ISSN NO. 2320-5407

Journal homepage: http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

CHARACTERIZATION OF INDIAN COMMON KRAIT (*BUNGARUS CAERULEUS*) VENOM

Dissertation submitted to

H. P. UNIVERSITY

In partial fulfillment of the requirements

For the award of the degree of

M.Sc. MICROBIOLOGY IV SEMESTER DEGREE EXAMINATION

MAY, 1988

ANJU DHIR

CENTRAL RESEARCH INSTITUTE

KASAULI

HIMACHAL PRADESH DISTRICT

INDIA

CONTENTS

Acknowledgement

I would like to express my deepest appreciation and gratitude to Late Dr. B. Das, Head of Antisera Department of Central Research Institute, Kasauli, Himachal Pradesh, India for his keen supervision and continuous encouragement. He offered deep experience, unlimited support, valuable suggestions and sincere advice. It was a great honor to work under his supervision. To him, I owe a great deal of gratitude and thanks.

Anju Dhir

List of Tables

List of Figures

INTRODUCTION

Snakes are the limbless vertebrates and belong to the class of living creatures known as reptiles which include all the living orders of crocodiles, turtles, tortoises and lizards.These reptiles came into existence long before the mammals and birds were born on this earth. Fossil remains of snakes have been found in the later cretaceous and early tertiary periods in the world's history. Herpetologists agree that the snakes and lizards share a common ancestor having labial glands on both jaws. Only fossil record available in this direction has been found in case of the marine snake like lizards. Underwood, 1969 also has the opinion that the origin of snakes precedes the modern lizards. The snakes have originated from lizards or some prelizard like ancestors.

Snakes are scattered all over the world with the exception of Newzeland where they have never known to exist. Snakes like heat, it means life and vitality to them, and therefore the snakes are most abundant in the tropical and subtropical regions of the globe, in forests and places with thick vegetation. This type of habitat is favored by snakes as it gives necessary warmth to quicken their sluggish vital forces. When the temperature of the air is fairly warm snakes may be seen at any hour of the day or night.

The venomous snakes are classified according to their morphological characteristics and comprise five families: crotalidae e.g. pit vipers; viperidae e.g. vipers; elapidae e.g. cobras and kraits; hydrophidae e.g. sea snakes and colubridae e.g. colubrids. The venomous snakes have a pair of venom glands which are lying on either side of the skull beneath the skin, situated just under and behind the eyes. The venom glands may be true salivary glands with or without digestive enzymes. Without the venom it may be very difficult or even impossible for snakes to obtain their food.

About 2000 different types of snakes exist and nearly 400 are known to be venomous. Nearly 20,000 people die every year from snake bite in India (Ahuja & Singh, 1954). Dr. K.C.Chughfrom P.G.I. in 1988 has also reported that the global annual mortality rate from snake bite is about 40,000; out of this nearly 10,000 deaths occur in India, which is highest as compared to any other country in the world. The common four speciesresponsible for these casualties belong to two families: *viperidae* and *elapidae.* The venom of elapids is neurotoxic and death occurs due to respiratory failure. The Indian common krait and cobra belong to this family. Whereas the venom of vipers cause hemorrhagic effect and death occurs due to cardiac failure – Russel's viper and *Echis carinatus* belong to this family.

The snakes are frequently found in and around the human dwellings. In autumn they find some cozy place in the thatch, under the roof or floor, down a hole, in a stack of timber, dung heap or in fodder, in order to hibernate in winter season. Moreover, the snakes are highly evolved creatures and do not depend much on oxygen like most of the other animals. Due to this quality and by virtue of their specifically modified organs they are able to penetrate into dense overgrown vegetation of the tropics where the smaller reptiles are found in less number.

The snakes may take a heavy toll of life every year but in nature they keep the growth of fast breeding creatures in check. In hot countries insects and rodents are a nuisance to the farmers as they damage the crops. Owing to the long tapering bodies, the snakes are capable to penetrate into the innermost dwellings of these animals to feed upon them. Thus they act as the part of nature's agent in checking their rapid increase. The snakes are also a valuable source of venom which is used in various research fields as the snake venoms retain many of their biological activities over long periods of time.

It was not until the 17th century that systematic studies of the venoms were made in a manner that may be regarded as positive. In 1664, Francesco Redi demonstrated for the first time that the snake venom must be injected under the skin to produce their characteristic effects as certain venoms were found to be harmless when taken by mouth. Systematic studies on the biological action of

snake venoms were undertaken by Flexner in 1863-1954at the Rockfeller institute in New York. The reports from these studies described the effects of various snake venoms in producing hemolysis and other toxic effects. But the chemists were among the first to consider the venom with interest, and they defined their composition. They saw the effect of common reagents on snake venom and tried to extract the toxic constituents. They observed that certain reagents destroyed the venom while others caused precipitation of the toxic components.

In 1938, a crystallisable protein 'crotoxin' was separated from venom of *Crotalus durissus terrificus* (Slotta and Frankel Conrat, 1938). After this work many reports about procedures for purifying snake venom enzyme appeared. The older methods for isolation and purification of the venom components include heat and pH treatment, alcohol fractionation, free boundary electrophoresis and ultracentrifugation. But these methods are not applicable today because these are nonspecific and inefficient. Nowadays, new methods are in use which are most efficient, more specific and quick. The modern methods are like sieve chromatography, gradient elution on columns of ion exchangers and hydroxyl apatite, preparative polyacrylamide gel electrophoresis and isoelectric focusing etc. These modern methods employ mild conditions of pH, temperature and solvents so that the biological activity of venom is retained.

AIM

Much progress has been made for isolating the constituents of snake venom in a pure and biologically active form in the past years. Many workers have worked on the venom of Naja, Russel's viper, Agkistrodon, Crotalus and Echis from time to time. But the venom of Indian common krait (*Bungarus caeruleus*) has not been studied by many workers. The venom of *B. caeruleus* is highly lethal - LD_{50} is 10-12 µg for 70-75 Kg. man (Ghose, 1980 and Lee, 1984). As many factors work in conjunction when a person gets envenomated, some of these lethal factors include the hemolytic and neurotoxic factors along with proteases, hyluronidases and phospholipases. Therefore there is need to study these specific qualities of the Indian common krait venom.

- An attempt has been made in the present study to observe the toxicity of this venom through different routes of inoculation.

- The hemolytic activity of Indian common krait venom has also been observed using red blood cells from different species of animals in order to characterize this venom.

REVIEW OF LITERATURE

Reptiles have been reported on this planet before the mammals were born. Crocodiles, lizards, tortoises, and snakes now comprise some of these animals on the earth. Snakes belong to the class reptilian and are classified under the order Ophidia.It can be inferred that the poisonous snakes have originated from the non-venomous lizard like ancestors and then evolved into different lines of poisonous snakes. This fact is based on the dental structure, tongue and the osteolological similarity between the lizard like ancestors and the snakes (Carroll, 1975).

In the ancient history, the evil spirit of the earth has been depicted by the dragon which is a form of snake. The fossils of snakes have been found in the stratified rocks of the earth. Animal worship has always been encouraged in India and snake is one of the most worshipped animals. In Indian tantras and religion the snakes are symbol of powerful dark forces from the unknown world. There are numerous legends in India pertaining to snakes. Snake worship, snake venom, possible ill effects of venom and the use of snake venom as antidotes to certain pathological conditions are

being known to everyone (Deoras, 1970). In India every year two days are devoted to snake worship that is Nag Panchmi and Anant Chaturdashi.

The origin of the poisonous snakes is thought to be from the non-poisonous snakes which do not have the venom glands and fangs to vipers which have well developed venom injecting apparatus (Underwood, 1979). The venom glands have originated from the exocrine glands and these oral glands of snakes are probably 150-200 million years old (Kochva, 1978).

The snakes are creatures without limbs and external ears. They usually do not attack unless frightened. Their favorite meal is frogs, eggs of the birds and small snakes. Their digestive system, respiration system, circulatory and reproductive system are fully developed. Snake has forked tongue and it can smell the chemical particles in the surrounding with it.

The snakes have been classified mainly into venomous and non-venomous snakes. The classification of snakes is based on the morphological characters especially the head shields and the body scales. Their shape, number and position on the body of snake helps in differentiating them from each other. The snakes come under the order reptilia. The main poisonous snakes fall in two major families namely elapidae and colubridae. These two families are relatively close to each other on the basis of their osteology and venom injecting apparatus. The common terrestrial poisonous snakes of India include cobra, kraits, Russel's viper and green pit viper. The vipers have triangular head which is broader than the neck. On the other hand the head of the elapids is blunt and narrower than the neck.

***Bungarus caeruleus* / Indian Common Krait:** The Krait is called by different names in India, like in Gujrat it is known as Konotare, in Madras as Yannabarian, in Mysore as Gudinasa, in Maharashtra as Maniyar and in U.P., Orissa, Madhya Pradesh, Rajasthan and Punjab as Karayat (Deoras, 1970). The kraits are one of the common kinds of landsnakes and are highly poisonous. Krait is a small sized snake, gape is not very wide. The poison fangs are at the back of the jaw and are small as compared with those of other poisonous snakes. The kraits resemble many nonvenomous snakes in general appearance like oligodon and lycodon species of snakes. But both these snakes are deep brown in color with white cross strips and they do not have hexagonal dorsal scales. The ventral scales beyond the vent are paired. The distinguishing characters of krait are:

1) A krait has broad belly plates.
2) The head is covered with shields.
3) The tail is round
4) The central row of scales down the back is distinctly enlarged and is more or less hexagonal
5) There are only four shields along either side of lower lip
6) The plates under the tail are entire and not divided

The kraits are restricted to Southeast Asia and are found in India, Burma, Southern China, Malaysia and Formosa. There are twelve species of krait and among these ten are found in India. The common krait is found throughout India up to an elevation of 914.4 m. The snake is found in crevices between the rocks, bricks and in the fields. The krait is very timid and comes out of its habitat at night. It never attacks unless threatened. This snake does not produce hissing sound but in the early morning a shrill sound is produced. The average snake is about one meter. The male snake is longer and stouter than female (Parker, 1963).

The color of the common krait is usually shining blue black with white cross circular band like markings over the entire body. The grey, dark brown and black varieties are also found. The markings on the body are arranged in pairs or singly and they continue till the end of the tail and are absent in front near the head. The ventral side of the snake is white. The whole body is covered

over by scales. The total number of median hexagonal scales is 245 to 255, ventral scales are 198 to 208 and subcaudals are 44 to 50 and caudal scales are 11 in an average snake.
The head has no loreal scales, supra labial scales are 7, in which 3rd and 4th supralabial touches the eye. Postocular scales are 2, preocular scale is one and infralabial scales are four in number. It feeds on the frogs, toads, mice, rats and other snakes like keel back and blind burrowing snakes. The kraits cast off the skin after 70 to 85 days in summer and 135 to 205 days in winters and rainy season.
The kraits are found in pairs. If one is killed the search should be made for the other one. Mating takes place in rainy season from July to September. The snake is oviparous and the eggsare laid in April in safe and protected places like under the dead leaves or in the holes. The mother incubates eggs and the eggs hatch out in about 50 to 60 days. The snake is about 15 to 20 cm in length and purple in color at birth. It grows at the rate of 5 to 7 cm per year for the first two years (Gharpurey, 1962).
The venom of *Bungarus caeruleus* contains many toxic protein and non protein components. The venom exhibits yellow color due to the presence of L-amino acid oxidase (Tu, 1977). Kornalik etal, 1964 has proved that yellow colored venom contains 200 times more L- amino acid oxidase than the white venom. The venom of *B. caeruleus* has been fractionated by various workers from time to time (Basu et al, 1970). In 1974 Chatman, et al used cation exchange resin sulphopyopyl-sephadex for isolation of neurotoxins and cytotoxins from elapid venoms. In 1976, Lee et al isolated four highly toxic fractions namely 1-B2, 1-C, 3-A and 3-B from venom of Indian common krait [*B. caeruleus*] by CM cellulose column chromatography on sephadex G-50 and rechromatography on CM sephadex G-25. Among these fractions 3-A and 3-B were homogeneous on disc electrophoresis (Lee etal, 1978). Gaitonde in 1975 used column chromatography and electrophoresis in order to purify the neurotoxin from *B. caeruleus* venom. In 1985, Bougis et al used reverse phase high performance liquid chromatography to fractionate the polypeptide components from elapidae snake venom in a single step.
In the venom of *B. caeruleus* many protein components and enzymes are found. Some of them are enumerated here:

1) Hyaluronidase: The enzyme catalyses the cleavage of internal glycosidic bonds of certain acid mucopolysacharides of animal connective tissue. Hyaluronidase is also reffered as spreading factor as it facilitates the diffusion of toxin into tissues of an animal (Duran, 1936).

2) NAD- Nucleosidase: This enzyme hydrolyses the nicotinamide N- ribosidic linkages of NAD, and is present in venom of krait (Lee, 1976).

3) Phospholipase A / Hemolysin: The hemolysin or phospholipase A activity is about 34% in the venom of *B. caeruleus* (Kocholaty et al 1971). This enzyme was isolated by many workers like Ghosh & De, 1937; Doery and Pearson in 1961; Marinett in 1955; Kawauchi et al in 1971. The phospholipase A is active at alkaline pH range i.e. 7.5 to 8.5. The optimum temperature for its activity varies with different venoms from different species of snakes e.g. the optimum temperature for PLA of venom from *B. fasciatus* is 55^0C (Nair et al, 1976).

4) Acetylcholinesterase: Although it was first demonstrated in cobra venom (Iyengar etal, 1938) but the enzyme has also been separated from the venom of *B. multicinctus* by electrophoresis. The venom of *Bungarus* species contains nine times more amount of cholinesterase as compared with venom of cobra. The enzyme is active at alkaline pH and optimum temperature is 40^0C in case of

B. fasciatus venom. Earlier this venom was thought to be related with the neurotoxicity but now it has been experimentally proved that there is no correlation between the acetylcholinesterase and venom neurotoxicity (Lee, 1979).

5) Neurotoxin: Neurotoxin is one of the basic proteins in the venom and most toxic too. Neurotoxins are divided in two classes on the basis of their sites of action, namely postsynaptic and presynaptic. The presynaptic neurotoxins inhibit the release of acetylcholine and are much lethal than postsynaptic neurotoxins (Heilboronn et al, 1987). The postsynaptic neurotoxins bind to the depolarizing action of acetylcholine. Although it has been shown that the venom of *B. caeruleus* contain presynaptically acting neurotoxin (Chang et al 1973), but in 1978 Lee et al isolated four toxic principles from venom *B. caeruleus*. Then based on toxicity test in mice, time taken for death at higher doses and the effect on acetylcholine response of rat denervated diaphragm, it was concluded that the two fractions I-B2 and I-C belong to postsynaptically acting neurotoxins similar to **α**-bungarotoxin whereas III-A and III-B belong to presynaptically acting neurotoxin similar to β-bungarotoxin (Lee et al 1976).

6) Non Protein constituents: A variety of non-protein constituents are found in venom. These include some inorganic salts, including a variety of metal ions (Devi 1968 and Tu 1977). These metal ions are bound to the venom proteins and serve as cofactor for the function of these proteins as it has been seen that hemorrhagic and proteolytic activities decrease on removal of these ions. Among these non-protein constituents peptides having pyroglutamic acid as the amino terminal are important for toxicity of the venom.

The krait may bite on any part of the body although most of the bites have been reported on the lower extremities like foot, leg, toe etc., cases have also been reported with bites on head and ear lobes. One such case of a 19 year old boy in the month of October, 1987 was observed by the author personally. He had a snake bite on the head near the ear lobe. The snake was identified as *B. caeruleus* as the victim had courage to catch hold the snake and he brought the snake with him in a box. The victim died within 24 hours due to undue delay in reaching the institute's treatment centre and the symptoms were already in advanced stage.

Pharmacology

The venom of Indian common krait *(B. caeruleus)* is neurotoxic mainly, as has been proved by the clinical symptoms. Both the presynaptic and postsynaptic neurotoxins that are responsible for the respiratory paralysis which is peripheral in origin are found to be present in its venom (Kellaway et al 1932 and Lee et al 1976). The venom of Indian common krait *(B. caeruleus)* and Formossan krait is highly toxic when administered intravenously (Lee, 1979). This is so because the venom may penetrate the blood brain barrier in sufficient amount and the animal dies immediately. In 1974, Mebs et al and Bon in 1976 isolated caerulotoxin from venom of *B. caeruleus* and found that 35% of the total toxicity of venom was due to the neurotoxin and this inhibits the depolarization of neuromuscular transmission without reducing the response to acetylcholine (Ho and Lee, 1983). In contrast to the presence of postsynaptic toxins in the venom of elapid and sea snakes, the neurotoxin acting specifically on the motor nerve terminal have been found only in venom of *B. caeruleus* (Lee et al 1960), *B. multicinctus* (Chang and Lee,1963), *Notechis scutatus* (Karlson et al,1972) and *Oxyuranus scutellatus* (all are members of elapidae family). The neurotoxins are enzymatic in action due to the presence of PLA in venom.

It is not clear whether the neurotoxin acts from inside or outside of the axolemma of nerves, but it is certain that the toxin blocks the neuromuscular transmission resulting in respiratory paralysis

(Strong et al, 1976). The venom of *B. fasciatus* produces a marked fall in blood pressure very shortly after intravenous injection in rabbits and cats as compared to *B. caeruleus*. This fall in blood pressure was related to PLA activity (Lin Shiau et al, 1975; Lee et al, 1985). Although no such cardiotoxin was found in caerulotoxin.

Uses of snake venom

While the snake bites take a heavy toll of life, their venoms are important in research and medicinal field.

As enzyme:

The snake venom is one of the most concentrated and valuable source of enzymes in nature. Its component phosphodiesterase is used for structural studies of nucleic acids and dinucleotide coenzymes (Laskowski, 1966). Another enzyme L-amino oxidase is used in identifying optical isomers of amino acid and for preparing **α**- keto acids (Meister, 1952). Proteinases are used for amino acid sequence studies of peptides and proteins (Mella et al, 1967) and PLA2 is used in lipid research (Russel, 1980).

In Medicine:

The snake venom has been used in the field of medicine too. Heparin is a well known anticoagulant. It is used intravenously to treat the thrombotic defects in blood vessels (Kornalik and Hladovec, 1975). It was shown by Witham et al in 1953 that on injecting crotalidae venom into animal body a physiologically active substance is released which lowers down the blood pressure. The snake venom has also been used in treatment of tumors (Dewys et al, 1976; Hillgard and Thornes, 1976). The snake venom has proteolytic enzymes which can be used to breakdown the protein components.

Snake venom can reduce blood pressure as is found in case of venom from Brazilian pit vipers. Researchers have converted venom into a non-toxic angiotensin converting enzyme (ACE). It reduces the risk of kidney disease, stroke and diabetes.

Research is going on:

- the potential use of snake venom as explosive detector
- future use of venom in treatment of muscular dystrophy.
- the treatment of Alzheimer's disease that slowly destroys the nerves
- the use of venom to kill parasites like sandfly
- the use of venom as pain killer

MATERIAL & METHODS

Material:

Venom: The Indian common krait venom was obtained from the pooled stock in the lyophilized form from Central Research Institute Kasauli. The venom was kept at $+4^{o}$c in vacuum over phosphorus pentoxide in a dessicator.

Preparation of Venom Solution: The dry venom kept in weighing bottle was weighed on Metler single pan electric balance and one percent (10 mg/ml) stock solution of venom was prepared in sterile normal saline. The venom solution was centrifuged at 1.500 rpm for 10 minutes to remove the suspended material. The supernatant venom solution was sterilized by passing through

membrane filter of pore size 0.22 µ. This solution was stored at +4^{o}c for further use in the experiments. Further dilutions of venom were freshly prepared in normal saline.
Mice: The mice were obtained from controlled breeding in Institute's animal house

Method:
Experimental method was used throughout the present study.
Toxicity Test: Toxicity of the venom was tested by preparing a series of dilutions with 25% increment and injecting 0.2 ml into each of group of six albino mice weighing 18-20 gram by various routes of inoculation.
Readings in terms of deaths and survivals were taken after 24 hours after inoculation. The 50% end point i.e. LD_{50} was calculated according to Reed and Muench's method (1938). In this method it is assumed that animals dying at a stated dose would also have been killed by greater amounts of the agent and conversely those surviving would have survived small doses. An accumulated value for the animals is obtained by adding the number dying at a certain dilution [but in reverse direction in case of survivors]. In making calculations it is assumed that the doses used are equally placed on the logarithmic scale, and that the 50% end point falls somewhere in the middle of the range of dilutions used.

Drawing Graph between Log (venom dose) and Probit absorption:
For this following transformations were made:
1. Transformation of venom dose to log (venom dose)
2. Transformation of percent mortality to probit mortality.
3. Then graph was plotted by taking log (dose) on X-axis and the probit mortality on Y- axis.
4. 50% toxicity was observed directly by drawing a straight line from a point say 'p' (probit 5.0 on Y- axis) parallel to the X- axis which crosses the line of hemolysis at point say 'q' and then drawing a perpendicular from q to the X- axis at point T.
Now PQ = OT = log (LD_{50}).
5. Hence LD_{50} was calculated by finding out the antilog (PQ).

Hemolytic Activity (Das, 1970):
A series of ten two fold dilutions from 5 mg/ml of stock venom solution were prepared in duplicate to observe the direct and indirect hemolysis.
In one row of dilutions, 0.3 ml of normal saline was added to 0.3 ml of venom dilution. In the second row 0.3 ml of horse serum was added to 0.3 ml of each dilution. Now 3 ml of 1% RBC of the animal under test was added to both rows and incubated at 37^{0}C for one hour and after this kept at 40c for one hour. These vials were centrifuged at 1,500 rpm for 10 minutes and supernatant was collected in separate vials. The percent transmission of lysed cells in the supernatant was observed in spectrophotometer at 540 nm and this was compared with standard.
Standard: The 10 ml of 1% RBC were taken in a test tube and centrifuged at 1,500 rpm for 10 minutes. The supernatant was discarded and to the remaining cells 10 ml of distilled water was added and shaken to lyse the RBC. Now two fold dilutions of these lysed RBC were made and percentage transmission was observed at 540 nm.

Drawing a graph between log (venom dose) and probit absorption: Following transformations were carried out:
1. Transformation of venom dose to log (venom dose)
2. Transformation of percent extinction due to hemolysis to probit extinction

3. Then graph was plotted by taking log (dose) on X- axis, and the probit extinction on Y- axis
4. 50% hemolysis was observed by drawing a straight line from a point 'p' (probit 5.0 on Y- axis) parallel to the X- axis which crossed the line of hemolysis at point 'q' and then drawing a perpendicular from q to X- axis at point T.

Now OQ = OT log (HD_{50})

5. Hence HD_{50} was calculated by finding out the antilog (PQ)

RESULTS

The toxicity of Indian common krait *(B. caeruleus)* venom was observed in mice through different routes of inoculation. The results are being presented in Table 1-5. The bar diagrams have also been given as a confirmatory support to the table of toxicity test.

1. The first route to observe toxicity was subcutaneous and the inoculum was 0.5 ml. The results have been shown in table1.

Table1. Determination of LD_{50} of Indian common krait *(B. caeruleus)* venom by subcutaneous route

Venom Dose in µg/ml	Log(dose)	Percent Mortality	Probit Mortality	Result LD_{50}
25.00	1.3979	77.8	5.7655	
20.00	1.3010	33.3	4.5687	
16.00	1.2041	8.3	3.6184	21.38 µg
12.80	1.1072	0	0	
10.24	1.0103	0	0	

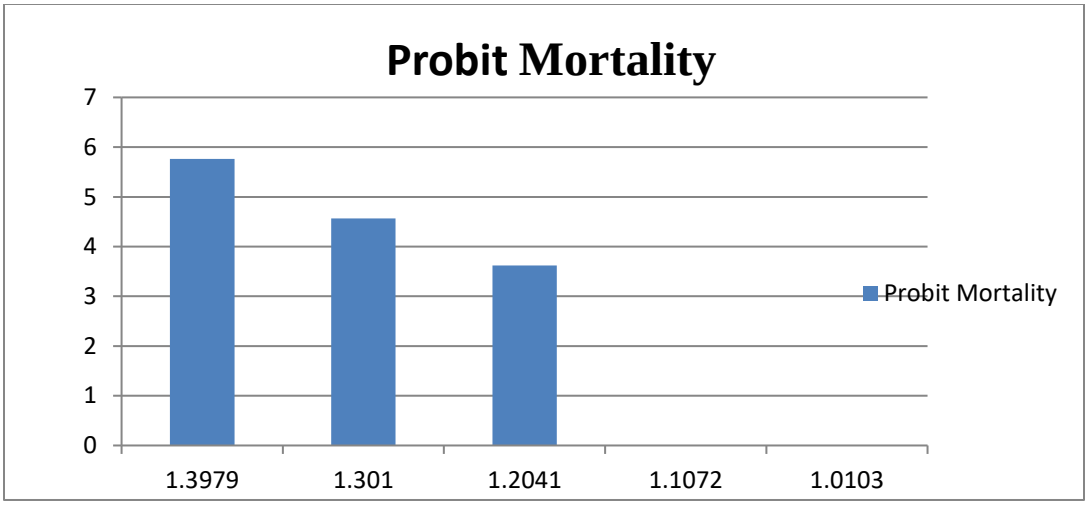


Figure1. Bar diagram of Probit mortality of Indian common krait *(B. caeruleus)* venom by subcutaneous route

From the above table & figure, one can observe that with the increase in dilution percent mortality decreases. As can be seen in subcutaneous route the venom was not much lethal. LD_{50} was calculated as 21.38 µg. The standard deviation was calculated as 0.7360 and co-efficient of variation as 3.4192.

2. The second route was intramuscular with 0.5 ml of inoculum and the results are presented below:

Table 2. Determination of LD_{50} of Indian common krait *(B. caeruleus)* venom by intramuscular route

Venom Dose in µg/ml	Log (dose)	Percent Mortality	Probit Mortality	Result LD_{50}
32.000	1.5051	100.0	8.0902	
25.600	1.4082	92.85	6.4611	16.60µg
20.480	1.3113	72.72	5.6033	
16.384	1.2143	44.44	4.8592	
13.1072	1.1173	15.38	3.9763	
10.4857	1.0193	0	0	

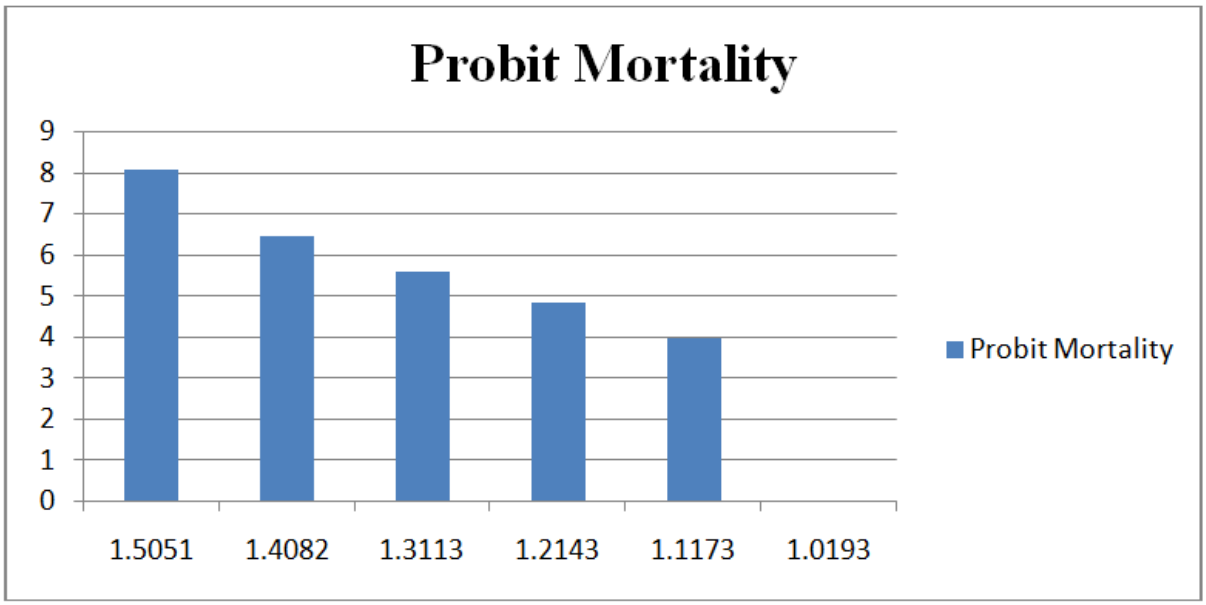


Figure2.Bar Diagram of Probit mortality of Indian common krait *(B. caeruleus)* by intramuscular route.

The observation from above table & figure showed that this route is slightly toxic than the S/C route. LD_{50} was calculated as16.60 µg. The standard deviation is 1.8898 and co-efficient of variation is 10.83

3. The toxicity of Krait venom was determined by inoculating 0.5 ml of inoculum through intraperitoneal route. The results are:

Table 3. Determination of LD_{50} of Indian common krait *(B. caeruleus)* venom by intraperitoneal route

Venom Dose in µg/ml	Log (dose)	Percent Mortality	Probit Mortality	Result LD_{50}
8.00	0.9031	100.00	8.0902	
6.40	0.8062	88.89	6.2160	4.892µg
5.12	0.7093	37.50	4.7076	
4.10	0.6128	16.67	4.0299	

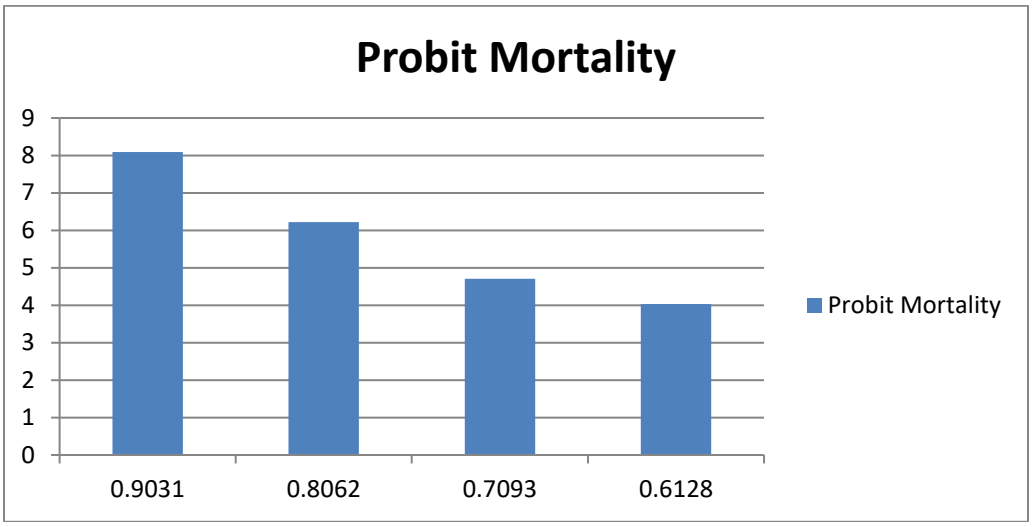


Figure 2. Bar diagram of Probit mortality of Indian common krait *(B. caeruleus)* venom by intraperitoneal route

In this case as can be seen from table & figure given above this route of inoculation is certainly more potent. LD_{50} was calculated as 4.892 µg. The standard deviation is 0.8861 and co-efficient of variation is 13.58. There is probit mortality at high dilution that is 0.6128 log dose, but further dilutions are not considered in the present study.

4. The toxicity of Krait venom was determined by inoculating 0.2 ml of inoculum through intravenous route. The results are:

Table 4. Determination of LD_{50} of Indian common krait *(B. caeruleus)* venom by intravenous route

Venom Dose in µg/ml	Log (dose)	Percent Mortality	Probit Mortality	Result LD_{50}
3.200	0.5051	100.00	8.0902	
2.560	0.4082	70.00	5.5244	2.198µg
2.0480	0.3113	36.36	4.6495	
1.6384	0.2143	13.33	3.8877	
1.3107	0.1173	0	0	

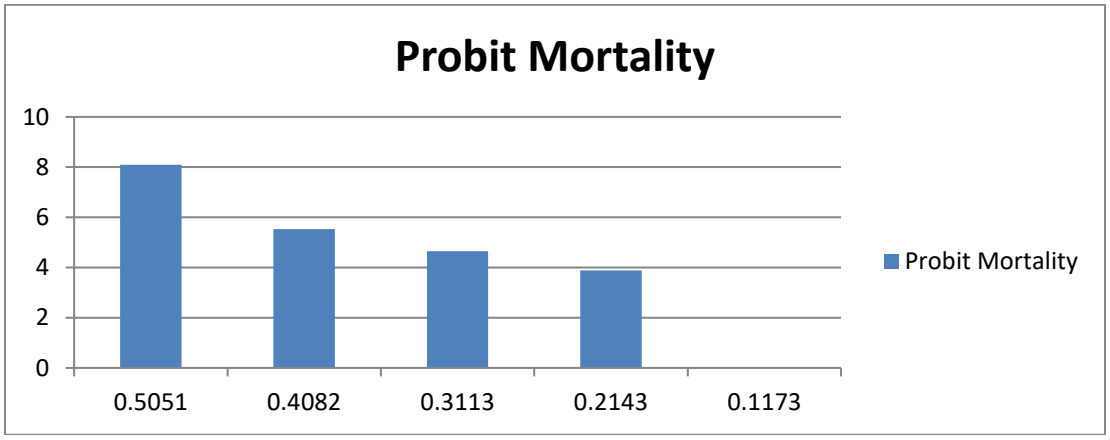


Figure 3. Bar diagram of Probit mortality of Indian common krait *(B. caeruleus)* venom by intravenous route

In intravenous route inoculum taken is small as compared to the above mentioned experiments as it was observed in pilot test that this route is lethal. In lethality it is just next to intracerebral route. The LD_{50} was calculated as 2.198µg. The standard deviation is 0.2970 and co-efficient of variation is 15.01. The above bar diagram was plotted to depict the results of the table, which shows the venom to be quite active by i/v route.

5. The toxicity of Krait venom was determined by inoculating 0.03 ml of inoculum through intracerebral route. The results are:

Table 5. Determination of LD_{50} of Indian common krait *(B. caeruleus)* venom by intracerebral route

Venom Dose in µg/ml	Log (dose)	Percent Mortality	Probit Mortality	Result LD_{50}
0.60	$\bar{1}$.7782	100.00	8.0902	
0.48	$\bar{1}$.6812	76.92	5.7356	
0.384	$\bar{1}$.5843	50.00	5.0000	0.3802 µg
0.3072	$\bar{1}$.4874	33.33	4.5684	
0.2457	$\bar{1}$.3904	11.76	3.8099	

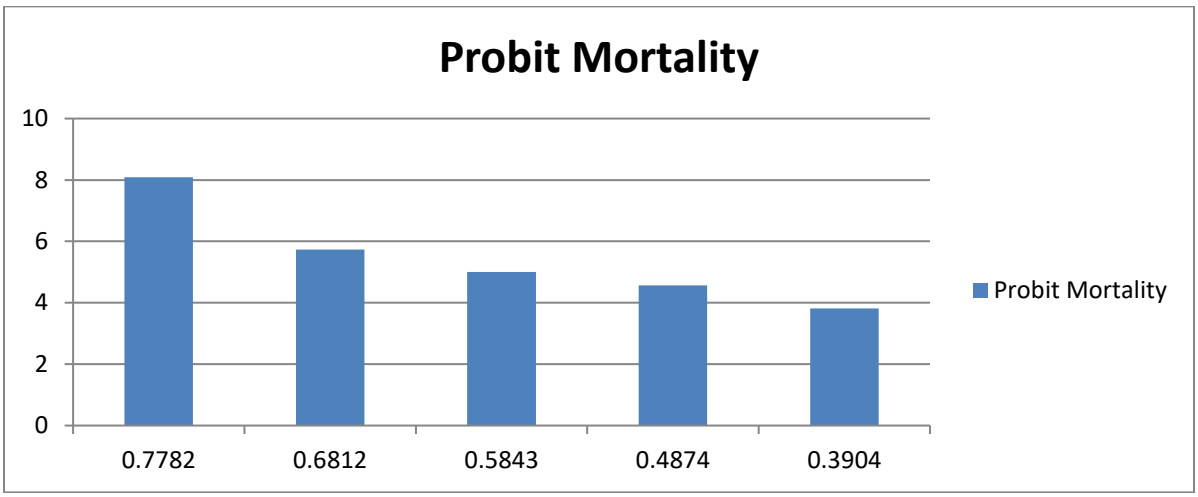


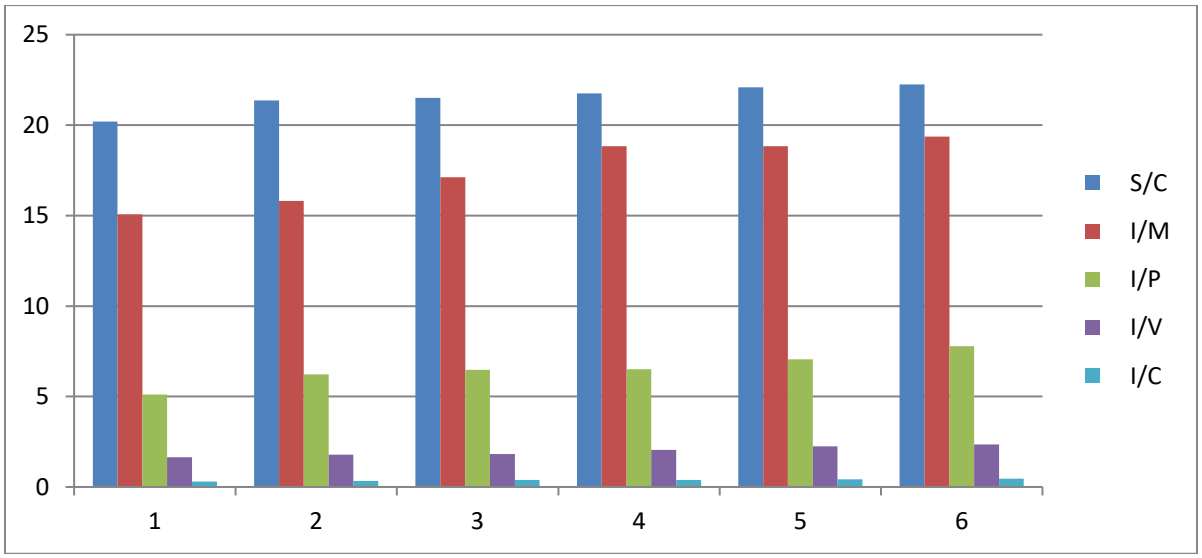
Figure 5. Bar diagram of probit mortality of Indian common krait *(B. caeruleus)* venom by intracerebral route

Ini/c route, LD_{50} was calculated as 0.3802 µg. This route was very effective and with very less inoculum and at very high dilutions also the mortality was observed. This was itself indicating to the neurotoxic nature of the common Krait venom. The standard deviation is 0.0515 and co-efficient of variation is 13.58. The bar diagram confirms the results of table 5 & one can visualize the high toxicity at very high dilutions of the venom.

Toxicity test of Indian common krait venom through each route of inoculation has been repeated six times and the comparative results have been presented below:

Table 6.Toxicity of Indian common krait *(B. caeruleus)* venom through different routes of inoculation

Routeof Inoculation	Volume of Inoculation	LD_{50} of venom µg						Average LD_{50} in µg
		1	2	3	4	5	6	
S/C	0.5	20.190	21.360	21.50	21.760	22.090	22.250	21.5250
I/M	0.5	15.070	15.820	17.120	18.840	18.840	19.360	17.4400
I/P	0.5	5.113	6.224	6.470	6.500	7.050	7.775	6.5220
I/V	0.2	1.638	1.781	1.813	2.048	2.242	2.355	1.9785
I/C	0.03	0.300	0.334	0.384	0.394	0.414	0.450	0.379



Abbreviations: s/c: subcutaneous; i/m: intramuscular; i/p: intraperitoneal;
i/v: intravenous; i/c: intracerebral

Figure 6. Comparative Results of various routes of inoculation

The results of above table and diagram show the toxicity of common krait venom through different routes of inoculation. The bar diagram given above shows the comparative results of all five routes of inoculation. Very high dose of venom is required in s/c route to be toxic. The Indian common krait venom was found most toxic through intracerebral route and least toxic through subcutaneous route. The order of toxicity is:

Intracerebral > intravenous > intraperitoneal > intramuscular > subcutaneous

This order of toxicity clearly indicates the nature of this venom that is, neurotoxic.

The test for toxicity determination of krait venom through intravenous route was repeated twenty times and the statistical analysis of this result is shown in table -7.

Table 7. Statistical analysis of the results of LD_{50} determinations of Indian common krait *(B. caeruleus)* venom

LD_{50} in µg (x)	Deviation from mean $(x- \bar{x})$	Deviation square $(x- \bar{x})^2$
1.2660	-0.5816	0.3382
1.6384	-0.2092	0.0438
1.6384	-0.2092	0.0438
1.6384	-0.2092	0.0438
1.6384	-0.2092	0.0438
1.6384	-0.2029	0.0438
1.6384	-0.2092	0.0438
1.7660	-0.0816	0.0666
1.7810	-0.0666	0.0044
1.8130	0.0346	0.0012
1.8840	0.0364	0.0013
1.8840	0.0364	0.0013
1.8910	0.0434	0.0019
2.0480	0.2004	0.0402
2.0480	0.2004	0.0402
2.0480	0.2004	0.0402
2.0480	0.2004	0.0402
2.0480	0.2004	0.0402
2.2420	0.3994	0.1555
2.3550	0.5074	0.2576
$\epsilon(x) = 36.9524$	$\epsilon (x- \bar{x}) = 0.0044$	$\epsilon(x- \bar{x})^2 = 1.3356$

Calculations:

Mean = $\epsilon(x) / n$
= 36.9524/20
= 1.8476

Standard Deviation $=\sqrt{\epsilon(x- \bar{x})^2 / n-1}$
$=\sqrt{1.3356/19}$
$=\sqrt{0.07029}$
=0.2651

Co-efficient of variation= S.D./Mean x 100
= 0.2651/1.8476 x 100
= 14.35

Range = Mean ± S.D.
= 1.8476 ± 0.2651
= 1.8476 + 0.2651 and =1.8476 – 0.2651
= 2.1127 and = 1.5825

Standard Deviation= S.D./√number of observations
= 0.2651/√20
= 0.2651/√4.472
= 0.0592799

95% confidential limit $= \bar{x} \pm t_{95}$ (S.E.)
= 1.8476 ± 2.080 (0.0593)
= 1.7243 to 1.9709

Characterization of Indian common krait venom was also carried out by estimating its hemolytic activity using red blood cells from different species of animals like mice, rat, guinea pig, rabbit, sheep, fowl, mule, horse, and human being. The results are presented below in tables 8- 16.

Table-8: Hemolytic activity of Indian common krait *(B. caeruleus)* venom using 1% mice red blood cells

Dose		Response		Results
Venom Dose	Log Dose	Percent Hemolysis	Probit Hemolysis	HD_{50}
166.67	2.2217	87	6.1264	4.571
83.33	1.9208	87	6.1264	
41.67	1.6198	78	5.7722	
20.83	1.3178	75	5.6745	
10.42	1.0178	75	5.6745	
5.2083	0.7167	64	5.3585	
2.6041	0.4157	37	4.6681	
1.30208	0.1145	13	3.8736	

No hemolysis in presence of normal saline without normal horse serum upto 166.67 µg/ml.

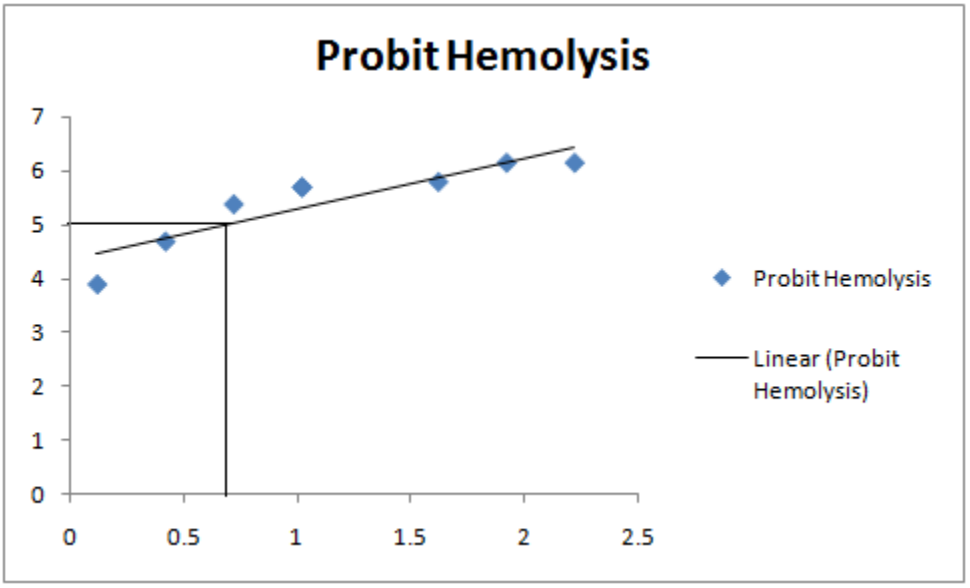


Figure-7. Hemolysis of 1% mice erythrocytes by Indian common krait *(B. caeruleus)* venom

Table-9: Hemolytic activity of Indian common krait *(B. caeruleus)* venom using 1% rat red blood cells

Dose		Response		Results
Venom Dose	Log Dose	Percent Hemolysis	Probit Hemolysis	HD_{50}
166.67	2.2217	52	5.0502	257.0
83.33	1.9208	32	4.6945	
41.67	1.6198	23	4.2612	
20.83	1.3178	22	4.2278	
10.42	1.0178	22	4.2278	
5.2083	0.7167	22	4.2278	

No hemolysis in presence of normal saline without normal horse serum upto 166.67 µg/ml

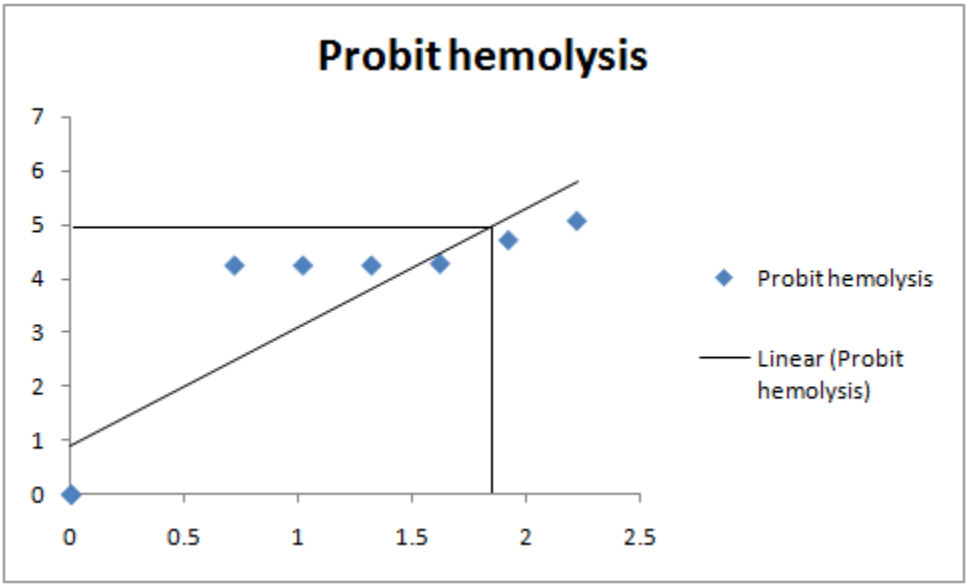


Figure -8: Hemolysis of 1% raterythrocytes by Indian common krait *(B. caeruleus)* venom

Table-10: Hemolytic activity of Indian common krait *(B. caeruleus)* venom using 1% guinea pig red blood cells

Dose		Response		Results
Venom Dose	Dose Log	Percent Hemolysis	Probit Hemolysis	HD_{50}
166.67	2.2217	99	7.3263	213.8
83.33	1.9208	99	7.3263	
41.67	1.6198	99	7.3263	
20.83	1.3178	98	7.0537	
10.42	1.0178	90	6.2816	
5.2083	0.7167	79	5.8064	
2.6041	0.4157	71	5.5534	
1.30208	0.1145	40	4.7467	

No hemolysis in presence of normal saline without normal horse serum upto 166.67 µg/ml

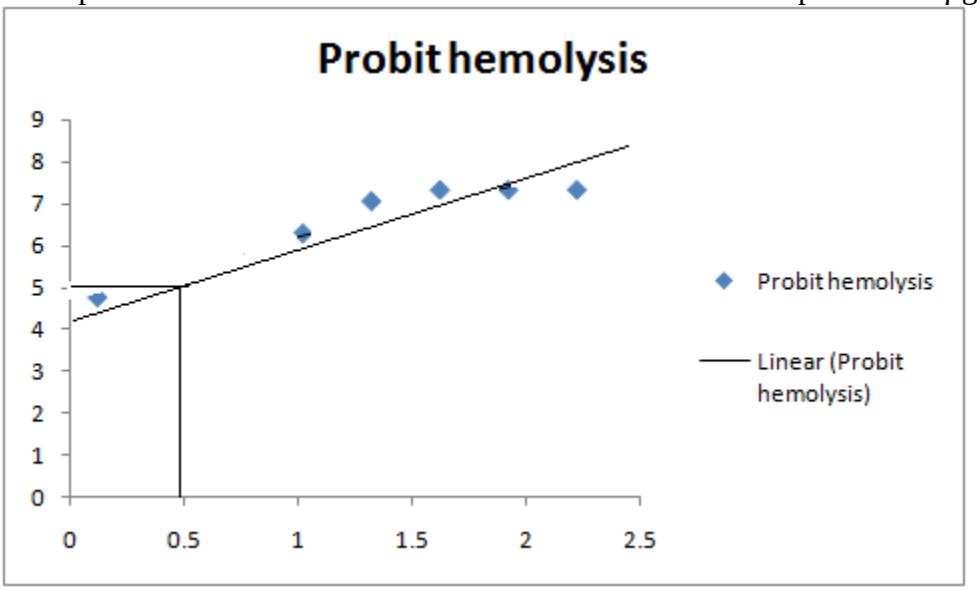


Figure -9: Hemolysis of 1% guinea pig erythrocytes by Indian common krait *(B. caeruleus)* venom

Table-11: Hemolytic activity of Indian common krait *(B. caeruleus)* venom using 1% rabbit red blood cells

Dose		Response		Results
Venom Dose	Log Dose	Percent Hemolysis	Probit Hemolysis	HD_{50}
166.67	2.2217	57	5.1750	239.9
83.33	1.9208	32	4.5323	
41.67	1.6198	11	3.7735	
20.83	1.3178	10	3.7184	
10.42	1.0178	6	3.4452	
5.2083	0.7167	5	3.3551	

No hemolysis in presence of normal saline without normal horse serum upto 166.67 µg/ml

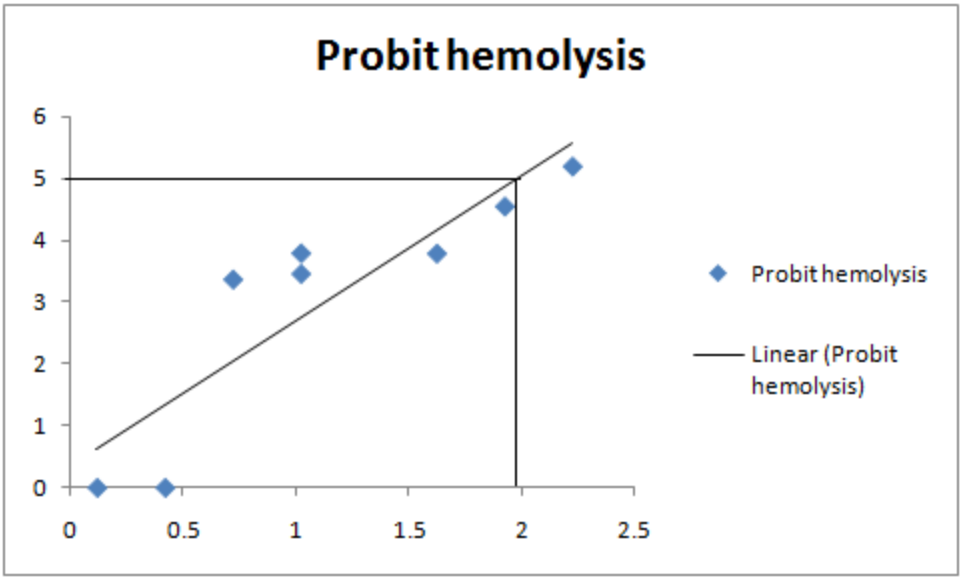


Figure -10: Hemolysis of 1% rabbit erythrocytes by Indian common krait *(B. caeruleus)* venom

Table-12: Hemolytic activity of Indian common krait *(B. caeruleus)* venom using 1% sheep red blood cells

Dose		Response		Results
Venom Dose	Log Dose	Percent Hemolysis	Probit Hemolysis	HD_{50}
166.67	2.2217	91	6.3408	56.23
83.33	1.9208	75	5.6745	
41.67	1.6198	44	4.7725	
20.83	1.3178	20	4.1584	
10.42	1.0178	5	3.3551	
5.2083	0.7167	1	2.6737	

No hemolysis in presence of normal saline without normal horse serum upto 166.67 µg/ml

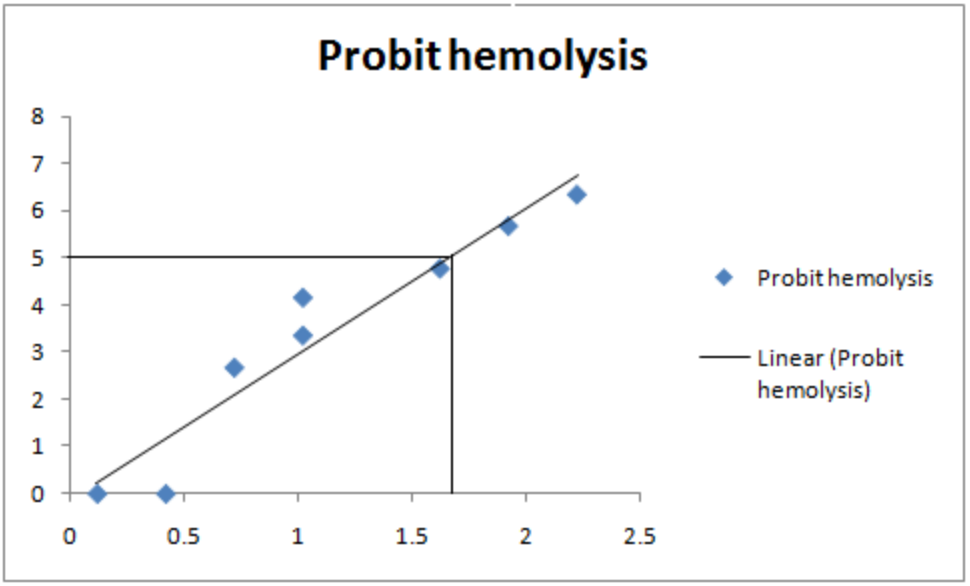


Figure -11: Hemolysis of 1% sheep erythrocytes by Indian common krait *(B. caeruleus)* venom

Table-13: Hemolytic activity of Indian common krait *(B. caeruleus)* venom using 1% fowl red blood cells

Dose		Response		Results
Venom Dose	Log Dose	Percent Hemolysis	Probit Hemolysis	HD_{50}
166.67	2.2217	15	3.9636	500.0
83.33	1.9208	10	3.7184	
41.67	1.6198	8	3.5949	
20.83	1.3178	7	3.5242	
10.42	1.0178	7	3.5242	
5.2083	0.7167	7	3.5242	

No hemolysis in presence of normal saline without normal horse serum upto 166.67 µg/ml

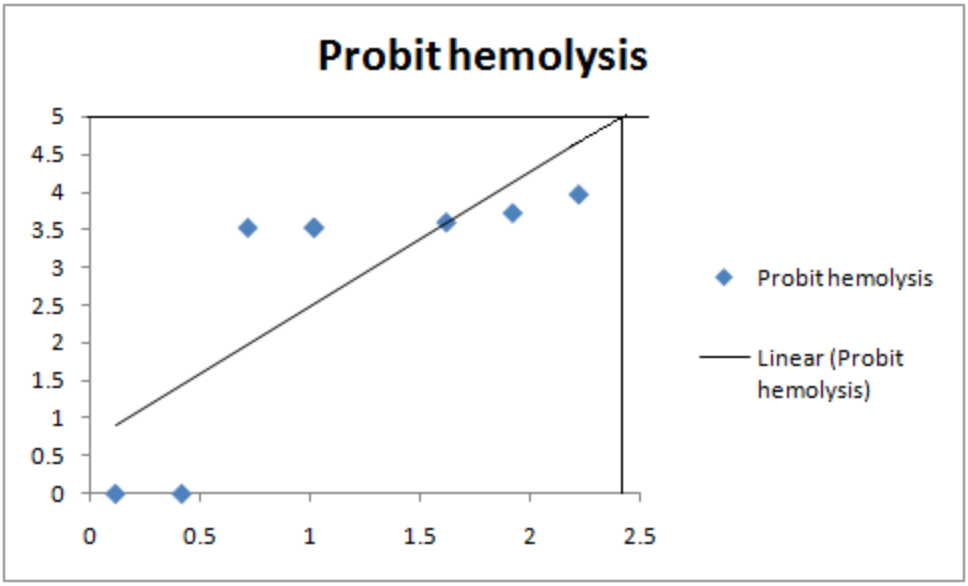


Figure -12: Hemolysis of 1% fowl erythrocytes by Indian common krait *(B. caeruleus)* venom

Table-14: Hemolytic activity of Indian common krait *(B. caeruleus)* venom using 1% mule red blood cells

Dose		Response		Results
Venom Dose	Log Dose	Percent Hemolysis	Probit Hemolysis	HD_{50}
166.67	2.2217	68	5.4677	138.0
83.33	1.9208	45	4.8743	
41.67	1.6198	24	4.2937	
20.83	1.3178	10	3.7184	
10.42	1.0178	2	2.9463	
5.2083	0.7167	2	2.9463	

No hemolysis in presence of normal saline without normal horse serum upto 166.67 µg/ml

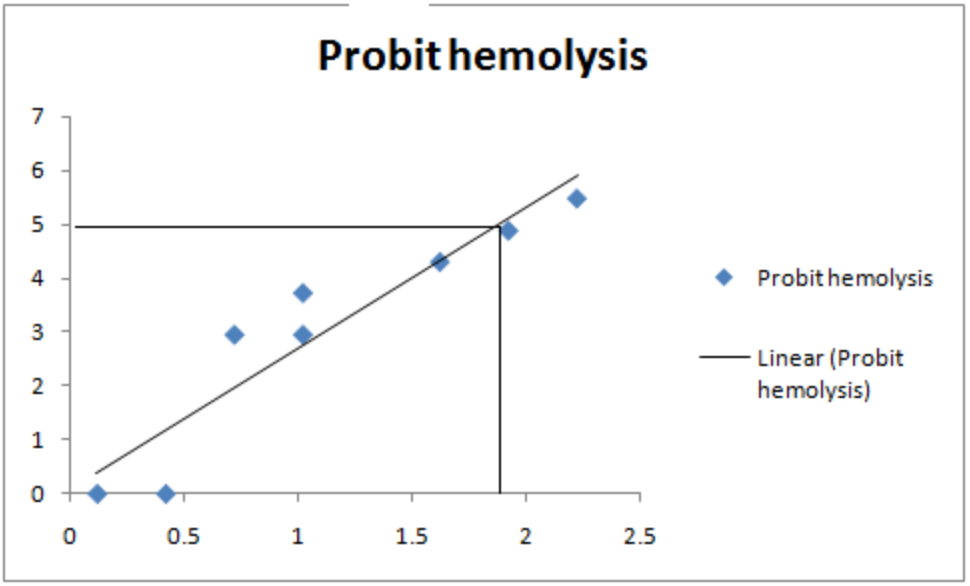


Figure -13: Hemolysis of 1% mule erythrocytes by Indian common krait *(B. caeruleus)* venom

Table-15: Hemolytic activity of Indian common krait *(B. caeruleus)* venom using 1% horse red blood cells

Dose		Response		Results
Venom Dose	Log Dose	Percent Hemolysis	Probit Hemolysis	HD_{50}
166.67	2.2217	77	5.7388	39.81
83.33	1.9208	70	5.5244	
41.67	1.6198	68	5.4677	
20.83	1.3178	45	4.8743	
10.42	1.0178	21	4.1936	
5.2083	0.7167	20	4.1584	

No hemolysis in presence of normal saline without normal horse serum upto 166.67 µg/ml

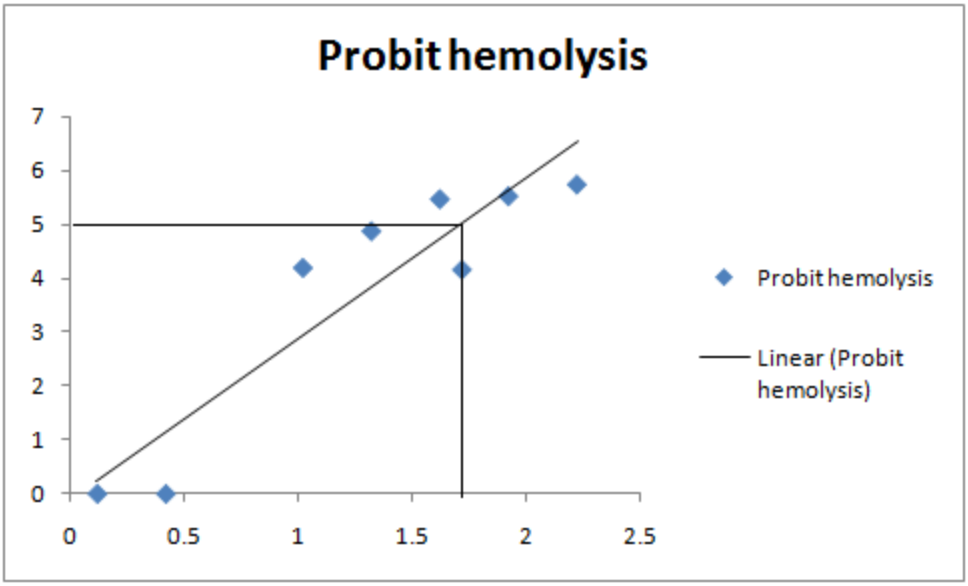


Figure -14: Hemolysis of 1% horse erythrocytes by Indian common krait *(B. caeruleus)* venom

Table-16: Hemolytic activity of Indian common krait *(B. caeruleus)* venom using 1% human red blood cells

Dose		Response		Results
Venom Dose	Log Dose	Percent Hemolysis	Probit Hemolysis	HD_{50}
166.67	2.2217	52	5.0502	177.8
83.33	1.9208	30	4.4756	
41.67	1.6198	26	4.3567	
20.83	1.3178	17	4.0458	
10.42	1.0178	15	3.9636	
5.2083	0.7167	15	3.9636	

No hemolysis in presence of normal saline without normal horse serum upto 166.67 µg/ml

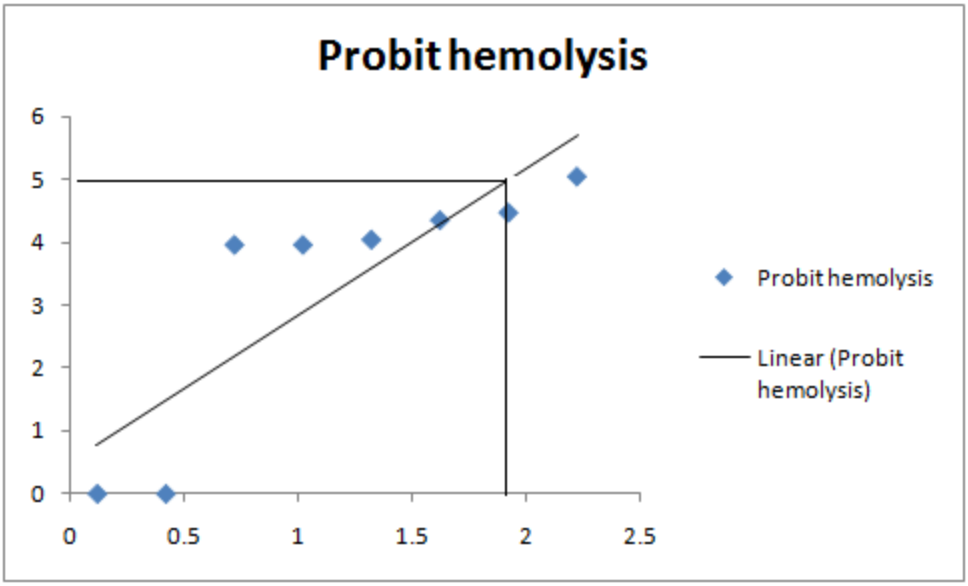


Figure -15: Hemolysis of 1% human erythrocytes by Indian common krait *(B. caeruleus)* venom

Table-17: Hemolytic activity of Indian common krait *(B. caeruleus)* venom using erythrocytes from different species of animals

Species of Animals	HD_{50} µg/ml
Guinea pig	2.138
Mouse	4.571
Horse	39.114
Sheep	56.230
Mule	138.0
Human	177.8
Rabbit	239.9
Rat	257.0
Fowl	500.0

From Table-17 it is evident that the erythrocytes from guinea pig and mice are more sensitive to Common Krait venom. But the hemolytic activity in the present study was observed using horse erythrocytes because of large source of red blood cells from a single animal.

The test for hemolytic activity of common krait venom has been repeated for twenty times using 1% horse erythrocytes. The statistical analysis on these results has been shown in Table-18.

Table-18: Statistical analysis of the results of HD_{50} determination

HD_{50} µg/ml x	Deviations from mean $X-\bar{X}$	Deviation square $(X-\bar{X})^2$
12.88	-26.234	688.22275
14.13	-24.984	624.20025
15.80	-23.314	520.22859
16.60	-22.514	506.88019
22.30	-16.814	282.71059
25.12	-13.994	195.83203
27.54	-11.574	133.95747
30.20	-8.914	79.45939
28.18	-10.934	119.55234
31.62	-7.494	56.160036
36.31	-2.804	7.862416
38.02	-1.094	1.196836
38.02	-1.094	1.196836
44.67	5.556	30.869136
50.12	11.006	121.13203
50.12	11.006	121.13203
56.23	17.116	292.95745
75.86	36.746	1350.26850
79.43	40.316	1625.37980
89.13	50.016	2501.60020
ϵ(x) = 782.28	**$\epsilon (x-\bar{x}) = 0$**	**$\epsilon (x-\bar{x})^2 = 9260.7389$**

Calculations:

Mean $= \epsilon(x) / n$

$= 782.28/20$

$= 39.114$

Standard Deviation $=\sqrt{\epsilon (x-\bar{x})^2 / n-1}$

$=\sqrt{9260.74/19}$

$=\sqrt{487.41}$

$=22.08$

Co-efficient of variation = S.D./Mean x 100

$= 22.08/39.114 \times 100$

$= 56.4$

Range = Mean ± S.D.

$= 39.114 \pm 22.08$

$= 39.114 + 22.08$ and $=39.114 - 22.08$

$= 61.194$ and $= 17.034$

Standard Error= S.D./√number of observations

$= 22.08/\sqrt{20}$

$= 22.08/\sqrt{4.472}$

$= 4.4934$

95% confidential limit $= \bar{x} \pm t_{95}$ (S.E.)

= 39.114 ± 2.080 (4.4934)
= 49.377 to 28.851

DISCUSSION

While classifying the medically important snakes, expert committee on snake venoms and antivenom sera have placed the Indian common krait (*Bungarus caeruleus*) in category-2 (uncommonly bites) of the poisonous snakes of South East Asia including India, Pakistan and Bangladesh. The other three species of poisonous snakes namely cobra, Russel's viper and saw scaled viper has been put in first category (commonly bites and cause death) (WHO, 1977). According to the expert committee, it seems that this species of snake is less important medically than the other three species of poisonous snakes of South East Asia. Therefore the venom of this species has been analysed to a lesser degree whereas the venom of common cobra has been analysed up to the level of amino acids.

In 1981 a coordination committee on venom and antivenom sera was held by the World Health Organization and the progress on the characterization of venom has been published in the form of an offset. In this publication also not much has been discussed on the characterization of common krait venom. This venom had not been included in the list of the 'International preparation of reference pools of snake venoms for characterization. Therefore it was decided to characterize the venom of this potentially dangerous snake in this study.To characterize this venom two properties have been studied here namely: lethal toxicity and hemolytic activity.

Lethal Toxicity: Lethal toxicity of common krait venom was studied in albino mice through different routes of inoculations. The results have been presented in Table-7. It can be observed from this table that the Indian common krait venom was found most toxic through intracerebral route. The order of lethal toxicity of krait venom has been found as shown below:

Intracerebral toxicity > intravenous toxicity > intraperitoneal toxicity > intramuscular toxicity > subcutaneous toxicity

This order of toxicity clearly indicates the nature of this venom. The more toxicity through intracerebral route provides an indication that this venom is affecting the central nervous system. Therefore the venom seems to be neurotoxic in nature. Though this venom has been found most toxic through intracerebral route yet to test toxicity intravenous route was employed as it was difficult to control the nonspecific deaths in intracerebral inoculation. Moreover, the quantity of inoculum (0.03ml) by the intracerebral route is so small that a slight variation in the lethal toxicity results. Therefore a handier intracerebral route of inoculation was used.

Scanty results on the lethal toxicity of this venom have been described in literature. Earlier it has been shown that the median lethal dose (LD_{50}) of this venom for a 20 g. mouse was 1.8 µg through intraperitoneal as well as through intravenous route (Minton, 1984). It was observed as 9.0 µg through subcutaneous route. Underwood in 1979 has shown this venom more toxic in mice through intravenous route and LD_{50} for a 20 g. mouse has been shown as low as 1.0 µ g. The results of intravenous toxicity in mice due to Indian common krait venom obtained in this study are in well agreement with those obtained by Minton. The mean LD_{50} of Indian common krait venom for 18-20 g mouse is 1.8475 (approximately 2 µ g), and is twice than the value given by Underwood. A detailed exercise on intravenous toxicity of common krait venom in mice has been shown in Table-7. It can be seen from these results that the mean of LD_{50} has been worked out as 1.8476 µg. The coefficient of variation of the results has been worked out as 14.35% which is well within the increment kept between the two consecutive doses of the dilution series of venom used for estimating toxicity. The 95% confidence limits are shown as 1.7243 to 1.9709. The value of LD_{50}

for Indian common krait venom estimated under the present study is within the range calculated by Minton.

Some workers have tried to explain the toxic action of common krait venom in experimental animals. The active principle mainly responsible for the toxicity of common krait venom is the neurotoxin which is a low molecular weight protein and it blocks the neuromuscular transmission postsynaptically (but does not bind to the nicotinic receptors site). The neurotoxin comprises 35% of the total lethal toxicity in venom. This is somewhat a novel neurotoxin different from the well studiedpresynaptically acting neurotoxins and β- neurotoxins of other elapid venoms which poses a neuromuscular blocking property (Cheymol, 1967) andblock peripheral nerve transmission. The purified neurotoxin specifically bind to the cholinergic receptor sites at the neuromuscular junction (Changeux et al, 1970).

The small molecular weight (6,000-8,000daltons) of these neurotoxins has a significant implication in the snake bite poisoning (Shipolini etal, 1974). The onset of systemic symptoms due to envenomation by neurotoxic venoms of cobra, krait, coral snake and sea snake, is extremely rapid, beginning within few hours and often instantaneously. Because of their small size neurotoxins the venommay diffuse into vital target organs much faster causing a quicker toxic action. The target of the presynaptically acting neurotoxin has not been identified as yet, but probably it might be either some specific structure in the nerve terminal which participates in the mechanism of acetylcholine release or energy supply (like that of mitochondria) for the activation or synthesis of the transmitters. In any case, all the neurotoxins shown to have presynaptic activity arephospholipase A. As phospholipase A is thought to be involved in neurotoxic action of the venom, therefore its presence might enhance the neurotoxicity of this venom. A venom usually contains more than one toxic component. Each of the toxic component need not be lethal to the same extent and they have to be characterized by its lethal property.

It has been observed in this study that the mice injected with common krait venom intravenously showed progressive symptoms of weakness followed by paralysis of skeletal muscles and death occurred by respiratory paralysis/ failure (Lee etal, 1985).

The toxicity of Indian common krait venom in mice has some similarity to the toxicity of this venom to human victims of common krait bite. The symptoms observed in human being bitten by Indian common krait were similar to those observed in mice, with addition of profuse salivation, ptosis and flaccid paralysis. Death occurred due to respiratory failure. Though the true curare like action has not yet been established like that in cobra venom, yet this venom is a true neurotoxin in its action as was observed in mice as well as in human beings.

Death of an experimental animal is not necessarily due to a single component and species of animal vary considerably in their response to pharmacologically active substance like common krait venom. The venom extracted from different species of snakes or same species from different geographical locations differ in their venom potency.

Hemolytic activity: The krait venom also contains hemolytic component known as hemolysin and it has been analysed in this study. For hemolytic activity of common krait venom, it was essential to select the species of animals for the red bloodcells which would be most suitable for this purpose. Erythrocytes from nine species of animals were taken depending upon availability. The results of 50% hemolytic dose have been shown in table-17. It can be seen from the table that the erythrocytes from guinea pigs in presence of horse normal horse serum are most sensitive to the action of krait venom and erythrocytes from fowl are least sensitive. Moreover erythrocytes from none of these species of animals showed hemolysis without the addition of normal horse

serumupto 166.67µ g of venom. This clearly indicates that the common krait venom is indirectly hemolytic and it doesn't contain the direct lyric factor like the venom of *Najanaja.*

The indirect lytic factor or PLA present in the Indian krait venom is active only in presence of phospholipids (lecithin) present in normal horse serum (Roy, 1945). The phospholipase A converts the phospholipids into lysophospholipidswhich is a strong hemolytic agent (Tu, 1977).

The lysophospholipidacts on the cell membrane of the erythrocyte producing leakage of hemoglobin into the suspending medium.Though the erythrocytes from mice and guinea pigs were found to be more sensitive to the action of Indian common krait venom than the erythrocytes from horse yet the latter was used in the further analysis of hemolytic activity of venom because the horse is a large source of erythrocytes and serum. The horse can be bled any number of times without much distress to the animal. The same horse could be utilized throughout the study for a period of nearly two years. This could not have been possible in case of smaller laboratory animals like mice or guinea pig. The change in the source of cells could have added unnecessary variation in the results. Thus the horse erythrocytes could be standardized once for all and 1% suspension of these cells were taken for further study which took nearly two years. The results of hemolytic activity of Indiancommon krait venom on horse erythrocytes have been presented in Table-17.

It can be observed from this table that a uniform gradation in the results has been obtained. The uniformity in the result can also be seen from the graphs and bar diagrams. With the help of these figures it was possible to calculate the 50% hemolytic dose most accurately. The test on hemolysisof Indian common krait venom using horse erythrocytes was repeated twenty times and statistical analysis of these results have been carried out. It can be seen from the analysis that the mean HD_{50} of this venom is 39.114 µg for 1 ml of 1% horse erythrocytes and the 95%confidence limits of HD_{50} was calculated as 20.526 to 49.377µg. The coefficient of variation 56.40% is well within the range of estimation.The experiment conducted to observe the hemolytic activity of Indian common krait venom did not show hemolysis without adding normal horse serum in any of the erythrocyte sample from an animal species. This proved that the venom does not possess direct lytic factor. May be it is not contributing much in envenomation. Only after adding normal horse serum the venom showed hemolysis.

The reaction involves:

phospholipase A converts the phospholipids into lysophospholipids (a strong hemolytic agent

↓

acts on the cell membrane of the erythrocyte

↓

leakage of hemoglobin

REFERENCES

1. Underwood G. (1969): cited by Kochva (1987)
2. Ahuja, M. L. and Singh, G.(1954): Snake bite in India. Ind. Jour. Med. Res. (42), pp. 685
3. Chugh K.C. (1988): Snake bites are highest in India. Indian Express 6th May 1988.
4. Redi F. (1664): Osserrazieni in terenoallevipere. Stella Florence. Quoted by Leak C.D. (1968) in development of knowledge about venoms. pp. 8.

5. Flexner S. and Noguchi H. (1902): Snake venom in relation to hemolytic, bacteriolysis and toxicity. Jour. Exp. Med. 6, 613.
6. Slotta K. and Frankel Conrat (1938): cited by Lee (1977): Handbook of experimental pharmacol 52, pp.1.
7. Ghose K.C. (1980): About snakes. I.M.J.R. (74), pp. 75.
8. Lee C.Y. (1984): Snake venoms. Toxicon 22, pp. 698 – 669.
9. Carrol R.L. (1975): Dermo-Triassic D Lizards from the Karoo. Cited by Kochva (1987); Toxicon 25(1), pp. 65.
10. Deoras P.J. (1970): General information. Snakes of India - The land and people. National Book Trust India New Delhi.
11. 58. Underwood G. (1979): Toxins from *B. caeruleus* venoms. Venomous snakes in India. Handbook Exptl. Pharmacol. 52, pp. 202.
12. Kochva E. (1978): Oral glands of Reptilia. Biol. of Reptilia (Ed. C. Gans) (8), pp. 43. Cited by Kochva et al.
13. Parker H.W. (1963): Reproduction and Development. Snakes. Page 135. Robert Hale ltd., London
14. Gharprey K. C. (1962): General information. Snakes of India and Pakistan 16. Popular Parkashan Bombay.
15. Tu A.T. (1977): Phospholipase A2 venoms, Chem. Mol. and Biol., Johan – Willey and sons. New York, London, Sydney, Toronto.
16. Kornalik F. and Moster R.W.P. (1964): A comparative examination of yellow and white venoms of *V. crumodytes*. Toxicon (2), pp. 175.
17. Basu A. S., Parker R. and O'Connor, R. (1970): Disc electrophoresis of glycoproteins in snake venoms. Toxicon (8), pp.279.
18. Chatman, V. B. and DiMari S. J. (1974): Use of sulphopropyl-sephadex chromatography and disc gel electrophoresis for the fractionation and identification of elapid venoms. Toxicon: 12(4), pp. 405-414.
19. Lee C., Chan Y. M. and Mebs D. (1976): Chromatographic separation of venom of *B. caeruleus* and pharmacological

characterization of its components. Toxicon (14), pp. 451.
20. Lee C. Y. and Tsai M.C. (1978): Further studies on neurotoxins isolated from venom of *B.*
21. Gaitonde B. B. and Kankonkar S. R. (1975): Preparation of cobra venom toxoid using gamma radiation. Bull. Haff. Inst.(3),2. Abst.Trop. Dis. Bull.74(10), pp. 866.
22. Duran R.F. (1936): The invasion of body by animal poisons. Science (83), pp. 286.
23. Kocholaty V.F., Ledfeved E.B., Daly J.G. and Billings T.A. (1971): Toxicity and some enzymatic properties and activities in the venom of crotalidae, viperidae and elapidae. Toxicon (9), pp. 131.
24. Ghosh B.N. and De S.S. (1938): I.J.M.R. (25),779.
25. Doery M.H. and Pearson E.J. (1961): Hemolysin in Australian snake venoms observation on the hemolysins of the venom of some Australian snakes and the separation of phospholipase A from venom of *P. porphyricus*. Biochem. Jour. (78), pp. 820.
26. Marinette G.B. (1965): The action of phosppholipase A on lipoproteins. Biochem. Biophys. Acta. 98, pp. 554.
27. Kawauchi S., Iwanaga S., Samejima Y., Suzuki T. (1971): Isolation and characterization of 2 PLA's from venom of *Agkistrodonhalysblomhoffii.*
28. Nair B.C, Nair G. and Elliot V.B. (1976): Temperature stability of PLA activity. II – variations in optimum temperature of PLA2 from various snake venoms. Toxicon 14, pp. 43.
29. Iyengar N.K., Sehra K.B., Mukerji B. and Chopra R.N. (1938): Cholinestrase in cobra venom. Curr. Sci. of India (7), pp. 51.
30. Chang C.C., Chuang, C.Y., Lee (1955). Role of cardiotoxin and phospholipase A in the blockade of nerve conduction and depolarization of skeletal muscle induced by cobra.Br. J. Pharmac. (1972), 44, 752-764.
31. Lee C.Y. (1979): Snake venom. Handb. Exptl. Pharm. (52), pp.360. Springer VerlagNewYork.

32. Heilboronn E., Jiang M. S., Zhou H., Hoggblad J. and Rydqvist (1987): Neurotoxins from Agkistrodon – *Halys* and *B. fasciatus* venom. Cited by Russell (1980).
33. LeeC.Y., Chen Y.M.,KarlssonE. (1976) Postsynaptic and musculotropic effects of notexin, a presynaptic neurotoxin from the venom of *Notechis scutatus scutatus* (Australian tiger snake). Toxicon,14(6),pp. 493-494
34. Devi A. (1968): Protein and nonprotein constituents of snake venoms. Venomous animals and their venoms (1), 128. [Eds. W. Bucherl, E.E. Buckley, V. Deulefeu].
35. Kellaway C. H., Cherry R. C. and Williams F.E. (1932): The peripheral action of Australian snake venoms II- the curare like action in mammals. Aust. J. Exp. Biol. Med. Sci. (10), pp. 181.
36. Mebs D., Lee C.Y., Ewanaga S. (1974): Chemical and Pharmacological characterization of toxic polypeptides from four elapid venoms, Animals, Plant and Microbial Toxins. Vol.1, pp. 17.
37. Bon, C. (1976): Caerulotoxin: An acidic neurotoxin from venom of *B. caeruleus* which blocks postsynaptically the neuromuscular transmission without binding to cholinergic receptor site. Bull. Inst. Pasteur Lille (74), pp. 41.
38. Ho C. L. and Lee C.Y. (1983): Mode of neuromuscular blocking action of caerulotoxin. Toxicon (21)2, pp. 310.
39. Lee C.Y., Chang C.C. and Su C.(1960): Effect of group specific reagents on toxicity and curare like activity of elapid venom. Jour. Formossan Med. Assoc. (59), pp. 75.
40. Chang C.C., Lee C.Y. (1963): Isolation of neurotoxin from venom of *B. multicinctus* and their modes of neuromuscular blocking action. Archs. Int. Pharmacodyn (44), pp. 241
41. Karlson E., Baker d., Rydon L. (1972): Purification of a presynaptic neurotoxin from venom of Australian tiger snake *N. scutatus*. Toxicon 910), pp. 405.
42. Strong P. N., Goarke J., Oberg G. and Kelly R. S. (1976): B-bungarotoxin – a presynaptic

toxin with enzymatic activity. Pro. Natn. Acad. Sci. USA 73, pp.178.
43. Lin S. S. Y., Huang M.C. and Lee C.Y. (1975): A study of cardiotoxic principles from the venom of *B. fasciatus* venom, Toxicon (130), pp.189.
44. Lee C.Y., Lee S.Y., Chan Y.M. and Lin W.W. (1985): On the causes of death due to toxic phospholipase A2 from snake venom. Toxicon 23, pp. 586
45. Laskowaski M.S. (1966): Modes of action of purified toxins from elapid venoms on neuromuscular transmission. Mem. Inst. But. (33), pp. 555.
46. Meister A. (1952): Enzymatic preparation of **α**- keto acids. Jour. Biol.Chem. 197, pp. 309.
47. Mella et al (1967): cited by lee (1979)
48. Russel F.E. (1980); Venoms- Snake venom poisoning, pp.214.J. B. Lippinettcompany.
49. Kornalik F. and Hladovoc J. (1975): The effect of defibrinating enzyme isolated from Echiscurinatus on experimentathronbosis. Thrombosis Res. (7), pp. 611. Cited by Russell (1980).
50. De Wys, W.D. Kwaan H.C. and Bathina S. (1976): Effect of defribrination on tumor growth and response to chemotherapy. Cited by Russell (1980).
51. Hillgard P. and Thornes R.D. (1976): Anticoagulant in the treatment of cancer. Europ. Jour. Cancer. (12), pp. 755.
52. Reed L.J. and Muench H.C. (1936): A simple method of estimating 50% end points. Am. Jou. Hyg. 27, pp. 493
53. Dass B., Chatterjee S.C. and Devi P. (1970): Hemolytic activity of Russell's viper venom. I.J.M.R. (58)399.
54. World Health organization (1977): Manual of International statistical classification of disease injuries and cause of death. Vol. 1, pp. 594.
55. *Minton* SA, Weinstein SA. Protease activity and lethal toxicity of *venoms* from some little known rattlesnakes. Toxicon. *1984*; 22(5):828-30.
56. Underwood, G. (1977): Classification &distribution of venomous snakes in the

world. Snake Venoms. Lee, C.Y. (editor). Handb. Expl. Pharmacol.52:15-40.
57. Cheymol (1967): Action neuromuscular de treiesvenins D' hydrophoiides. Toxicon (5), pp. 111.
58. Lester H.A. (1970): Postsynaptic action of cobra toxin at the myoneural junction. Nature (227), pp. 727.
59. Changeux et al (1970): Proc. Nat. Acad. Set. USA (67), pp. 1241.
60. Shipolini R. A., Bailey G.S. and Banks B.E.C. (1974): The separation of a neurotoxin from the venom of *N. melanoluca* and primary sequence determination. Curr. Jour. Biochem. 42, pp. 203.
61. Roy A.C. (1945): Lecithin and venom hemolysis. Nature 155, pp. 696.