Biochemical and biological characterization of *Naja kaouthia* venom from North-East India and its neutralization by polyvalent antivenom

Diganta Das\(^a\), Nanjaraj Urs\(^b\), Vilas Hiremath\(^b\), Bannikuppe Sannanaik Vishwanath\(^b\) and Robin Doley\(^a,\)*

\(^a\)Molecular Toxinology Laboratory, Department of Molecular Biology and Biotechnology, Tezpur University, Tezpur 784028, Assam, India; \(^b\)Department of Studies in Biochemistry, University of Mysore, Mysore 570006, Karnataka, India

*Correspondence to: Robin Doley, Email: doley@tezu.ernet.in, Tel: +91 37 12275412; FAX +91 37 12267005

Received: 13 June 2013; Revised: 30 October 2013; Accepted: 06 November 2013; Published: 06 November 2013

© Copyright The Author(s). First Published by Library Publishing Media. This is an open access article, published under the terms of the Creative Commons Attribution Non-Commercial License (http://creativecommons.org/licenses/by-nc/2.5). This license permits non-commercial use, distribution and reproduction of the article, provided the original work is appropriately acknowledged with correct citation details.

ABSTRACT

This study describes biochemical and biological properties of *Naja kaouthia* (Indian monocled cobra) venom of North-East India. The LD\(_{50}\) of the crude venom was found to be 0.148mg/kg and neurotoxicitic symptoms like paralysis of lower limbs and heavy difficulty in breathing at sub-lethal dose in mice was observed. The venom exhibited PLA\(_2\), indirect hemolytic and myotoxic activities but showed weak proteolytic and low direct hemolytic activities. It did not exhibit any hemorrhage when injected intradermally to mice. Anticoagulant activity was prominent when recalcification, prothrombin and activated partial thromboplastin time were tested on platelet poor plasma. Rotem analysis of whole citrated blood in presence of venom showed delay in coagulation time and clot formation time. Fibrinogen of whole citrated blood was depleted by venom when analyzed in Sonoclot. Crude venom at 10µg and after 16hr of incubation was found to degrade \(\alpha\) chain of fibrinogen.

Neutralization study showed that Indian polyvalent antivenom could neutralize some of the biochemical and biological activities as well as its fibrinogenolytic activity.

KEYWORDS: *Naja kaouthia*, haemostasis, thromboelastometry, myotoxicity, polyvalent antivenom

INTRODUCTION

Snakebite envenoming is a neglected tropical disease (WHO), which requires immediate attention. It is estimated that globally 2.5 million people are bitten by snakes each year with ~85,000 deaths (Gutierrez et al, 2010); in India, approximately 35,000 to 40,000 people die of snakebites annually (Chippaux, 1998; Kasturiratne et al, 2008). According to recent National Mortality Survey data, the incidence of snakebite cases is likely to be more than 50,000 per year in India (Mohapatra et al, 2011). However, these data may be far from the truth as most of the incidences happen in rural areas and these deaths mostly remain unreported. In India the “Big Four”, *Naja naja*, *Bungarus caeruleus*, *Daboia russelii* and *Echis carinatus* are considered to be medically important snakes and are responsible for most of the deaths. Recently, it has been reported that hump-nosed pit viper (*Hypnale hypnale*) from Kerala, is capable of causing lethal envenomation (Joseph et al, 2007). Hence, in addition to the “Big Four”, there might be other medically important snakes in specific geographical locations, which need attention. This is important for clinical diagnosis for treatment and for production of effective antivenoms. In India, polyvalent antivenom is raised against the “Big Four” venoms but these snakes may not be present throughout the country. Moreover, administration of this polyvalent antivenom has well documented limitations (Offerman et al, 2001; Laloo and Theakston, 2003; Williams et al, 2007).

*Naja kaouthia* is recognized phenotypically with the presence of O-shaped or monocellate hood pattern. They are widely distributed in Nepal, North East India, Bangladesh, Myanmar, Thailand and Peninsular Malaysia (Whitaker, 1978; Viravan et al, 1992; Mukherjee and Maity, 2002).
According to WHO, it belongs to Category 1 of venomous snakes. The symptoms of cobra bite are general neurotoxicity leading to flaccid paralysis and death by respiratory failure, and also severe hypertension (Agarwal et al, 2006; Halesha et al, 2013). Symptoms of coagulopathy have also been reported in victims of *Naja kaouthia* of Asian origin (Khandelwal et al, 2007). The *Naja kaouthia* venom of North-East India origin has not been explored though venom of West Bengal (India) origin have been studied extensively (Mukherjee and Maity, 2002; Lalloo and Theakston, 2003; Mukherjee, 2007; Debnath et al, 2010; Sekhar and Chakraborty, 2011). Hence, some work on biochemical and biological characterization of the *Naja kaouthia* venom and its in vitro neutralization by Indian polyvalent antivenom has been undertaken previously.

**MATERIALS AND METHODS**

**Reagents and kits**

sPLA₂ assay kit was procured from Cayman Chemical Company (MI, USA). NEOPLASTINE® CL PLUS and APTT reagent were obtained from STAGO (France). AGAPEE kit for CK/LDH analysis was purchased from AGAPEE diagnostics (Switzerland), Glass beads gbACT+ kit was obtained from Sienco, Inc. (USA). Polyvalent antivenom manufactured by Bharat Serums and Vaccines Limited (India) was purchased locally. Bovine plasma fibrinogen was obtained from Sigma-Aldrich and all other reagents used were of analytical grade and were either from Merck or Sigma-Aldrich, (USA).

**Animals**

Male Swiss albino mice of 40±3gm were obtained from central animal facility, University of Mysore. All animal were housed in well ventilated cages and experiments were carried out according to the Animal Ethical Committee Protocol (University of Mysore, Mysore, India, Proposal no. UOM/IAEC/25/2011).

**Collection of snake venom, preparation and storage**

Adult *Naja kaouthia* were captured from Jamugurihat, district Sonitpur, Assam, North-East India in the, month of May from its natural habitat and venom was extracted by allowing the snake to bite into a sterile beaker covered with para-film. The crude venom was immediately desiccated using dehydrated silica gel and stored in -20°C until further use. The permission for milking of snakes was obtained from Principal Chief Conservator of Forest (Wild Life) and Chief Wild Life Warden of Assam, India (WL/FG.27/tissue Collection/09 dated 07/10/2011).

**Determination of protein content**

Total protein content of *Naja kaouthia* venom was determined according to Lowry’s method using BSA as standard (Lowry et al, 1951).

**Phospholipase A₂ (PLA₂) activity**

PLA₂ activity was assayed using sPLA₂ assay kit according to the manufacturer’s protocol (Cayman Chemical Company, MI, USA). Briefly, in a 96-well microtitre plate, 10µl of venom (0.1mg/ml), 10µl DTNB (5, 50-dithio-bis-(2-nitrobenzoic acid)) and 5µl assay buffer were added. The reaction was initiated by adding 200µl of substrate solution (dihexanoyl Thio-PC). After gentle shaking, the optical density was measured every minute at 405nm using MultiSkan GO multi plate reader (Thermo Scientific, USA) for 10min. Assay buffer was used as blank and bee venom PLA₂ (0.01mg/ml) was used as a positive control. Tests were carried out in triplicate and mean values were taken. The activity was expressed as micromoles of dihexanoyl Thiol-PC hydrolyzed per min per mg of enzyme.

**Caseinolytic assay**

Digestion of casein was evaluated according to the method of Ouyang and Teng (Ouyang and Teng, 1976). Briefly, 1% (w/v) casein in 20mM Tris-Cl, pH 7.4, was incubated with various amounts of venom protein (1, 5, 10, 50 and 100µg) for 1hr at 37°C. Reaction was stopped by addition of ice cold 10% (v/v) TCA and centrifuged for 10min at 5000rpm (Thermo Scientific, USA, Heraeus Multifuge XIR). The digested protein in the supernatant was determined according to Lowry’s method (Lowry et al, 1951). Tyrosine curve was used to determine the protease activity and one unit of protease activity is defined as n mole equivalent of tyrosine formed per min per ml.

**LD₅₀ determination**

Toxicity of the venom was analyzed according to the method of Meier and Theakston (Meier and Theakston, 1986). Briefly, various amount of freshly dissolved venom (0.05 to 1mg/kg) in saline was injected intraperitoneally to eight male Swiss albino mice in a final volume of 150µl and the controls were injected with saline alone. The animals were carefully monitored for 24hr and their survival time was recorded and LD₅₀ was determined.

**Edema inducing activity**

The procedure of Yamakawa et al, (Yamakawa et al, 1976) as modified by Vishwanath et al, (Vishwanath et al, 1988) was followed. Mice weighing 20–30gm were injected with varying amount of venom (2–15µg) in a total volume of 20µl saline into intra plantar surface of right hind foot pad. Respective left foot pad received 20µl of saline and served as vehicle. Control mice were injected with 20µl saline into intra plantar surface of both hind foot pads. After 45min the mice were anesthetized (barbitone, 30mg/kg, i.p.) before sacrifice and hind limbs were removed at the ankle joint and weighed individually. The increase in weight due to edema is expressed as the ratio of the weight of edematous limb to the weight of vehicle (saline injected) limb x100. The amount of venom required to cause an edema ratio of 120% (20% above the basal level) is defined as minimum edema dose (MED).

**Hemorrhagic activity**

Hemorrhagic activity was assayed as described by Kondo et al, (Kondo et al, 1960). Various amount of venom (2–15µg) in 30µl saline were injected intradermally into mice and control mice received saline instead of venom sample. After 3hr, mice were sacrificed using anesthesia (barbitone, 30mg/kg, i.p.). The dorsal surface of the skin was removed and the inner surface was observed for hemorrhagic lesions. *E. carinatus* venom was used as positive control. The minimum hemorrhagic dose (MHD) is defined
as the concentration of venom that induce a hemorrhagic spot of 1 cm diameter from the spot of injection.

**In-vitro myotoxicity**

For myotoxicity, release of serum creatine kinase (CK) and lactate dehydrogenase (LDH) in the blood were determined using AGAPPE kit (AGAPPE diagnostics, Switzerland). Group of six male albino mice were injected (i.m) with 15 µg crude venom (40 µl) and control received 40 µl of saline. After 3 hr, mice were anesthetized and 0.5 ml of blood samples was drawn using cardiac puncture. The serum obtained by centrifugation was diluted with saline at 1:20 ratio. The CK and LDH activity were measured in 10 µl of plasma according to the manufacturer’s protocol and were expressed in Units/liter (U/l). The results are mean ±SD of three experiments.

**Collection of Blood and Platelet Poor Plasma (PPP) preparation**

Fresh goat blood was collected in citrated tube (0.11M tri sodium citrate) at 1:9 ratios (citrate: blood) from local butcher’s shop. Human blood was collected from healthy donors (27 Yr) who had not taken any medication for last 48hr. 9 ml of blood was drawn with 20 gauge 3/4” needle and immediately transferred to a plastic tube containing 1 ml of 0.11M tri sodium citrate (Suntravat et al, 2010). The tubes were centrifuged at 3000rpm (Thermo Scientific, USA, Heraeus Multifuge X1R) for 15mins to separate the red blood cells (RBC) and platelet poor plasma (PPP) and used within 4hr of collection.

**Direct and indirect hemolytic activity**

The RBC pellet obtained from the blood (as described above) was washed 4–5 times and re-suspended in 0.9% (w/v) saline to a final concentration of 10% (v/v). Various amount of venom were incubated for 60min at 37°C with 150 µl of 10% RBC to a final volume of 2ml with 0.9% (v/v) NaCl. The tubes were centrifugation at 5000rpm (Thermo Scientific, USA, Heraeus Multifuge X1R) for 10min and the absorbance of the supernatant was measured at 540nm in a MultiSkanGO, UV-Vis spectrophotometer (Thermo Scientific, USA). The hemolysis caused by dH2O was considered as 100%. For Indirect hemolytic, 20 µl of egg yolk substrate solution was added to the reaction mixtures at the time of incubation and hemolysis was measured as described for direct hemolytic activity. The results are mean ±SD of three experiments.

**Fibrinogenolytic activity**

Fibrinogenolytic activity was assayed according to the method of Ouyang and Teng, using bovine fibrinogen (2mg/ml) dissolved in 50mM Tris HCl buffer, pH 7.5, 0.15M NaCl (Ouyang and Teng, 1976). To 300 µl of dissolved fibrinogen, various amount of venom in 150 µl of buffer was incubated for different time intervals at 37°C. The incubated mixtures were then run on a 12.5% (w/v) SDS-PAGE according to the method of Laemmli (Laemmli, 1970). Staining was done with 0.25% (w/v) Coomassie brilliant blue R250 and destained till the protein bands were visible.

**In-vitro coagulant assays**

Recalcification time

Recalcification time of human PPP was measured using coagulation analyzer (STAGO, France). Various amount of venom in 50 µl of PBS was pre-incubated with 50 µl of human PPP at 37°C for 3 min and 50 µl of 25 mM CaCl2 was added to initiate the clot formation. The clotting time with PBS was considered as normal clotting time. The results are mean ±SD of three experiments.

**Activated partial thrombin time (APTT) test**

Activated partial thrombin time was determined using APTT reagent obtained from STAGO (France) according to the manufacturer’s protocol on a coagulation analyzer (STAGO, France). Various amount of venom in 50 µl of PBS was pre-incubated with 50 µl of human PPP at 37°C for 1 min and 100 µl of PT reagent was added to initiate the clot formation. The clotting time with PBS was considered as normal clotting time. The results are mean ±SD of three experiments.

**Whole citrated blood analysis**

**Thromboelastometry analysis**

To quantify the CT (clotting time, in seconds), CFT (clot formation time, in seconds) and MCF (maximum clot firmness, in mm) of the whole citrated blood, Rotem® Analyzer (ROTEM® Pentapharm GmbH Diagnostic Division; Munich, Germany) was used. For the analysis, blood samples from healthy volunteers were collected in 0.11M tri sodium citrate at 9:1 (blood: citrate) ratio. Various amount of venom in 20 µl of PBS was mixed with 20 µl of 200mM CaCl2, to this reaction mixture, 320 µl of whole citrated blood was added and clot formation was observed over 30min. Clot formation function with only PBS was considered as control. The results are mean ±SD of three experiments.

**Sono clot analysis**

A glass bead activated test tube (gbACT+ Kit obtained from Sienco, Inc, USA) was used to monitor clot detection, clot rate and platelet function (clot retraction) in a Sono clot Coagulation and Platelet Function Analyzer (Sienco, Inc, USA). Various amount of venom in 20 µl of PBS was added to 320 µl of citrated human blood followed by 20 µl 200mM CaCl2. The head assembly of the analyzer was closed 10s after the start button was pressed. Data were acquired and analyzed with Signature Viewer software (Sienco, Inc.). The results are mean ±SD of three experiments.

**Neutralization studies**

For neutralization studies, various amount of polyvalent antivenom was pre-incubated with 1 µg of *Naja kaouthia* venom in a final volume of 20 µl for 1 hr at 37°C and assays were performed as described above. The percentage inhibition was calculated by considering the activity in absence of polyvalent antivenom as 100%. The results are mean ±SD of three experiments.
RESULTS

Biological characterization

The biochemical and biological activities of the crude venom are listed in Table 1. The median lethal dose (LD₅₀) was found to be 0.148mg/kg when injected intraperitoneally to experimental mice. When sub-lethal dose of venom was injected to mice, neurotoxic symptoms like difficulty in movement; breathing and frequent drinking of water were observed followed by death after 40min. The amount of CK and LDH released after injection of 15µg of venom was found to be 6.605U/l and 26.38U/l respectively in the plasma. The CK was 10 times more than observed for the control mice (0.63U/l), however, the LDH was found to be only 3U more. The minimum edema dose (MED) of the venom was found to be 11.25µg. No direct hemolytic activity was observed up to 10µg of venom but when the amount was increased up to 100µg, it exhibited 1.4% RBC hemolysis. For indirect hemolytic activity, 23% hemolysis was observed for 1µg of venom. The venom showed weak proteolytic activity when tested on casein. The amount of tyrosine liberated was 0.14±0.02 moles by 100µg of venom in 1min. PLA₂ activity of the venom was 7.58±0.02 moles by 100µg of venom in 1min when assayed using sPLA₂ assay kit. However no haemorrhagic spot was observed when 3µg of venom was injected intradermally (Figure 1).

In-vitro coagulation activities

The venom showed anticoagulant activity in dose dependent manner. When recalcification time of human plasma was tested with 1µg venom, the plasma did not form clot (Figure 2). Prothrombin time increased dose dependently and at 0.1µg, clot formation was not observed up to 500s whereas the normal clotting time was 126.5sec (Figure 2). The APTT test on plasma did not increase significantly up to 500sec whereas the clot rate decreased which might be due to depletion of fibrinogen. However, up to 1µg the platelet function was found to be normal but at 10µg the platelet function was not observed (Table 2). Lower amount of venom did not show any digestion of fibrinogen (data not shown). However, when the amount of venom was increased to 10µg, clear digestion of α chain of fibrinogen was observed after 16hr of incubation (Figure 3A).

Neutralization studies

Effect of polyvalent antivenom on some of the biochemical and biological properties of Naja kaouthia venom are shown in Table 3. At 1:1 ratio, the polyvalent antivenom could neutralize the venom activity.

Table 1. Some biochemical and biological activities of Naja kaouthia venom

| Parameters                  | Activity          |
|-----------------------------|-------------------|
| LD₅₀                        | 0.148 mg/kg       |
| PLA₂ activity assay         | 7.9±0.24*         |
| Direct hemolytic assay (100µg venom) | 1.4±0.51%         |
| Indirect hemolytic assay (1µg venom) | 23.0 ±3%          |
| Caseinolytic activity (100µg venom) | 0.14±0.02*         |
| Creatine kinase (CK) (15µg i.m. injection) | 6.6±0.2 U/l       |
| Lactate dehydrogenase (LDH) (15µg i.m. injection) | 26.3±2.3 U/l      |
| Minimum edema dose (MED)    | 11.2±0.18 µg      |
| Haemorrhagic activity (up to 15µg) | NA                |

*Normal CK and LDH values are 0.63 U/l and 23.39 U/l respectively; Ψmol of diheptanoyl Thiol-PC hydrolyzed/min/mg; *µ moles of tyrosine formed/min; NA= No Activity. Results are mean ±SD (n=3)

©The Authors | Journal of Venom Research | 2013 | Vol 4 | 31-38 | OPEN ACCESS

Figure 1. Haemorrhagic activity of Naja kaouthia venom. A. Control (30µl of saline), B. Naja kaouthia venom (15µg), C. Saw scaled viper venom (3µg) (Positive control), the arrow indicates site of injection.

Figure 2. Dose dependent anticoagulant activity of Naja kaouthia venom on human plasma. Effect of crude venom on Recalcification time, Prothrombin Time test (PT) and Activated Partial Thrombin Time test (APTT). The results are mean ±SD of three experiments.
Table 2. Anticoagulant activity of Naja kaouthia venom on whole citrated blood. Results are expressed as mean ±SD of three experiments.

| Parameters                                      | PBS          | Crude venom (µg/ml) |
|-------------------------------------------------|--------------|---------------------|
|                                                 | 0.1          | 1.0                 | 10                   |
| Thromboelastometry analysis                     |              |                     |
| Coagulation time (CT) (s)                       | 503±10       | 634±15              | >1200                | >1200                 |
| Clot formation time (CFT) (s)                   | 87±3         | 266±10              | NCF                  | NCF                   |
| Maximum clot firmness (MCF) (mm)                | 65±2         | 61±1.3              | NCF                  | NCF                   |
| Sonoclot analysis                               |              |                     |
| Activated clotting time (ACT) (s) (range: 128–213) | 176±5.2      | 215±7.4             | 243±6.3              | 591±10                |
| Clot rate (CR)(range: 9.0–35)                   | 23±0.5       | 23±0.32             | 16±0.21              | 1.2±0.2               |
| Platelet function (range: 3–5)                  | 2.8±0.01     | 3.8±0.02            | 3.3±0.01             | 0                     |

*NCF: No clot formation, the results are expressed as mean ± SD

Figure 3. A. Fibrinogenolytic activity of Naja kaouthia venom. SDS-PAGE of bovine fibrinogen (reduced) after incubation with 10µg crude Naja kaouthia venom at various time intervals. B. Inhibition of fibrinogenolytic activity of Naja kaouthia by polyvalent antivenom. The venom:polyvalent antivenom (1:1, w/w) mixture was pre-incubated for 1hr at 37°C. This mixture was incubated with 300µl of fibrinogen (2mg/ml) for 24hr and aliquots were withdrawn at different time interval and fractionated in 12.5% (w/v) SDS-PAGE. Lane 1. Undigested fibrinogen (control). Lane 2. Fibrinogen incubated with only venom; Lane 3. After 0.5hr; Lane 4. 1hr; Lane 5. After 2hr; Lane 6. After 4hr; Lane 7. After 8hr; Lane 8. After 16hr; and Lane 9. After 24hr.

DISCUSSION

The patho-physiological effect post-snakebite envenomation varies greatly among the various species and even within species due to variation in the venom proteins and biological activities (Glenn et al, 1983; Minton and Weinstein, 1986; Daltry et al, 1996; Saravia et al, 2002; Menezes et al, 2006). These variations affect the classical manifestation of envenomation and require specific consideration for treatment. Hence understanding the biochemical and biological properties of snake venom from a particular geographic location is important.

The LD₅₀ of the Naja kaouthia venom was found to be 0.148mg/kg, whereas those for cobra venoms of Thailand and Kolkata origin were reported to be 0.23mg/kg and 0.7mg/kg, respectively (Mukherjee and Maity, 2002; Leong et al, 2012). Though the route of injection was different (Kolkata origin venom given via tail vein injection) in these experiments, the lethal dose of North East origin venom was less than that of the other geographical locations suggesting it might be more lethal. However, the comparative study with indistinguishable experimental conditions would be necessary to differentiate these venoms. In mice the venom did not induce haemorrhagic activity and venom of Kol kata origin is reported to be devoid of such activities. The haemorrhagic is mainly caused by metalloproteases, which are abundantly found in viper venom (Kamiguti et al, 1996; Chakrabarty et al, 2000; Mukherjee, 2008). Moreover, the edema inducing activity was not found to be significant. Hence this venom might not induce inflammation and tissue damage at the site of bite. Interestingly, the venom at 100µg showed only 1.4% hemolysis of RBC, whereas at the same amount Kolkata venom activity is reported to be 39.0% (Mukherjee and Maity, 2002). The membrane damaging activity is mainly contributed by the low molecular weight proteins which might be absent in this venom. The indirect hemolytic activity of the venom in presence of the egg yolk is due to PLA₂ enzymes. The lysophospholipids and free fatty acids formed during the catalysis of phospholipids by PLA₂ enzyme exhibits this activity as they are lytic in nature (Condrea et al, 1964). The presence of various PLA, isoenzymes and neurotoxins in Naja kaouthia venom have been not neutralize the PLA₂ activity of the venom but at 1:100 ratios, 97.38 ± 4.8% inhibition was observed. Inhibition of the indirect hemolytic activity of venom was also observed similar to the PLA₂ activity. When the concentration of the polyvalent antivenom was increased by 100 times, indirect hemolytic activity was completely neutralized. Recalification time of the venom was neutralized up to 49.34% at 1:1 ratio and with 10 times increase in polyvalent antivenom, 92.03% neutralization was observed. Similarly, the APTT and PT was also brought to the normal clotting time when the polyvalent antivenom was 10 times excess of the venom concentration. Moreover, degradation of α chain of fibrinogen by venom was inhibited by polyvalent antivenom at 1:1 ratio (Figure 3B).
reported by various workers (Joubert and Taljaard, 1980; Meng et al, 2002; Qiumin et al, 2002; Doley et al, 2004). When the crude venom was analyzed for the PLA₂ activity using diheptanoyl thiolester PC as substrate, the amount of substrate hydrolyzed product was 7.9±0.24 μmol/min/mg suggesting the presence of enzymatically active PLA₂ in the venom. PLA₂ is one of the major constituent in the elapid venom, which confers multiple toxicity to the prey or victim such as membrane damaging, neurotoxicity, edema and prolongation of coagulation time (Kini and Evans, 1989; Doley et al, 2004). Hence the myotoxicity, neurotoxicity and edema induced by this venom are due to the presence of large amount of PLA₂ enzyme in the venom. The observed differences in the biochemical and biological activities in the venoms of Indian origin might be due to variation in the venom composition and content due to difference in geographical locations. Both venoms were collected during summers; however, in the present study, the ages of the snakes were unknown as they were captured from the wild. Detailed analysis of *Naja kaouthia* venoms from different locations of India need to be carried out to decipher the differences in the venom composition as well as the presence of unique toxins.

Snake venoms affect the haemostasis process of victim/prey either by prolonging or shortening the clotting time. ELapid venoms are anticoagulant in nature due to the presence of large amount of strong and weak anticoagulant PLA₂ enzymes. Moreover, non-enzymatic protein from elapid venom like Cardiotoxin from *Naja nigrigollis crawshawii* and Hemextin A and hemextin AB complex from *Hemachatus haemachatus* venom are also reported to be anticoagulant in nature (Kini et al, 1988; Banerjee et al, 2005). The venom significantly delayed the recalcification time, PT and APTT of plasma under *in vitro* condition, which is due to strong anticoagulant proteins present in the venom. The plasma did not form clot at 0.01, 0.1 and 1 μg concentration of venom when tested for recalcification time, PT and APTT, respectively. This suggests that the anticoagulant activity of the venom is most likely to affect all the pathways. Venom PLA₂ enzymes inhibit activation of FX to FXa which leads to disruption in the formation of prothrombinase complex, which is required for blood coagulation (Stefansson et al, 1990; Kerns et al, 1999; Kini, 2005). The higher amount of venom required in case of PT and APTT for non-coagulation of blood might be due to the addition of extra phospholipids during these tests; however, this needs to be verified. The venom proteins, especially the PLA₂ enzymes, hydrolyze the phospholipids which are required for the prothrombinase complex formation. The Sonoclot and Rotem analysis also demonstrated that the *Naja kaouthia* venom is anticoagulant in nature. The whole citrated blood analysis by sonoclot clearly indicated the depletion of fibrinogen in the reaction when pre-incubated with venom. The lower value of MCF by Rotem analysis indicates decreased platelet number or function, decreased fibrinogen level or fibrin polymerization disorders, or low activity of factor XIII. Recently, Nk α metalloprotease, which cleaves the α-chain, as well as a low molecular protein with fibrinogenolytic activity have been reported (Wijeyewickrema et al, 2007; Deb Nath et al, 2010). The weak proteolytic activity towards casein and higher amount of venom and time required for complete degradation of α chain of bovine serum fibrinogen might be due to presence of these proteins in lower amount. Hence anticoagulant activity of *Naja kaouthia* might not be only due to degradation of phospholipids or α chain of fibrinogen but action of different venom proteins which might be acting enzymatically or non-enzymatically on coagulation factors and complexes.

Polyspecific antivenom is currently used by the medical practitioners for the treatment of snakebite patients in India. The Indian polyspecific antivenom is prepared using the venoms of four major poisonous snake species viz: *Naja naja*, *Daboia russelii*, *Echis carinatus* and *Bungarus caeruleus*. In most of the cases, it has been observed that the efficacy is highly reduced when antivenoms raised against venom from a particular geographic region is used to treat victims from another region (Shashidharamurthy et al, 2002; Shashidharamurthy and Kemparaju, 2007). The polyspecific antivenom could neutralize some of the biochemical and biological activity partially at 1:10 ratio (venom: polyclonal antivenom) and complete neutralization was observed when the dose of the polyspecific antivenom was increased to 10 fold. The partial inhibition might be due to the antibodies of *Naja naja* proteins present in the polyspecific antivenom, which recognizes the *Naja kaouthia* venom proteins. Present study documents that the polyspecific antivenom can neutralize some of tested biochemical and biological activities of *Naja kaouthia* venom under *in vitro* condition.

**Table 3. In vitro neutralization of whole venom activity by polyspecific antivenom**

| Activity                  | % inhibition by polyspecific antivenom |
|---------------------------|---------------------------------------|
|                           | 1:1    | 1:10  | 1:100 |
| PLA₂ activity             | 0      | 40.0±5.0 | 97.38±4.8 |
| Indirect hemolytic        | 11.96±2.12 | 68.15±0.15 | 100    |
| Recalcification time      | 49.34±5.01 | 92.03±3.0 | 96.52±2.81 |
| PT                        | 36.44±5.8  | 78.19±3.86 | 99±1.76 |
| APTT                      | 32.33±6.44 | 92.1±5.83 | 100    |
| Fibrinogenolytic (α chain present) | 100    |

* The results are expressed as mean±SD (n=3)  
Values indicate % inhibition at each venom: antivenom (µg:µg) ratio

©The Authors | Journal of Venom Research | 2013 | Vol 4 | 31-38 | OPEN ACCESS
ACKNOWLEDGEMENTS

We acknowledge DBT, Govt of India, for grant under twin-
ing programme for NER India, Tezpur University for the
start-up grant and DBT, UGC and DST, New Delhi, for vari-
ants grants to the department. We also thank the anonymous
reviewers for their valuable comments and suggestions that
have improved the manuscript.

COMPETING INTERESTS

None declared.

LIST OF ABBREVIATIONS

CFT; clot formation time
CT; Coagulation time
MCF; Maximum clot firmness
ACT; Activated clotting time

REFERENCES

Agarwal R, Aggarwal AN and Gupta D. 2006. Elapid snakebite as
a cause of severe hypertension. J Emerg Med, 30, 319–320.
Banerjee Y, Mizuguchi J, Iwanaga S and Kini RM. 2005. Hemextin
AB complex—a snake venom anticoagulant protein complex that
inhibits factor VIIa activity. Pathophysiol Haemost Thromb, 34,
184–187.
Chakraborty D, Datta K, Gomes A and Bhattacharyya D. 2000.
Haemorrhagic protein of Russell’s viper venom with fibrinolytic
and esterolytic activities. Toxicon, 38, 1475–1490.
Chippaux JP. 1998. Snake-bites: appraisal of the global situation.
Bull World Health Organ, 76, 515–524.
Condrea E, Mammon Z, Aloof S and Devries A. 1964. Susceptibility
of erythrocytes of various animal species to the hemolytic and
phospholipid spitting action of snake venom. Biochim Biophys
Acta, 84, 365–375.
Daltry JC, Wuster W and Thorpe RS. 1996. Diet and snake venom
evolution. Nature, 379, 537–540.
Debnath A, Saha A, Gomes A et al. 2010. A lethal cardiotoxic-
cytotoxicytotoxic protein from the Indian monocellate cobra (Naja kaou-
tha) venom. Toxicon, 56, 569–579.
Doley R, King GF and Mukherjee AK. 2004. Differential hydroly-
sis of erythrocyte and mitochondrial membrane phospholipids by
two phospholipase A2 isoenzymes (NK-PLA2-I and NK-PLA2-
II) from the venom of the Indian monocellate cobra Naja kaouthia.
Arch Biochem Biophys, 425, 1–13.
Glenn JL, Straight RC, Wolfe MC and Hardy DL. 1983. Geo-
graphical variation in Crotalus scutulatus scutulatus (Mojave
rattlesnake) venom properties. Toxicon, 21, 119–130.
Gutierrez JM, Williams D, Fan HW and Warrell DA. 2010. Snake-
bite envenoming from a global perspective: Towards an inte-
grated approach. Toxicon, 56, 1223–1235.
Halesha BR, Harshavardhan L, Lokesh AJ, Channaveerappa PK
and Venkatesh KB. 2011. A study on the clinico-epidemiological
profile and the outcome of snake bite victims in a tertiary care
centre in southern India. J Clin Diagn Res, 7, 122–126.
Joseph JK, Simpson ID, Menon NC et al. 2007. First authenticated
cases of life-threatening envenoming by the hump-nosed pit
viper (Hypnale hypnale) in India. Trans R Soc Trop Med Hyg.
101, 85–90.
Joubert FJ and Taljaard N. 1980. Snake venoms. The amino acid
sequences of two Melanoleuca-type toxins. Hoppe Seyler's Z
Physiol Chem, 361, 425–436.
Kamiguti AS, Hay CR, Theakston RD and Zuzul M. 1996. Insights
into the mechanism of haemorrhage caused by snake venom met-
alloproteinases. Toxicon, 34, 627–642.
Kasturiratne A, Wickremasinghe AR, de Silva N et al. 2008. The
global burden of snakebite: a literature analysis and modelling
based on regional estimates of envenoming and deaths. PLoS
Med, 5, e218.
Kerns RT, Kini RM, Stefansson S and Evans HJ. 1999. Targeting
of venom phospholipases: the strongly anticoagulant phospholipase
A(2) from Naja nigricollis venom binds to coagulation factor Xa
to inhibit the prothrombinase complex. Arch Biochem Biophys,
369, 107–113.
Khanderwal G, Katz KD, Brooks DE, Gonzalez SM and Ulshney
CD. 2007. Naja Kaouthia: two cases of Asiatic cobra envenoma-
tions. J Emerg Med, 32, 171–174.
Kini RM. 2005. Structure-function relationships and mechanism
of anticoagulant phospholipase A2 enzymes from snake venoms.
Toxicon, 45, 1147–1161.
Kini RM and Evans HJ. 1989. A model to explain the pharmacolog-
ical effects of snake venom phospholipases A2. Toxicon, 27,
613–635.
Kini RM, Haar NC and Evans HJ. 1988. Non-enzymatic inhibi-
tors of coagulation and platelet aggregation from Naja nigricollis
venom are cardiotonins. Biochem Biophys Res Commun, 150,
1012–1016.
Kondo H, Kondo S, Ikezawa H and Murata R. 1960. Studies on the
quantitative method for determination of hemorrhagic activity of
Habu snake venom. Jpn J Med Sci Biol, 13, 43–52.
Laemmlı UK. 1970. Cleavage of structural proteins during the
assembly of the head of bacteriophage T4. Nature, 227, 680–685.
Lalloo DG and Theakston RD. 2003. Snake antivenoms. J Toxicol
Clin Toxicol, 41, 277–290.
Leong PK, Sim SM, Fung SY et al. 2012. Cross neutralization of
Afro-African cobra and Asian krait venoms by a Thai polyvalent
snake antivenom (Neuro Polyvalent Snake Antivenom). PLoS
Negl Trop Dis, 6, e1672.
Lowry OH, Rosebrough NJ, Farr AL and Randall RJ. 1951. Protein
measurement with the Folin phenol reagent. J Biol Chem, 193,
265–275.
Meier J and Theakston RD. 1986. Approximate LD50 determina-
tions of snake venoms using eight to ten experimental animals.
Toxicon, 24, 395–401.
Menezes MC, Furtado MF, Travaglia-Cardoso SR, Camargo AC
and Serrano SM. 2006. Sex-based individual variation of snake
venom component among eighteen Bothrops jararaca siblings.
Toxicon, 47, 304–312.
Meng QX, Wang WY, Lu QM et al. 2002. A novel short neurotoxin,
cobrotoxin c, from monocellate cobra (Naja kaouthia) venom:
isoulation and purification, primary and secondary structure deter-
mination, and tertiary structure modeling. Comp Biochem Phys-
iol C Toxicol Pharmacol, 132, 113–121.
Minton SA and Weinstein SA. 1986. Geographic and ontogenic
variation in venom of the western diamondback rattlesnake
(Crotalus atrox). Toxicon, 24, 71–80.
Mohapatra B, Warrell DA, Suraweera W et al. 2011. Snakebite
mortality in India: a nationally representative mortality survey.
PLoS Negl Trop Dis, 5, e0108.
Mukherjee AK. 2007. Correlation between the phospholipids
domains of the target cell membrane and the extent of Naja
kaouthia PLA(2)-induced membrane damage: evidence of dis-
tinct catalytic and cytotoxic sites in PLA(2) molecules. Biochim
Biophys Acta, 1770, 187–195.
Mukherjee AK. 2008. Characterization of a novel pro-coagulant
metalloprotease (RVBCM) possessing alpha-fibrinogenase and
tissue haemorrhagic activity from venom of Daboia russellii rus-
selli (Russell’s viper): evidence of distinct coagulant and haem-
orrhagic sites in RVBCM. Toxicon, 51, 923–933.
Mukherjee AK and Maity CR. 2002. Biochemical composition,
lethality and pathophysiology of venom from two cobras—Naja
naja and N. kaouthia. Comp Biochem Physiol B Biochem Mol
Biol, 131, 125–132.
Offerman SR, Smith TS and Derlet RW. 2001. Does the aggressive
use of polyvalent antivenin for rattlesnake bites result in serious
acute side effects? West J Med, 175, 88–91.
Ouyang C and Teng CM. 1976. Fibrinogenolytic enzymes of
Trimeresurus mucrosquamatus venom. Biochim Biophys Acta,
420, 298–308.
Qiumin L, Qinxiong M, Dongsheng L et al. 2002. Comparative study of three short-chain neurotoxins from the venom of Naja kaouthia (Yunnan, China). J Nat Toxins, 11, 221–229.

Saravia P, Rojas E, Arce V et al. 2002. Geographic and ontogenic variability in the venom of the neotropical rattlesnake Crotalus durissus: pathophysiological and therapeutic implications. Rev Biol Trop, 50, 337–346.

Sekhar CC and Chakrabarty D. 2011. Fibrinogenolytic toxin from Indian monocled cobra (Naja kaouthia) venom. J Biosci, 36, 355–361.

Shashidharamurthy R, Jagadeesha DK, Girish KS and Kemparaju K. 2002. Variations in biochemical and pharmacological properties of Indian cobra (Naja naja naja) venom due to geographical distribution. Mol Cell Biochem, 229, 93–101.

Shashidharamurthy R and Kemparaju K. 2007. Region-specific neutralization of Indian cobra (Naja naja) venom by polyclonal antibody raised against the eastern regional venom: A comparative study of the venoms from three different geographical distributions. Int Immunopharmacol, 7, 61–69.

Stefansson S, Kini RM and Evans HJ. 1990. The basic phospholipase A2 from Naja nigricollis venom inhibits the prothombinase complex by a novel nonenzymatic mechanism. Biochemistry, 29, 7742–7746.

Suntravat M, Nuchprayoon I and Perez JC. 2010. Comparative study of anticoagulant and procoagulant properties of 28 snake venoms from families Elapidae, Viperidae, and purified Russell’s viper venom-factor X activator (RVV-X). Toxicon, 56, 544–553.

Viravan C, Looareesuwan S, Kosakarn W et al. 1992. A national hospital-based survey of snakes responsible for bites in Thailand. Trans R Soc Trop Med Hyg, 86, 100–106.

Vishwanath BS, Kini RM and Gowda TV. 1988. Purification and partial biochemical characterization of an edema inducing phospholipase A2 from Vipera russelli (Russell’s viper) snake venom. Toxicon, 26, 713–720.

Whitaker R. 1978. The Venomous Snakes. Common Indian Snakes. The Macmillan Co., (India).

Wijeyewickrema LC, Gardiner EE, Shen Y, Berndt MC and Andrews RK. 2007. Fractionation of snake venom metalloproteinases by metal ion affinity: a purified cobra metalloproteinase, Nk, from Naja kaouthia binds Ni2+-agarose. Toxicon, 50, 1064–1072.

Williams DJ, Jensen SD, Nimorakiotakis B, Muller R and Winkel KD. 2007. Antivenom use, premedication and early adverse reactions in the management of snake bites in rural Papua New Guinea. Toxicon, 49, 780–792.

Yamakawa K, Nozaki M, and Hokoma Z. 1976. Fractionation of Sakishima habu (Trimeresurus elegans) venom and lethal hemorrhagic and edema forming activity of the fraction. In: Ohsaka A, Hayashi K, and Sawai Y (Eds). Animal Plant and Microbial Toxins. New York Plenum Press. pp 97–109.