13ug/kg	Naja naja	Kidney	0	+	+	+	-	-	-	-	-	-	-	-	-
13ug/kg	Naja naja	Lungs	0	+	+	+	-	-	-	-	-	-	-	-	-
13ug/kg	Naja naja	Spleen	0	+	+	+	-	-	-	-	-	-	-	-	-
13ug/kg	Naja naja	Muscle	0	+	+	+	-	-	-	-	-	-	-	-	-

Discussion

The characterization of biological activities of *Naja naja* snake venoms is important not only to elucidate the pathophysiology of envenomation, but also to seek new approaches for the treatment of patients. Da Silva and Aird (2001) demonstrated using samples from 49 coral snake (*Micrurus sp*.) populations, representing 15 nominal taxa that prey preference appears to be a more significant determinant of venom enzymatic composition than phylogeny and selectively neutral genetic events.

The present results obtained with *Naja naja* venom from Tamil Nadu showed remarkable differences concerning the lethal, edematogenic, hemolytic and myo-toxic activities of other Naja kauothia venoms, such as these. Considering its lethality, *Naja naja* venom is highly toxic (13µg/g) when compared with i.p. LD50 s from other cobra venoms, e.g. *Naja kauothia* (0.7mg/kg), (Sashidharamurthy *et al.*, 2002, Mukherjee and Maity,2002).

Our results demonstrated that the injection of *Naja naja* venom into the subcutaneous tissue of mice induced an intense dose-dependent edematogenic response (MED 0.27 µg /mice), in comparison with MED from other elapidic

venoms: *Naja kouthia*, 4.8/mice(Mukherjee Maity,2002), M. frontalis, 5.72 mg/mice; M. lemniscatus, 10.8mg/mice and M. ibiboboca, 20.5 mg/mice (Sanchez *et al*., 1992; Casais de Silva, 1995), and M. spiixi, M. averyi, and M. hemprichii venoms also induce edematogenic effects (Barros *et al*.,1994).

Naja naja venom showed an intense indirect hemolytic activity on human red blood cells. This activity may be related to the presence of phospholipase A2 in this venom, similarly to other snake venom (Nget-Hong and Ponnudurai, 1992; Otero *et al.,* 1992; Rosso *et al*., 1996).

Muscular lesion could also be confirmed by the pronounced increase of serum CK levels, which was released after venom inoculation probably by the action of venom myotoxins. Increased levels of serum CK and/or the presence of myonecrosis are also induced by other venoms (Gutie´rrez *et al*., 1983; 1986;; Arroyo *et al*., 1987; Barros *et al.,* 1994) in addition to other elapidic venoms, e.g. *Oxyuranus scuttelatus* (Harris and Maltin, 1982), *Naja naja*, *Bungarus fasciatus* and *Dendroapis jamesoni* (Homma and Tu, 1971; Ownby and Colberg,1988).

Low levels of caseinolytic activity and no hemorrhagic, coagulant or dermonecrotic activities could be observed even if high concentrations of venom were employed. According to Tu (1977a), elapidic venoms have little or no proteolytic activity. Several authors verified the absence of either coagulant (Sanchez *et al*., 1992; Tan and Ponnudurai, 1992a) or hemorrhagic activities in *Micrurus* snake venoms (Gutie´rrez *et al*., 1983; Otero *et al*., 1992; Sanchez *et al*., 1992; Tan and Ponnudurai, 1992b). On the other hand, *M. averyi and M. fulvius fulvius* venoms induced hemorrhage (Tan and Ponnudurai, 1992c; Barros et al., 1994), and a hemorrhagic type phospholipase A2 toxin was isolated from M. frontalis venom (Francis *et al*., 1997).

The eletrophoretic profile of *Naja naja* revealed the presence of at least 12 bands, *Naja naja* venom bands between 84 and 29 kDa., in agreement with the data shown by Alape-Giro´n *et al.*, (1994), who studied the electrophoretic mobility of venom components from *Naja naja* species. Comparative studies of *Micrurus* venoms using either chromatographic profiles (Da Silva *et al*., 1991) or enzymatic activities (Aird and Da Silva, 1991; Tan and Ponnudurai, 1992a) evidenced that components similar to a-neurotoxins and phospholipases A2 (PLA2) are present in almost all venoms tested. a-neurotoxins are 7–8 kDa basic proteins and PLA2 (either acidic or basic) have at least one subunit of 14– 16 kDa (Rosso et al., 1996). Since *Naja naja* venom presents low molecular weight bands not more prominent which are not recognized by antiserum on Western blotting, it is likely that *M. altirostris* venom contains a novel a-neurotoxin with more potent lethal activity. Inter- and

intraspecific variations on electrophoretic profile were also observed in other *Micrurus* venoms (Tan and Ponnudurai, 1992a; Alape-Giro´n *et al.*, 1994; Casais de Silva, 1995).

The commercial antiserum produced by Instituto Butantan was prepared by immunization of horses with a pool of *Naja naja* venoms (Raw *et al.*, 1991). This has raised questions about the effectiveness of this antiserum in cases of envenomation caused by other *Naja naja* species, whose venoms are not included in the venom pool used for equine immunization. Antibodies present in anti-elapidic serum showed similar ELISA antibody titres against both homologous (M. frontalis) and heterologous (M. altirostris) venoms, and also recognized several antigens of both venoms when they were analyzed by Western blotting. However, components below 29 kDa of *Naja naja* venom were weakly recognized by anti-elapidic serum, with exception of 16 kDa component, recognized the probable a-neurotoxins and PLA2 regions usually find in naja venoms.

The antivenoms showed different neutralizing efficacy against *Naja naja* venom and individual proteins of *Naja naja* venom. The whole venom antiserum was very effective against *Naja naja* venom compare than commercial antivenom. The cocktail antiserum of individual proteins of *Naja naja* venom is very effectively neutralize the *Naja naja* venom(10LD50=2ml) by both invitro and in vivo methods.

Conclusion

In conclusion, the preparation of Monovalent antiserum could increase the survival time of mice (when the cocktails of antiserum and venom are pre incubated before intraperitoneal injection and antiserum administered after different time intervals (2,4,6,8,10 and 12hrs of venom injected) to reduce the lethality and myotoxicity effect of the venom. The present investigation will support scientifically the use of preparation of Monovalent antiserum and production of monoclonal antibodies for treating snakebite and diagnosis of snake venoms.

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Protein Pattern of Common Poisonous Snake Venoms Cobra (*Naja naja*), Krait (*Bungarus caeruleus*), Russell's Viper (*Daboia russelli*) And Saw-Scaled Viper (*Echis carinatus*)

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Introduction

Snake venoms are complex mixtures, produced by a specialized sero-mucous gland and inoculated by a specialized apparatus. Most of the constituents are proteins, with low molecular weight compounds such as peptides and nucleosides, metal ions are also present. The death of the prey is due to respiratory or circulatory failure caused by various neurotoxins, cardiotoxins, coagulation factors, and other substances acting alone or synergistically. The various enzymes injected into the prey start the digestion of the tissue. Snake venoms and other toxic secretions contain a large number of pharmacologically highly active substances with a specific mode of action (Meier, 1991). Snake venoms are either colourless or yellowish, depending on the amount of I-amino acids oxidase present, and enzyme with riboflavin as part of its prosthetic group. The most important pharmacologically active constituents are proteins. There are two groups to be distinguished; enzyme and nonenzymic polypeptides (Meier, 1991). Over 90% of the solid snake venom components are proteins or peptides possessing toxic or biological effects. The nonprotein fraction of snake venoms consists of inorganic anions and cations of low-molecular weight substances like amino acids, small peptides, lipids, nucleosides and nucleotides, carbohydrate, and amines (Stocker, 1991). The venom composition may be varying depending on age, (Moreno *et al*., 1988) geographic origin, (Stocker,1991) and individual snake, (Taborska and Kornalik,1985) as concluded from toxicity determination and measurements of enzyme activities.

This work explains about Protein patterns of four Indian snake's venom, namely *Naja naja* (cobras), *Bungarus caeruleus* (Krait), *Dobaia russelii* (Russell's viper) and *Echis carinatus* (Saw-Scaled Viper). The protein patterns of these venoms are studied and compared with proteins markers (known molecular weight).

Snakes and venomous snakes

Snakes are cold-blooded vertebrate. They are classified in class Reptilia, order Squamata, suborder Serpentes. About 346 species are recognized and classified in 11 families in India. Venomous snakes are identified in only three families: Elapidae, Viperidae, and Colubridae. There are about 70 species belongs to Elapidae and Viperidae out of which (8) *Bungarus Spp*, (4) *Naja Spp* and (3) *Viper Spp* are venomous. Mildly venomous snakes are coming under the family Colubridae which are rear fang snakes. The distinctions between mildly venomous and venomous are variable due to envenomation by a rear fanged.

Important venomous snakes in India

The most important venomous snakes in India are belongs to two family namely Elapidae and Viperidae.

Sub Family Elapidae

Snakes in this family have short permanently erect fangs. This venom is highly toxic and often fatal to man. Elapidae venom contains potent neurotoxins and also, like many other snake venoms, several varieties of proteins, both enzymes and non-enzymes. The main toxic constituent is neurotoxin, which is the major cause of death from respiratory paralysis. Among these, there are four species of the genus *Naja* (cobras): *Naja naja*, *Naja kaouthia*, *Naja oxiana* and *Naja sagittifera* and eight species of the genus *Bungarus* (kraits): but common species are *Bungarus caeruleus* and *Bangarus fasciatus* and the king cobra (Ophiophagus hannah). Other Elapidae snakes in India include coral snakes of the genus *Calliophis* and *Sinomicrurus macclellandi* They have potent venoms but rarely bite humans

Family Viperidae

The snakes in this family have long fangs, which are normally folded up against the upper jaw but are erected when the snake strikes. They are divided into 2 subfamilies: the true vipers (Viperinae) and pit viper (Crotalinae). The Crotalinae have a special sense organ, the pit organ, to detect their warm-blooded prey. This is situated between the nostril and the eye.

Subfamily Viperinae

There are three species in India namely Russell's Viper (*Daboia russelii*) and Saw scaled Viper *(Echis carinatus*) Levantine Viper *(Macrovipera lebetina*). The Russells' viper is motionless and relies on camouflage to avoid detection but

will hiss loudly and strike quickly when threatened. The venoms are complex and composed of several toxins, which are responsible for hemolysis, procoagulant activity, rhabdomyolysis and neurotoxicity. Pain is moderate at the bite site and later at regional lymph nodes. Rare neurotoxic symptoms included drowsiness, syncope, visual disturbances, and pituitary necrosis. Bite-site symptoms of swelling and necrosis are less severe than with pit viper bites. Common sites of bleeding are fang marks, gums, nose, skin, the gastrointestinal tract, kidneys, central nervous system and renal failure is multifactorial. Renal damage may require peritoneal dialysis or hemodialysis (Cox, 1991). Their venom is quite toxic and potentially lethal. Mortality from their bites is fairly high.

Subfamily Crotalinae

The snakes in this subfamily gave a prominent loreal pit located between the nostril and the eye that leads to a thermosensitive organ used in finding prey. There are twenty-three species and subspecies reported in India and one the important venomous snake is Hump nosed pit viper (*Hypnale hypnale*). Like Russell's viper, the venom has hemotoxic activity. The bite site is painful. There is severe necrosis. haemorrhagic blisters are common, petechiae, gingival bleeding and haematuria are seen, but renal failure is rare. Morbidity is high, but mortality among hospital-treated patients is rare in India. Clinical manifestation consists of pain and early edema at the bite site. Ecchymosis, haemorrhagic blistering and coagulopathies can spread to the entire limb, cephalic and peripheral. Renal failure and central nervous system haemorrhages are rare but have been reported.

Proteins and polypeptides in snake venoms

A) Enzymes in snake venoms

Most of the proteins in venom are water-soluble enzymes. Many of these enzymes are hydrolases and possess a digestive role such as proteinases, exo and endopeptidases and phosphodiesterase. At least 26 enzymes have been detected in snake venoms. Of these 12 enzymes are found in all venoms and venom contents differ significantly related to snake species. Enzymes may be subjected to great variations depending on the condition of snake (e.g., age, nutrition, sex, living space or circumstances). Enzyme levels of viperid and crotalid venoms are in the range 80-90% of total dry matter, whereas the corresponding range for elapid venoms is 25-70% (lwanaga and Suzuki, 1979). Like in the majority of animal proteins, structure, immunological properties and biological behaviour of snake venom enzymes are species specific. Accordingly, the interaction of venom protein and their targets in prey organism depends on specific features of both snake and prey species.

B) Nonenzymatic snake venom proteins

1) Neurotoxins

The venom of snake species may contain agents that affect nerve functions of the prey animal, causing cramps, convulsions, or paralysis. Snake venom neurotoxins can be grouped into two major categories;

a) **Postsynaptic neurotoxins**

These toxins found predominantly in elapid venom. These neurotoxins are low-molecular weight basic proteins. They were divided in two types by this basis of their sizes and disulfide linkages. Short neurotoxins contain 60-62 amino acids residues and 4 disulfide crosslinked bridges and most of long neurotoxins consist of 70-74 amino acid residues and 5 disulfide bridges or sometime 4 disulfide bridges. The mode of action of both types is the same. The block neuromuscular transmission by binding specifically with high affinity to the acetylcholine receptor in the postsynaptic membranes of skeletal muscles prevents the binding of chemical neurotransmitter acetylcholine and thereby blocks the excitation of muscles. However, short neurotoxins block at the neuromuscular junction leads to flaccid paralysis. This action similar to that of d-tubocurarine so called curare- like or curare mimetic or curariform toxins or α-neurotoxin, whereas the block of d-tubocurarine is easily reversible by physostigmine, the effect of most - α - neurotoxins is virtually irreversible or slowly reversible.

b) Presynaptic neurotoxins

The presynaptic neurotoxins are mostly phospholipase A_2 and they exert the catalytic function of this type of enzyme. The significance of phospholipid cleavage for the neurotoxic effect is not yet fully understood. All of these neurotoxins found in elapid and some viperid snake venoms have a basic phospholipases A_2 in common that may be complexed with acidic, basic or neutral proteins units. The mode of action is two types:

a) Highly toxic phospholipase A_2 which that inhibit transmitter release from nerve terminals

b) Toxins which enhances transmitter release (dendrotoxins)

2) Cytotoxins

Cytotoxins are toxic polypeptides consisting of 60 to 62 amino acid residues with four intramolecular disulfide bonds. The pharmacological actions of cytotoxins comprise hemolysis, cytolysis, depolarization of muscle membrane and specific cardiotoxicity.

a) Enzymes found in snake venoms

Phospholipase A_2, L-Amino acid oxidase, phosphodisesterase, 5–Nucleotidase, phosphomonoesterase, Deoxyribonuclease, Ribonuclease, Adenosine triphosphatase.

b) Enzymes found in crotalid and viperid venoms

Endopeptidase, Arginine ester hydrolase, Kininogenease, Thrombin like enzyme, Factor X activator, Prothrombin activator

c) Enzyme mainly in elapid venoms

Acetylcholinesterase, Phospholipase B, Glycerophosphatas

d) Enzymes found in some venoms

Glutamic-pyruvic transminase, Catalase, Amylase, Beta-Glucosaminidase, Lactate dehydrogenase, Heparinase like enzyme. (Iwanaga and Suzuki ,1979).

3) Myotoxins

In severe cases of envenomation, Myonecrosis can cause permanent tissue damage, producing loss of fingers and toes, legs and arms. But in less severe cases, the muscle can be regenerated. Myotoxins are snake venom polypeptides that induce skeletal muscle contraction or produce local myonecrosis or myoglobinuria. Myonecrosis, although common in most cases of snake envenomation, is most pronounced with Crotalidae and Viperidae venoms and can also be observed with Hydrophiidae and Elapidae envenomation.

4) Cardiotoxins

Cardiotoxins are single-chain polypeptides. Chemical and structural related to the neurotoxins. All of these toxins are highly basic polypeptide consisting of about 60-62 amino acids residues with four disulfide linkages in the molecules. They have little affinity for nicotinic AchR but act on cell membrane. Cardiotoxins have little lytic effects on a wide range of cells, so they have other names as direct lytic factor, cytotoxins, membrane-disruptive polypeptide or membrane toxin. These have effects including systolic contractions, hemolysis, cytolysis, and muscle depolarization (Harvey,1991). Other non-enzymatic snake venom proteins act as proteinase inhibitors or represent structure analogues of proteinase inhibitors, bradykinin-potentiating peptides, choline esterase inhibitor, phospholipase inhibitors, nerve growth factors, lectins, proteins affecting platelet functions and proteins acting on the complement systems.

C) Nonproteinous snake venom components

The nonprotein portion of the venom is much smaller than the proteins. In general, it is biologically less active. Included in the nonprotein fraction are metal ions, inorganic anions, and some small organic molecules such as peptides, lipids, nucleosides, carbohydrates and amines (Stocker, 1991). Since all snake venoms contain multiple components with different mechanisms of action, the pathogenesis developing after a bite is a very complex nature. It is not only dependent on the qualitative composition, but also on their quantitative distribution of different components in particular venom.

Materials and Methods

Collection of Snake venoms

The lyophilized snake venoms cobra (*Naja naja*), krait (*Bungarus caeruleus*), Russell's viper (*Daboia russelii)*saw-scaled viper (*Echis carinatus*) and have been obtained from Irula Snake-Catcher's Industrial Co-operative Society, Chennai and preserved in desiccators at 4°C for future use with necessary permission has been obtained from Tamil Nadu Forest Department (WL1/7/06 dated 22.11.2006). Venoms are dissolved in 0.9% saline and centrifuged at 2000 rpm for 10 min. The supernatant was stored at 4°C for further experiments. Venom concentration was expressed in terms of dry weight.

Protein Estimation

The concentration of venoms was determined by the method of Lowry *et al*.(1951). The method and reagents used are given below:

Reagents used

a) **Bovine Serum Albumin** (stock solution of 1mg/ml) – protein standard

i) **Solution A** (1% Copper sulphate)

ii) **Solution B** (2% Sodium potassium tartarate)

iii) **Solution C** (2% Sodium carbonate in 0.1N NaoH)

a) the solutions are mixed in the ratio of C:B: A as 98:1:1. Out of which 4.5 ml of this mixture was pipetted into clean, marked glass tubes. The standard BSA was added to the tubes at concentrations of 10ug, 20ug,40ug,60ug,80ug and 100ug. 1µl of test samples were added to each marked tube and the tubes were incubated at room temperature for 10minutes.

b) 0.5 ml of Folin's reagent (1:1 solution of Folin's Ciocalteau reagent in distilled water) was added to the tubes. The tubes were incubated in the dark for 30 minutes and the optical density was read at 640 nm. The readings from protein standards were used to a plot a standard graph and to estimate value of the test sample.

SDS-PAGE analysis of big four snake venoms

The venom was analyzed by 12% SDS-PAGE (Laemmli, 1970) to study the protein pattern. Highly and partially purified venom samples will be subjected to SDS-PAGE analysis for comparison. The samples are mixed with Laemmli sample buffer (10%SDS, 10%w/v, Beta mercaptoethanol, 50% sucrose, 0.02% bromophenol blue), boiled for 5 min, and electrophoresed at a constant current of 30 mA. After electrophoresis, the gel was stained with Coomassie brilliant blue. Molecular weight standards will be co-electrophorized. Prominent bands of snake venom toxic proteins will be identified and eluted for immunization.

Monomer solution: 20% acrylamide and 0.8% N,N-ethylene bis acryl amide in distilled water. The Solution was filtered through Whatman filter paper and stored in brown bottles at 4C.

Separating gel buffer: 1.5M Tris-Cl, pH 8.8

Stacking gel buffer : 0.5M Tris Cl, pH6.8

Electrophoresis buffer(5x) 10% SDS, 10%(v/v) B- mercaptoethanol, 50% sucrose, 0.025% bromopenol blue in stacking gel buffer.

Proteins are separated using 10 – 15% separating gel and 5% stacking gels were used. Stacking gel was approximately 1/5 of the separating gel. Protein estimations were performed (Lowery et al.,1951) and equal amounts of total proteins were loaded in each well. Electrophoresis was carried out at room temperature with constant current of 20mA for stacking gel and 30 mA for separating gel. Gels were stained with staining solution (0.25g of Coomassie Brilliant Blue R-250 in 45% methanol, 10% acetic acid) overnight and destained with 45% methanol, 10% acetic acid solution until a clear background was obtained. Photographs were taken with Tracktel GDS-2 gel documentation systems (Vision systems, Germany).

Electro elution, Dialysis and concentration of snake venom toxic proteins

The proteins of the venom were electroeluted from the gel using the method of Hunkapiller and Lujan (1986). A preparative SDS–PAGE was run with venom. After the run, the gel was soaked in pre chilled KCl (0.4 M). The prominent venom protein bands were excised and gel slice will be minced into small pieces (less than 1 mm) with a sterile razor blade. The gel pieces were put into a dialysis bag with TE

buffer (10 mM Tris–HCl and 1 mM EDTA, pH 8) and the bag was kept in horizontal electrophoretic tank filled with TE buffer. Constant power supply (50 mA) was set and run for 6 h. After elution the sample was dialysed and concentrated by a Speed vac evaporator. The venom proteins were estimated and stored separately at 4^{o}C.

Results

4.1 Protein Estimation:

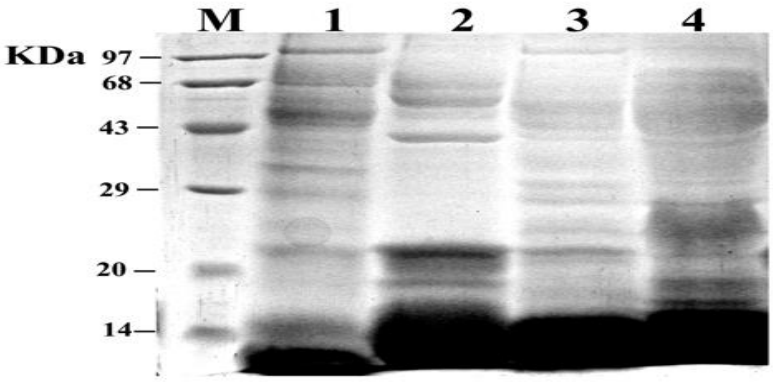
The concentration of protein in four venom samples was determined as;

- Spectacled cobra (*Naja naja*) = **79.3 µg/µl.**
- Russell's viper (*Daboia russelii*) = **64 µg/µl**.
- Common krait (*Bungarus caeruleus*) = **61.8 µg/µl.**
- Saw-scaled viper (*Echis carinatus*) = **72.3 µg/µl.**

SDS-PAGE protein profiles of snake venoms

The proteins profiles of common Spectacled cobra, Common krait, Russell's viper and saw scaled viper venoms were analyzed by SDS-PAGE are compared with the protein marker. Fig.1 shows that protein patterns of Cobra Common Krait, Russell's viper and saw scaled viper venoms. Four major bands with relative molecular masses (Mr) of approximately 92, 62, 48, 35, and 30, 20, 16kDa were detected and also detected additional bands with Mr values around 14 kDa. *Naja naja* venom presented higher number of protein bands. (Fig.1) and protein profiles confirmed by western blot analysis.

Figure 1: Protein profiles of 1) *Naja naja, 2) Common* krait, 3) *Russell's viper 4) Saw scaled viper* venoms by SDS PAGE analysis



Discussion

The SDS-PAGE analysis of venom proteins from common cobra (*Naja naja)* common krait (*Bungarus caeruleus*), Russell's viper (*Daboia russelli*), and saw-scaled viper (*Echis carinatus*) provides valuable insights into their molecular compositions. The comparison with protein markers allows for the determination of relative moleculare masses (Mr) of the detected bands.

The results, as depicted in Fig. 1, indicate distinct protein patterns for cobra and krait venoms. Interestingly, Cobra venom shows a higher number of protein bands compared to krait venom. This difference in banding patterns suggests variations in venom composition and molecular diversity among these snake species.

The presence of major bands in the higher Mr range (92 to 48 kDa) likely corresponds to larger proteins or protein complexes within the venoms. These proteins may include enzymatic components, neurotoxins, cardiotoxins, or other bioactive molecules responsible for the venom's toxic effects on prey or humans.

The detection of additional bands in the lower Mr range (around 30 to 14 kDa) indicates the presence of smaller proteins, peptides, or fragments in the venoms. These smaller components may contribute to specific venom activities such as neurotoxicity, hemotoxicity, or local tissue damage upon envenomation.

The higher number of protein bands observed in cobra venom suggests a more complex venom composition compared to krait venom. This complexity could be attributed to the diverse array of toxins and bioactive molecules produced by cobra species, which are known for their potent venom and diverse venom arsenal.

Overall, the SDS-PAGE and western blot analyses provide a visual representation of venom protein profiles, highlighting the molecular diversity and complexity of snake venoms. Further studies combining proteomic techniques with functional assays are necessary to characterize individual venom components and their biological activities, aiding in the development of targeted antivenom therapies and venom-neutralizing strategies.

Conclusion

Analysis of venom proteins from *Naja naja*, *Bungarus caeruleus, Daboia russelii,* and *Echis carinatus* reveals distinct molecular compositions and protein profiles among these venomous snake species. The comparison with protein markers enables the determination of relative molecular masses of the identified bands, shedding light on the diversity and complexity of snake venoms. Overall, the present

analysis serves as a valuable tool for studying venom protein profiles, providing essential insights into the molecular diversity, composition, and complexity of snake venoms. These insights are crucial for advancing our understanding of snake envenomation and improving clinical management through the development of more effective antivenom treatments.

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Proteomics of Snake Venoms

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Snake venom is a complex mixture of proteins and enzymes that vary widely across different snake species. These venom components are typically encoded by genes in the snake's genome. Venom genes have evolved over millions of years and are often highly specialized, allowing snakes to effectively immobilize or kill their prey.

Snake venoms typically consist of a mixture of 20 to >100 components, of which the majority (>90%) are peptides and proteins, with the dominant bioactivities including neurotoxicity, haemotoxicity and cytotoxicity, depending on the snake species. Venom composition varies widely between species and even within the same species. Other factors, such as environmental conditions, age, sex or type of prey available, can also affect venom composition.

There are several types of genes involved in snake venom production:

Toxin Genes: These genes encode the proteins and enzymes that make up the toxic components of venom. Examples include neurotoxins that affect the nervous system, hemotoxins that target blood cells, and cytotoxins that damage cells.

Accessory Genes: These genes are involved in the production, packaging, and delivery of venom. They may encode proteins that help transport venom toxins from the venom glands to the fangs, or they may be involved in regulating venom production.

Evolutionary Genes: Genes involved in the evolution of venom, such as those that control the expression and modification of venom components over generations. Evolutionary changes in these genes contribute to the diversity of venom types seen in different snake species.

Regulatory Genes: Genes that regulate the expression of venom genes. These genes determine when and how much venom is produced, often in response to stimuli such as prey presence or threat perception.

Understanding snake venom genes can provide insights into how venomous snakes have evolved and how their venom functions. It can also inform research aimed at

developing antivenoms and exploring potential medical applications of venom components.

Toxin genes

Snake venom toxin genes encode the proteins and enzymes that are responsible for the toxic effects of snake venom. These toxins are the key components that snakes use to immobilize, kill, or digest their prey, as well as defend themselves against predators. Snake venom toxins can be classified into several major groups based on their mode of action and biological effects:

Neurotoxins: These toxins target the nervous system of prey or predators. They can block neurotransmission, leading to paralysis or neuromuscular blockade. For example, α-neurotoxins found in elapid (cobra, mamba) venom interfere with acetylcholine receptors, causing paralysis.

Hemotoxins: Hemotoxic toxins affect the blood and circulatory system. They can cause hemorrhage, disrupt blood clotting, or lead to tissue necrosis. Many viperid snakes (e.g., pit vipers) produce hemotoxins such as metalloproteinases and serine proteases.

Cytotoxins: These toxins damage cells and tissues, leading to local tissue destruction and inflammation. Phospholipases A2 (PLA2) are common cytotoxic components found in snake venom. They can disrupt cell membranes and cause cell death.

Cardiotoxins: These toxins target the cardiovascular system, affecting heart function and blood vessels. They can cause cardiac arrest, arrhythmias, or hypotension. Certain snake venoms contain cardiotoxic components like cardiotoxins and phospholipase A2 enzymes.

Myotoxins: Myotoxic toxins target muscle tissues, leading to muscle damage, necrosis, and pain. They can also affect muscle function and mobility. Bothropic snakes, for example, produce myotoxins that contribute to the local effects of envenomation.

Other Toxins: Snake venoms can contain a variety of other toxins with diverse effects, including enzymes (e.g., hyaluronidases, phosphodiesterases), lectins, and peptides (e.g., bradykinin-potentiating peptides). These toxins may have roles in aiding venom spread, disrupting immune responses, or modulating prey physiology.

Snake venom toxin genes are typically located in the snake's genome and are often organized in clusters or families. These genes can undergo evolutionary changes, including gene duplications, mutations, and selection pressures, leading to the diversification and adaptation of venom composition in different snake species. Understanding the structure and function of these toxin genes is crucial for studying venom evolution, developing antivenoms, and exploring potential therapeutic applications of venom components.

Accessory genes

Accessory genes associated with snake venom play critical roles in the production, processing, and delivery of venom components. These genes are not directly involved in encoding toxins but are essential for the synthesis, storage, and secretion of venom. Here are some key aspects of accessory genes related to snake venom:

Venom Gland Development: Accessory genes regulate the development and differentiation of venom glands in snakes. They control the formation of specialized structures within the glands that facilitate venom production and storage.

Venom Synthesis: These genes are responsible for the synthesis of non-toxic proteins and enzymes involved in venom production. For example, they may code for proteins involved in post-translational modifications of venom toxins or enzymes that aid in venom processing.

Venom Packaging: Accessory genes encode proteins that assist in the packaging and storage of venom components within secretory vesicles. They ensure that venom toxins are properly folded, modified, and stored until they are released during envenomation.

Secretion Mechanisms: Genes associated with venom secretion regulate the mechanisms by which venom is delivered into prey or predators. This includes genes involved in vesicle trafficking, exocytosis, and venom ejection through specialized delivery systems such as fangs or stingers.

Regulation of Venom Production: Accessory genes also play a role in regulating the expression of venom genes. They respond to internal and external stimuli, such as feeding, hormonal cues, or threat perception, to modulate venom production levels and timing.

Evolutionary Adaptations: These genes can undergo evolutionary changes, including gene duplications, deletions, and mutations, leading to variations in venom

production and composition among snake species. Adaptive evolution of accessory genes contributes to the diversification and specialization of venom across snake lineages.

Understanding the function and regulation of accessory genes is crucial for comprehensively studying snake venom biology. These genes work in conjunction with toxin genes to ensure the efficient production, storage, and delivery of venom, ultimately shaping the potency and effectiveness of snake venoms in prey capture and defense.

Evolutionary genes

Evolutionary genes related to snake venom refer to the genetic mechanisms and processes that drive the diversification, adaptation, and evolution of venomous traits in snakes over time. These genes play crucial roles in shaping the complexity, specificity, and efficacy of snake venoms. Here are some key points about evolutionary genes of snake venom:

Gene Duplication and Diversification: One of the fundamental processes driving the evolution of snake venom is gene duplication. Venom genes undergo duplication events, leading to the formation of gene families with multiple copies of related genes. This allows for the diversification of venom components, as duplicated genes can evolve independently, acquiring new functions or modifying existing ones.

Positive Selection: Evolutionary pressures such as predation, prey preference, and defense drive positive selection on venom genes. Mutations that enhance venom efficacy, specificity, or adaptability are favored and become fixed in populations over time. Positive selection acts on coding regions of venom genes, leading to amino acid substitutions that alter toxin properties.

Gene Convergence and Convergence: Convergent evolution plays a significant role in the evolution of venomous traits across snake lineages. Distantly related snake species may evolve similar venom compositions or target similar physiological pathways through independent evolutionary paths. Convergent evolution is often driven by shared ecological roles or selective pressures.

Gene Regulation and Expression: Evolutionary changes in regulatory elements control the expression of venom genes. Enhancers, promoters, and transcription factors that modulate venom gene expression can evolve, leading to differences in venom production levels, timing, and tissue specificity among species.

Evolutionary changes in gene regulatory networks contribute to the fine-tuning of venom traits.

Horizontal Gene Transfer (HGT): While less common than other mechanisms, horizontal gene transfer can contribute to the evolution of venom genes in snakes. HGT events involve the transfer of genetic material between species, potentially introducing novel venom components or modifying existing ones. However, the extent and significance of HGT in snake venom evolution are still areas of active research.

Co-evolution with Prey and Predators: The evolutionary dynamics of snake venom genes are influenced by interactions with prey and predators. Co-evolutionary arms races between venomous snakes and their prey or predators drive continual adaptations in venom composition and functionality. Changes in venom genes may reflect responses to evolving prey defenses or predator immune systems.

Phylogenetic Patterns: Comparative genomics and phylogenetic analyses reveal patterns of gene evolution and divergence across snake species. Phylogenetic trees constructed based on venom gene sequences can elucidate evolutionary relationships, gene orthology, and gene family expansions or contractions.

Understanding the evolutionary genes of snake venom is essential for unraveling the mechanisms driving venom diversity, functionality, and adaptation. It provides insights into the genetic basis of venom evolution, the roles of selection pressures, and the ecological and evolutionary dynamics of venomous snake species.

Regulatory genes

Regulatory genes associated with snake venom play crucial roles in controlling the expression, production, and modulation of venom components. These genes are involved in fine-tuning venom production, responding to environmental cues, and ensuring the efficient deployment of venom during predatory or defensive encounters. Here are some key aspects of regulatory genes of snake venom:

Gene Expression Regulation: Regulatory genes control the transcription and translation of venom genes. They include transcription factors, enhancers, repressors, and other regulatory elements that bind to specific DNA sequences within venom gene promoters or regulatory regions. By modulating gene expression, regulatory genes regulate the synthesis of venom components.

Environmental Stimuli Response: Regulatory genes are sensitive to various environmental stimuli that trigger venom production. These stimuli can

include feeding cues, threat signals, hormonal changes, and temperature fluctuations. Regulatory genes integrate these signals and activate or suppress venom gene expression accordingly.

Hormonal Regulation: Hormones play a key role in regulating venom production in snakes. Regulatory genes involved in hormonal signaling pathways, such as those related to stress response, reproductive cycles, and metabolic states, influence venom gland activity and venom composition. For example, glucocorticoids can stimulate venom production in response to stress.

Developmental Regulation: Regulatory genes control the development and maturation of venom glands during embryogenesis and postnatal growth. They ensure the proper differentiation of glandular tissues, the formation of venom reservoirs, and the establishment of venom delivery mechanisms, such as fangs or specialized venom apparatus.

Seasonal Variation: In some snake species, regulatory genes exhibit seasonal variations in expression, leading to changes in venom composition or production levels throughout the year. Seasonal factors such as temperature, humidity, prey availability, and mating behaviors can influence the activity of regulatory genes and venom production patterns.

Evolutionary Plasticity: Regulatory genes can undergo evolutionary changes, including gene duplications, mutations, and selection pressures. Evolutionary plasticity in regulatory regions allows for the adaptation of venom production to changing ecological conditions, prey preferences, and predator interactions across evolutionary time scales.

Gene Networks and Pathways: Regulatory genes function within complex gene regulatory networks and signaling pathways. They interact with other genes involved in venom production, storage, and delivery, forming interconnected networks that govern venom gland physiology and venom composition.

Understanding the role of regulatory genes in snake venom biology is crucial for deciphering the molecular mechanisms underlying venom production, adaptation, and diversity. It provides insights into the dynamic regulation of venom synthesis in response to internal and external cues, contributing to the evolutionary success of venomous snakes in their ecological niches.

Composition of snake venom

There are more than 100,000 venomous animal species identified in the world. Each of these species is capable of producing venoms that often contain upwards to 100 different molecules. Animal venoms are used effectively for defense and predation. Animal venoms are not composed of single toxins but cocktails of complex chemical mixtures of pharmacologically active components including proteins, peptides, and enzymes with specific biological activities, as well as some non-protein compounds such as carbohydrates, lipids, metal ions and other, as yet, unidentified substances. Figure 1 illustrates the composition of snake venom.

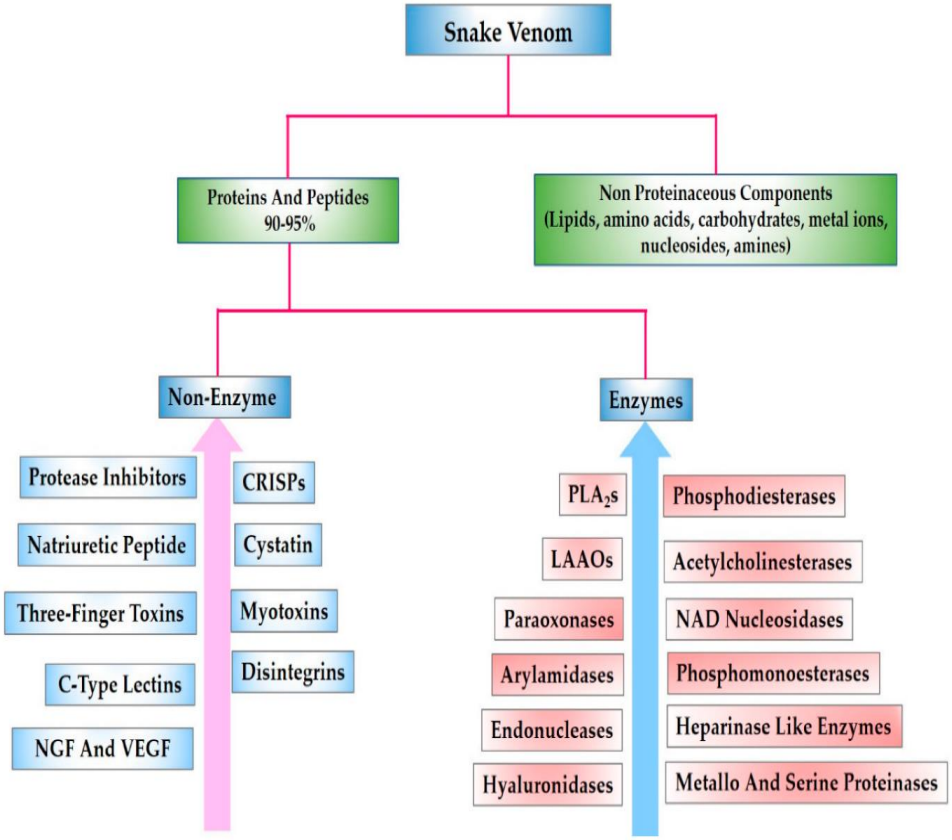

Figure 1: Composition of snake venom

The venom gland represents a modified salivary gland of venomous snakes for producing and storing venom toxins. Venom glands in the "Big Four" snakes, an advanced group of snakes, are situated in the temporal region behind the eye. The venom apparatus, which is considered as the evolutionary successor of Duvernoy's gland of colubrid snakes, is comprised of a highly specialized primary venom gland,

a duct with an accessory gland, muscles for squeezing the venom, and fangs for delivering the toxic venom. The primary function of the venom is to immobilize the prey and also aid in the predigestion of prey. Evidence has also been presented for the involvement of specific components of venom in prey re-localization [2].

Although otherwise poisonous, when used in the right proportions or structurally engineered, several venom peptides can be used directly as therapeutic drugs or as drug leads. These peptides are of great value, due to their diversified and distinct pharmacological activity, and high affinity and selectivity towards their receptors. Table 1 shows some important peptides and their biological significance (1).

Peptide	Mechanism of Action	Biological Significance/Therapeutics
3FTX (neurotoxin)	Selective inhibition of nAChRs at neuromuscular junction and interfere with nerve transmission.	Tool to decipher structural and functional details of nAChRs. α-cobratoxin is under clinical trial for drug-resistant HIV strains, treatment of multiple sclerosis, muscular dystrophy, myasthenia gravis and amyotrophic lateral sclerosis.
3FTX (cardiotoxin)	Membrane perturbation by electrostatic and hydrophobic interactions with the cell membranes.	Under scientific investigation for cancer inhibitory studies and potential use as anti-microbial agent.
Disintegrin	Selectively bind to integrin receptors present at the surface of platelet and other cells.	Tirofiban and Eptifibatide are under clinical use as antithrombotic agents. These compounds were developed from the snake venom disintegrns echistatin and barbourin. Contortrostatin is in preclinical studies for the inhibition of platelet aggregation and prostate cancer.
Kunitz-type inhibitor	Inhibition of serine proteases (e.g., plasmin, kallikrein, trypsin). Interferes with the blood coagulation cascade and fibrinolysis.	A plasmin inhibitor Textilinin-1 is in preclinical studies as antibleeding agent.
Natriuretic peptide	Interaction of Nps with guanylyl cyclase receptors leads to an increase of cylic guanosine monophosphate (cGMP), and affects subsequent signalling cascade. Nps can interfere the renin-angiotensin system by inhibiting the angiotensin converting enzyme.	These peptides serve as tool to understand NP biology. Cenderitide was under clinic studies for cardiovascular disease. However, its clinical development was terminated by Capiricor (US pharmaceutical company) in 2017.
BPPs	Inhibit the function of angiotensin converting enzyme, and raise the level of bradykinin.	Captopril and its analogue are under clinical use for the treatment of hypotension. These compounds were developed from the snake venom BPP.
Crotamine	Interacts electrostatically with DNA. Penetrates membranes via heparan sulphate proteoglycans binding.	Carrier for biomolecules, tool for cancer studies.
Sarafotoxin	Vasoconstriction via endothelin receptors.	Molecular probe to better understand endothelial system and related diseases.
Waglerin	nAChR antagonist.	Anti-wrinkle cosmetic cream SYN-AKE is available in the market. The active ingredient of this cream is a peptide mimic, which was designed using waglerin as a template.

Table 1: Some important components of snake venom and their biological significance

High-quality genomes of venomous snakes, combined with transcriptomics, will enable generation of a comprehensive catalog of venom-gland-specific toxin genes that can be used for the development of synthetic antivenom of defined composition using recombinant technologies.

19 'venom-ome-specific toxins' (VSTs) showed venom gland-specific expression, and these probably encode the minimal core venom effector proteins of cobra *Naja naja* and Figure 2 shows the toxins.

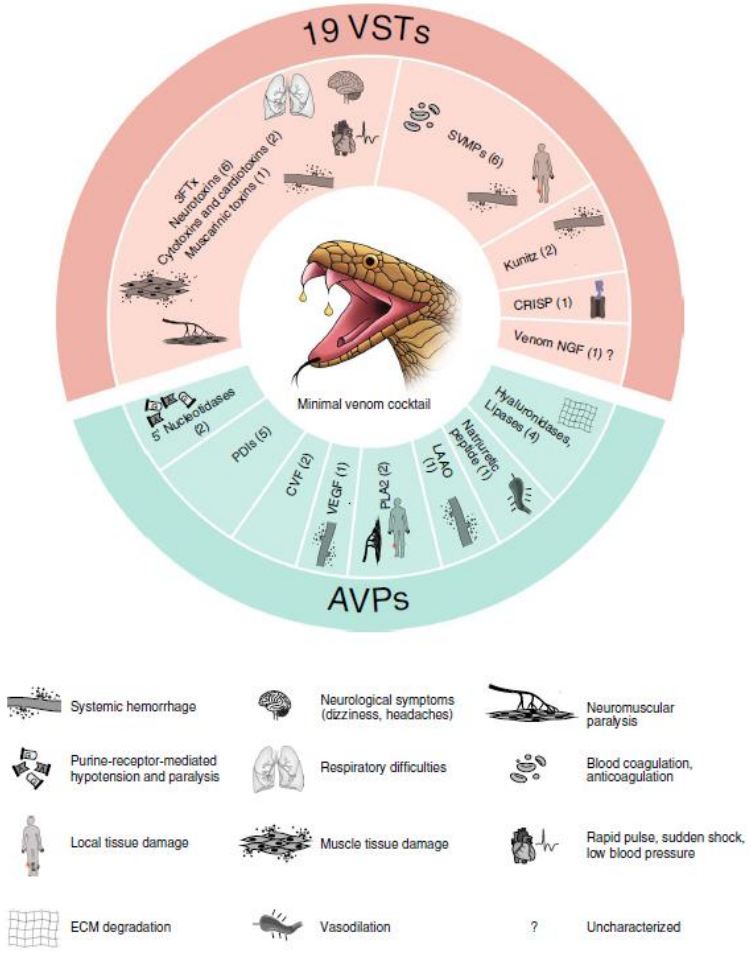

Figure 2: *Naja naja* minimal venom cocktail. The venom-ome-specific toxins (VSTs), accessory venom proteins (AVPs) and their primary physiological targets. ECM, extracellular matrix; PDIs, protein disulfide isomerases [3].

Some of the venom proteins exhibit enzymatic activities, whereas several others are non-enzymatic proteins and peptides. Other components in the snake venom are nucleosides, metallic cations, carbohydrates and very low levels of free amino acids and lipids with less biological activity. Sodium is the most abundant cation in snake venom, but its role is unknown. Zinc is needed for activation of anticholinesterase (acetylcholinesterase inhibitor) and calcium is required for phospholipase activity. The presence of cadmium was found to inhibit biological processes in specific enzyme activities. Variation in snake venom composition is found between species, subspecies or even in the same snake specimen. Snake venom variation is associated with many factors including phylogeny, geographic distribution, age, sex, and diet.

Enzymatic Proteins from Snake Venoms

Snake venoms are cocktails, which exhibit enzymatic activities. Most commonly, snake venoms possess enzymes such as proteolytic enzymes, arginine ester hydrolase, thrombin-like enzymes, hyaluronidase, phospholipase A2, acetylcholinesterase, nucleases (RNase, DNase, and Phosphodiesterase), and L-amino-acid oxidase (LAAO). Snake venoms are known as some of the richest sources of these enzymes.

Non-Enzymatic Proteins from Snake Venoms

Studies in the past few decades have revealed that snake venoms are also rich in non-enzymatic proteins. In the opposite of the snake venoms enzymatic proteins, non-enzymatic proteins contribute generally to immobilization of prey. In general, they act on specific membrane receptors, ion channels or plasma proteins, which cause disruption of the physiological processes of the prey by leading to neurotoxic and cardiotoxic effects. They are categorized into many protein families depending on their amino acid sequences and protein folding. Among the well-known non-enzymatic proteins families in snake venoms are (i) cysteine-rich secretory proteins (CRISPs) or helveprins; (ii) snaclecs (C-type lectins and related proteins); (iii) proteinase inhibitors; (iv) nerve growth factors; (v) bradykinin-potentiating peptides; (vi) natriuretic peptides; (vii) three-finger toxins; (viii) sarafotoxins; (ix) cobra venom factors; (x) vascular endothelial growth factors; (xi) vespryns; (xii) waprins; and (xiii) veficolins [81–84]. Within each family, the non-enzymatic proteins and peptides share remarkable similarities in their primary, secondary, and tertiary structures, but they may differ from each other in their pharmacological effects [4].

Amplification of snake venom toxicity by some endogenous signaling pathways

Snake venoms enhance their lethality by co-opting prey signaling systems, disordering and amplifying the prey's inflammation and cell survival machinery. Identifying key venom targets and their endogenous counterparts for inhibition should be applicable to most medically important snake species, and simultaneously address the fundamental matter of the recipient's biological response to the venomous insult (Figures 3 ,4, and 5).

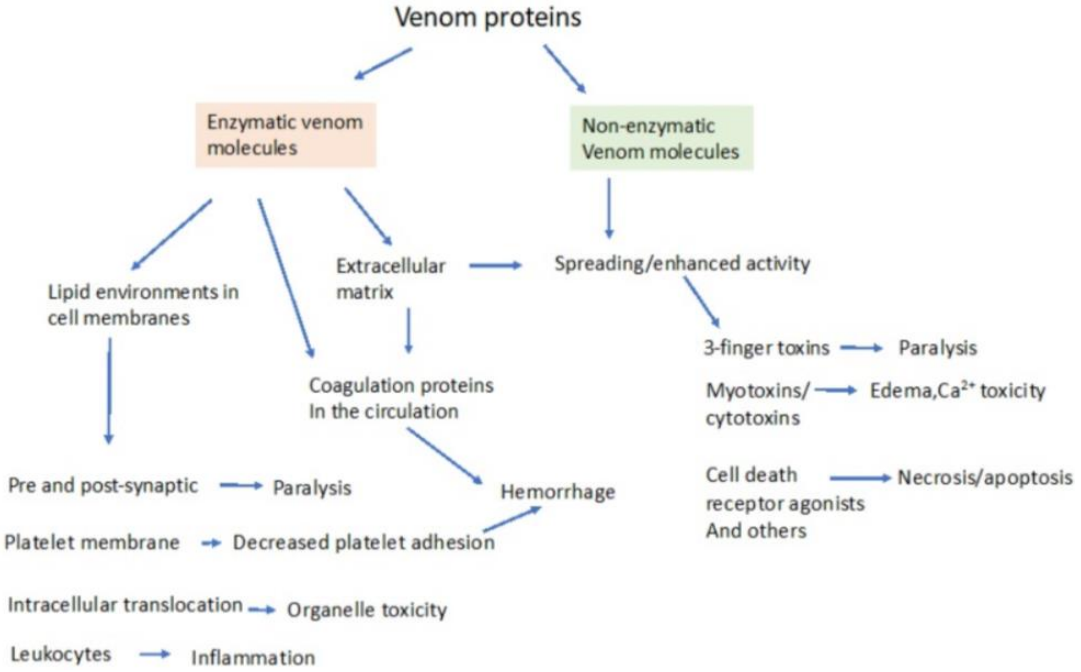

Figure 3: General targets of major snake venom proteins divided into venoms that have intrinsic enzymatic activity and those that are non-enzymatic. Enzymatic venom proteins are typically hydrolases such as PLA2, serine proteases, metalloproteases, or hyaluronidases, releasing biologically active products that act on the extracellular matrix, on membrane proteins, on membrane-based signaling molecules or inside cells. Examples of non-enzymatic venom components include the curare-like 3-finger toxins from kraits, potassium channel blocking dendrotoxins and pore-forming myotoxins. Enzymatic destruction of the extracellular matrix by metalloproteases and hyaluronidases enhance venom spread and amplify toxicity. Other, direct acting, non-enzymatic protein toxins no doubt exist in yet to be characterized venoms. Further, venomproteins may simultaneously have enzyme-based and non-enzyme-based toxicities, such as components of PLA2 heterodimers, blurring these distinctions. Considerable cross-talk between enzymatic and non-

enzymatic venom components may exist, for example non-enzymatic svPLA2s may dimerize and activate endogenous catalytic PLA2 proteins [5].

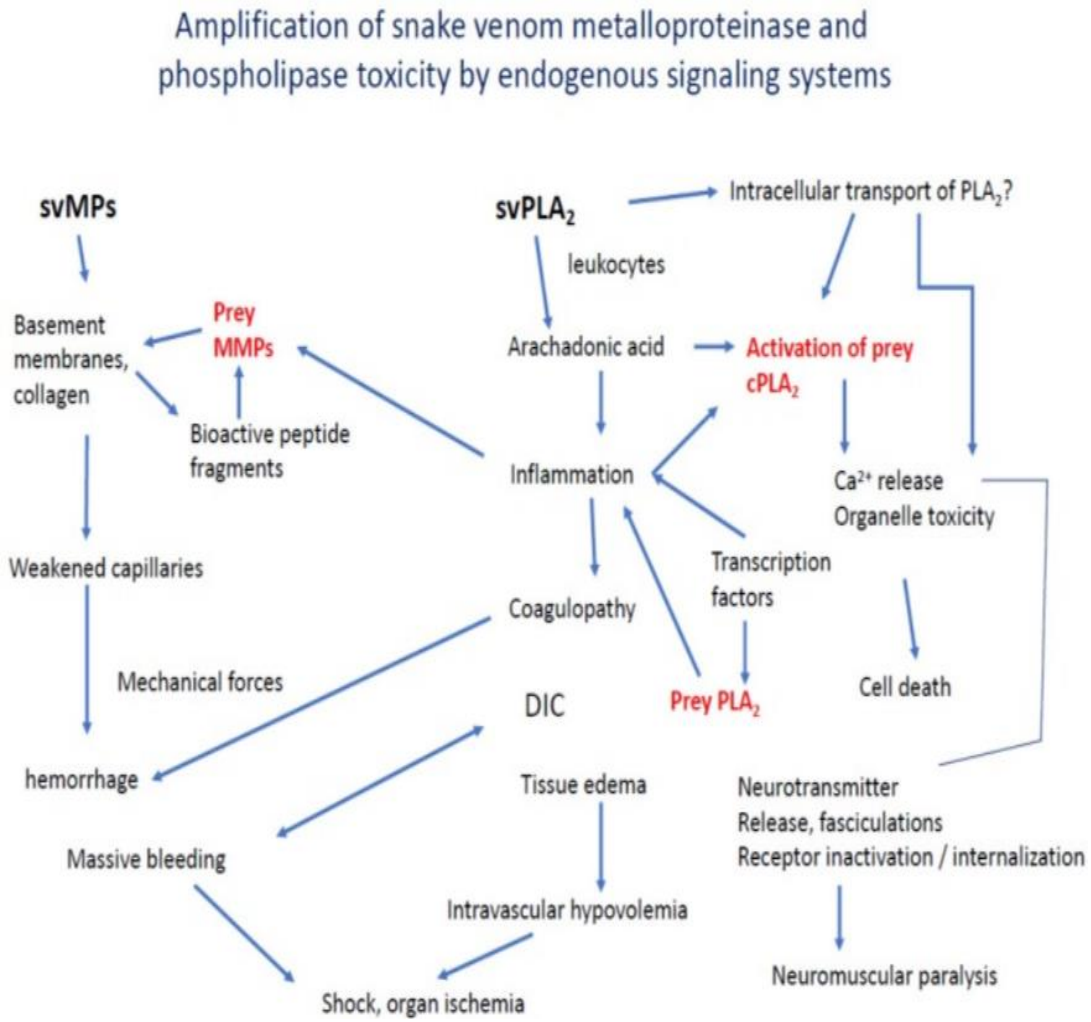

Figure 4: Interaction of the effects of enzymatic venom components to produce and amplify immediate and long-term toxicity for immobilizing prey and deterring predators. cPLA2, cytosolic/endogenous PLA2s. cPLA2 = cytosolic/endogenous PLA2. MMP = endogenous, inducible matrix metalloproteases, DIC = disseminated intravascular coagulation, snake venom metalloproteases (svMP) [5].

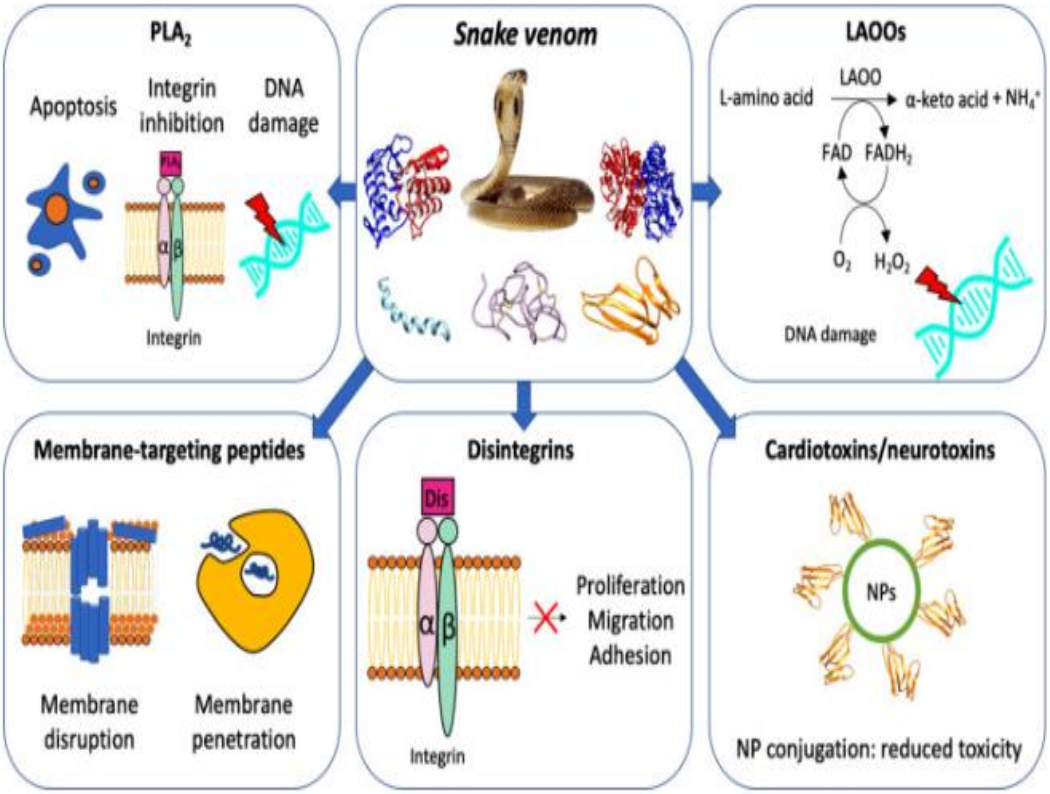

Figure 5 : Some of the targets of snake venom proteins [6].

Snake venom protein constituents may present different biological activities that affect physiological processes such as neurotransmission, the complement system and homeostasis (Figure 6). These venoms can act in more than one system at the same time and they may present antigenic effects.

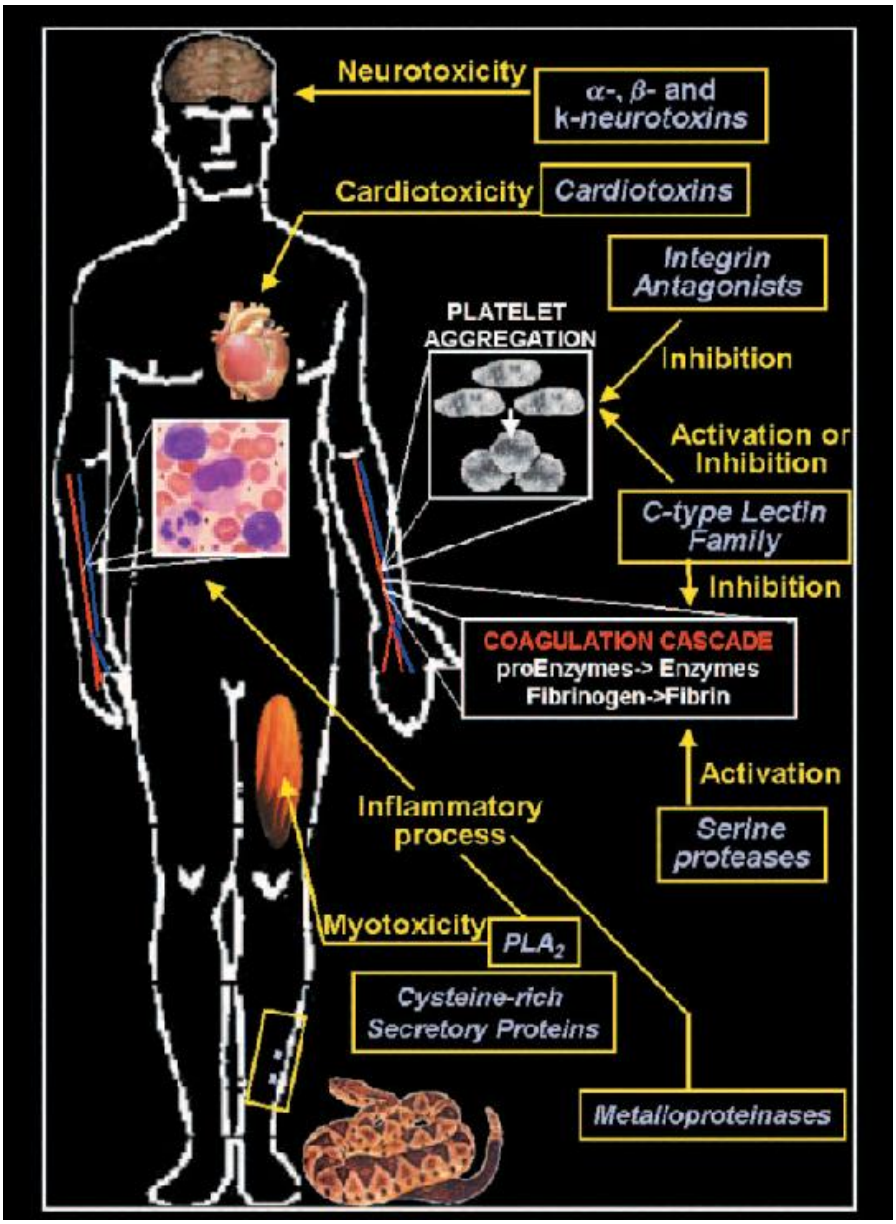

Figure 6: The biological effects of some snake venoms during the envenomation process [7].

Snake Venom Molecules of Commercial use

At the end of the last millennium, the development of therapeutic drugs made a significant improvement to the understanding of the mechanisms of action and structure–function relationship of important biological molecules. The broad spectrum of snake venom activities, including their biochemical, toxicological, physiological and pharmacological profiles, results from the action of their constituents. Therefore, snake venom are of biological interest as a potential source of active compounds. These molecules could act as (or be used as a prototype for) (i) therapeutic agents ; (ii) research tools for use in the diagnosis of several diseases ; and/or (iii) in basic research for understanding physiological and pathological processes.

Each component of snake venom contributes to the overall toxicity, mode of action, and clinical manifestations of envenomation. The specific composition and potency of venom can vary widely among different snake species, reflecting adaptations to their prey, predators, and environmental niches.

In conclusion, the composition of snake venom is a complex and diverse mixture of proteins, peptides, enzymes, and other bioactive molecules that collectively contribute to the venom's potency and physiological effects. Understanding the intricacies of snake venom composition is crucial for several reasons:

Biomedical Significance: The study of snake venom components has led to the discovery of bioactive molecules with potential therapeutic applications. Venom-derived proteins and peptides show promise in drug development, particularly in areas such as pain management, cardiovascular disorders, and cancer treatment.

Antivenom Development: Knowledge of venom composition is essential for producing effective antivenoms. Antivenoms are created by immunizing animals with venom components, and a thorough understanding of venom composition helps in selecting the most appropriate antigens for vaccine production.

Evolutionary Insights: The diversity of venom components across snake species provides insights into the evolutionary adaptations and ecological roles of venomous snakes. Comparative studies of venom composition shed light on the evolutionary mechanisms driving venom diversity, including gene duplication, positive selection, and convergent evolution.

Clinical Management: Understanding the specific toxins and enzymes present in snake venoms is crucial for diagnosing and managing snakebite envenomation. Different venom components can lead to varying clinical manifestations, necessitating tailored treatment strategies based on venom composition.

Biotechnological Applications: Snake venom components have also found applications in biotechnology, such as in the development of enzyme inhibitors, diagnostic tools, and biopesticides. Their unique properties make them valuable resources for scientific research and technological innovation.

Overall, the complexity and diversity of snake venom composition highlight the intricate interplay between molecular biology, ecology, and human health. Continued research into venom components promises to unlock further therapeutic potentials and deepen our understanding of venomous organisms' biology and evolution.

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