Venom and Purified Toxins of the Spectacled Cobra (*Naja naja*) from Pakistan: Insights into Toxicity and Antivenom Neutralization

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Abstract. Geographical variations of snake venoms can result in suboptimal effectiveness of Indian antivenoms that are currently used in most South Asian countries. This study investigated the toxicity and neutralization profile of the venom and toxins from Pakistani spectacled cobra, *Naja naja*, using VINS polyvalent antivenom (VPAV, India), *Naja kaouthia* monovalent antivenom (NKMAV, Thailand), and neuro bivalent antivenom (NBAV, Taiwan). Cation-exchange and reverse-phase high-performance liquid chromatography fractionations followed by toxin identification through liquid chromatography–mass spectrometry (MS)/MS indicated that the venom comprised mainly of postsynaptic neurotoxins (NTXs) (long neurotoxins [LNTXs], 28.3%; short neurotoxins [SNTXs], 8%), cytotoxins (CTXs) (31.2%), and acidic phospholipases A₂ (12.3%). NKMAV is the most effective in neutralizing the lethal effect of the venom (potency = 1.1 mg venom/mL) and its LNTX (potency = 0.5 mg toxin/mL), consistent with the high content of LNTX in *N. kaouthia* venom. VPAV was effective in neutralizing the CTX (potency = 0.4 mg toxin/mL), in agreement with the higher CTX abundance in Indian cobra venom. All the three antivenoms were weak in neutralizing the SNTX (potency = 0.03–0.04 mg toxin/mL), including NBAV that was raised from the SNTX-rich Taiwanese cobra venom. In a challenge-rescue experiment, envenomed mice were prevented from death by a maximal dose of VPAV (intravenous 200 µL) but the recovery from paralysis was slow, indicating the need for higher or repeated doses of VPAV. Our results suggest that optimal neutralization for Pakistani *N. naja* venom may be achieved by improving the formulation of antivenom production to enhance antivenom immunoreactivity against long and SNTXs.

INTRODUCTION

Bites by venomous snakes can result in envenomation, a life-threatening disease that is prevalent in many tropical and subtropical countries. The exact epidemiology and the global burden measure of snakebite envenomation, however, remains elusive due to the lack of reliable information on incidence, morbidity, and mortality.^{1,2} Most figures available were obtained from fragmentary surveys conducted in limited regions, representing only a small fraction of the true epidemiology.³ Persistent underreporting of the mortality and morbidity of snakebite envenomation earned it the most neglected status among the World Health Organization listed tropical neglected diseases/conditions.⁴ The problem is particularly aggravated in South Asia, where human populations are heavily engaged in agricultural activities.⁵ In Pakistan, the annual mortality estimate following envenomation soars as high as 20,000.^{1,6} The hidden toll of suffering continues to affect the families of the deceased, and patients who survived with crippling deformity.

With today's medical advancement, snakebite envenomation is supposedly a preventable and treatable condition. Unfortunately over the years, various challenges remain unresolved, hindering the solution for envenomation in many countries.⁷ One of these challenges is pertaining to the production and distribution of an effective antivenom tailored to the region that requires it. Countries that do not have local antivenom manufacturing plant such as Sri Lanka, and those with limited local antivenom production such as Pakistan, resort to importing antivenom from India.^{6,7} The Indian antivenoms assume the “Big Four” formulation using venoms of the com-

mon cobra (*Naja naja*), common krait (*Bungarus caelurues*), Russell's viper (*Daboia russelii*), and saw-scaled viper (*Echis carinatus*), sourced from a restricted area in southeastern India. These snakes although can be found in Pakistan, their venom profiles can vary geographically within the same species as demonstrated in several other venoms, attributed mainly to ecological factor.^{2,8–10} By the same token, the antigenicity of toxins can vary substantially too, thus limiting the efficacy of the imported antivenom against local species.^{11,12} Here, a very pertinent concern arises when imported antivenoms are not vigorously evaluated against the venoms of local species. This contributes to uncertainty on the indication and dosing of the foreign antivenom, thereby exposing the patients to high doses of antivenom (and the risk of anaphylaxis) in which itself is probably ineffective to begin with.

In Pakistan, the landscapes differ greatly from fertile plains to deserts, forests, mountains, plateaus, and coastal lines. The extremely diverse bioclimatic and topographic profiles create multifarious habitats that cultivate unique fauna and flora with an exotic blend of Palaearctic, Indo-Malayan, and Ethiopian forms.¹³ These include many venomous snakes of great medical importance, some of which are shared with the Indian subcontinent (the Big Four aforementioned), while many more are unique endemic species or those overlap with the middle eastern and Himalayan species.¹⁴ As with most developing countries, snakebite envenomation in Pakistan occurs following increased human contact with snakes during agricultural activities.¹ This is particularly obvious with anthropometrically adapted snakes such as cobras (Elapidae: *Naja*).² At least two cobra species distribute in Pakistan: *N. naja*, the black spectacled cobra that distributes across the southern and eastern Pakistan including Punjab, Baluchistan, and Sind provinces, and *Naja oxiana*, the brown ox cobra restricted to northern Pakistan at areas of higher elevations.¹⁵ A previously known subspecies in the southern Pakistan, *Naja naja karachiensis*, has been synonymized with the species *N. naja* under the current systematics.¹⁶ Cobra envenomation is characterized

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by local tissue necrosis and neuromuscular paralysis that leads to respiratory failure. Depending on the amount of venom injected, paralysis following cobra bites can occur within several hours, with death ensues if breathing is not assisted. Antivenom is the only proven effective pharmacological therapy that can expedite the clearance of venom and shorten the duration of intubation.¹⁷ Unfortunately, this management approach is confronted by the limited availability of local antivenom, while Indian antivenoms (produced for treating the Big Four) have been used for a long time in the country. However, geographical variations of snake venoms should not be disregarded, as ramifications have been demonstrated in the discrepancies of venom toxicity and therapeutic response to antivenom of a single geographical source.^{2,18} This is particularly relevant in the case of cobra for two obvious reasons: first, cobra distributes widely in many parts of Pakistan and adapts well to human settlements, constituting a common cause of bites in the country; second, antivenom neutralization potency against cobra venom has been reported to be low, ostensibly due to the poor immunogenicity of small polypeptide toxins in the venom.¹⁹ In view of these, this study was conducted to investigate the immunoneutralization profile of the Pakistani *N. naja* venom, using different cobra antivenoms available in the region. The principal toxins of the venom were also purified and tested for toxin-specific antivenom neutralization. It is hope that the findings will contribute to the optimization of cobra antivenom production and use in the future.

MATERIALS AND METHODS

Venom and antivenoms. *Naja naja* venom from Pakistan was purchased from Latoxan (Valence, France) and both were pooled venom from adult snakes. Three antivenoms were used in this study: VINS polyvalent antivenom (VPAV), *Naja kaouthia* monovalent antivenom (NKMAV), and Taiwan neuro bivalent antivenom (NBAV). VPAV is a polyspecific antivenom prepared against the venoms of Indian cobra (*N. naja*), common krait (*B. caeruleus*), Russell's viper (*D. russelli*), and saw-scaled viper (*E. carinatus*) (all Indian origin), manufactured by VINS Bioproduct Limited, Hyderabad, India (batch no.: 01AS12041; expiry date: March 2016). NKMAV is a monospecific antivenom prepared against the venom of Thai *N. kaouthia* (Thai monocled cobra), manufactured by the Queen Saovabha Memorial Institute, Bangkok, Thailand (batch no.: NK00514; expiry date: October 2019). NBAV is a bispecific antivenom from Taiwan Central for Disease Control, Taipei, Taiwan ROC, raised against the venoms of *Bungarus multicinctus* and *Naja atra* of Taiwan origin (batch no.: FN10101; expiry date: April 2017). All antivenoms used were in the form of lyophilized F(ab')₂ derived from equine antisera. All the antivenoms were reconstituted in 10 mL normal saline before use.

Animals and ethics clearance. Mice used in this study were of albino Institute of Cancer Research (ICR) strain (20–25 g) supplied by the Animal Experimental Unit, University of Malaya. The protocol of animal studies was based on the Council for International Organizations of Medical Sciences guidelines on animal experimentation²⁰ and was approved by the Institutional Animal Care and Use Committee of the University of Malaya (ethics clearance number: 2014-09-11/PHAR/R/TCH).

Chemicals and materials. All chemicals and reagents used were of analytical grade. Ammonium bicarbonate, dithiothreitol

(DTT), and iodoacetamide were purchased from Sigma-Aldrich (St. Louis, MO). Mass spectrometry (MS) grade trypsin protease, Spectra™ Multicolor Broad Range Protein Ladder (10–260 kDa), and high-performance liquid chromatography (HPLC) grade solvents used in the studies were purchased from Thermo Scientific™ Pierce™ (Rockford, IL). RP-18 (5 µm) HPLC cartridge, LiChroCART® 250-4 LiChrospher® WP 300, and Millipore ZipTip® C₁₈ pipette tips were purchased from Merck Millipore (Billerica, MA). Resource S (1 mL) column was purchased from GE Healthcare (Stockholm, Sweden).

Isolation of the major toxins from *Naja naja* venom. The isolation of the toxins was conducted on sequential fractionation by two different types of chromatography using a Shimadzu LC-20AD High Performance Liquid Chromatography system (Kyoto, Japan).

Fractionation of venom using Resource S ion-exchange chromatography. Five-milligram lyophilized venom was subjected to cation-exchange chromatography using a Resource S column preequilibrated with 20 mM 2-(N-morpholino) ethanesulfonic acid (MES), pH 6.0 as eluent A. Elution was achieved with 0.8 M sodium chloride in 20 mM MES, pH 6.0 as eluent B, using a linear gradient flow of 0–30% B from 5 to 40 minutes followed by 30–100% B from 40 to 55 minutes. The flow rate was set at 1.0 mL/minutes, and the fractions were collected manually at absorbance of 280 nm.

Purification of venom toxins using reverse-phase chromatography. The concentrated fractions collected manually from cation-exchange high-performance liquid chromatography were buffer exchanged and subjected to further purification by reverse-phase high-performance liquid chromatography (RP-HPLC) with a C-18 column (5-µm pore size). The column was preequilibrated with 0.1% trifluoroacetic acid (TFA) in water and eluted with 0.1% TFA in acetonitrile, labeled B, using the following gradient established for venom fractionation in the laboratory²: 5% B for 10 minutes, 5–15% B over 20 minutes, 15–45% B over 120 minutes, and 45–70% B over 20 minutes. The elution rate was set at 1 mL/min. Protein fractions were collected manually at absorbance 215 nm, subsequently lyophilized and stored at –20°C until use.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis. The concentrated fractions obtained from RP-HPLC were reconstituted in ultrapure water and an aliquot was subjected to 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing condition as described by Laemmli.²¹ Spectra Multicolor Broad Range Protein Ladder (10–260 kDa) (Thermo Scientific Pierce) was used to calibrate the molecular masses of protein bands.

In-gel tryptic digestion of protein. Protein bands of interest were excised from Commassie Brilliant Blue-stained SDS-PAGE gel and subjected to reduction with DTT, alkylation with iodoacetamide, and in-gel digestion with MS grade trypsin protease according to the manufacturer's protocol (Thermo Scientific Pierce). The tryptic digested peptides were desalted with Millipore ZipTip C₁₈ pipette tips (Merck) according to the manufacturer's protocol to enhance the performance of MS.

MS analysis. The tryptic digested peptides (0.5 µL) and 0.5 µL α-cyano-4-hydroxycinnamic acid matrix were mixed and spotted on OPTI-TOF™ LC/MALDI insert plate (123 mm × 81 mm). MALDI-TOF/TOF (matrix-assisted laser desorption/ionization time-of-flight) was performed using AB SCIEX 5800 Plus Analyzer (Framingham, MA) equipped with a neodymium:

yttrium–aluminum–garnet laser (laser wavelength was 355 nm). The MALDI-TOF/TOF calibration mixtures (AB SCIEX) were used to calibrate the spectrum to a mass tolerance within 10 ppm. For MS mode, peptides mass maps were acquired in positive reflection mode and 800–4,000 m/z mass range were used with 100 laser shots per spectrum. The MS/MS peak detection criteria used were a minimum signal-to-noise ratio of 100. The raw mass spectra acquired were exported to AB SCIEX ProteinPilot™ Software search against all nonredundant National Center for Biotechnology Information Serpentes database (taxid: 8570, Serpentes). MS peak filter mass range 800–4,000 m/z was applied. Precursor and fragment mass tolerances were set to 100 ppm and 0.2 Da, respectively, and allowing one missed cleavage. Oxidation (M) was set as a variable modification, and carbamidomethylation (C) was set as a fixed modification. The protein score intervals (confidence interval) above 95% were considered as confident identification.

Estimation of the relative protein abundances of venom toxins. The relative abundance of individual protein fraction from ion-exchange HPLC or RP-HPLC was estimated based on the ratio of peak area measured using Shimadzu LCsolution Software. The total area under the curve for each chromatogram (ion exchange or RP) was represented as 100%. The final abundance of a purified toxin was the product of multiplying the percentage of the peak areas implicated based on its sequential elution profiles using the two chromatographic columns.

Enzymatic assay for phospholipases A₂. The enzymatic activity of phospholipases (PLA₂) was tested on egg yolk substrate using an acidimetric method described by Tan and Tan.²² The activity of the enzyme was expressed in micromoles of fatty acid released/min/mg enzyme.

Determination of lethality of *N. naja* venom and its purified toxins. The determination of intravenous (IV) and subcutaneous (SC) median lethal dose for venom or toxin was modified from an *in vivo* assay reported recently from the same laboratory.²³ The venom or toxins were injected IV (via tail caudal vein) or SC (via loose skin over the neck) into albino mice of ICR strain (20–25 g) at appropriate doses ($N = 4$ per dose). The mice were allowed free access to food and water *ad libitum*, and the survival ratio of mice at each dose was recorded at 48 hours post injection. Median lethal doses (LD₅₀) were then estimated using the probit analysis method.²⁴

Neutralization of *N. naja* venom and its purified toxins through incubation with antivenoms. This was adapted from the venom/toxin–antivenom immunocomplexation approach reported from the same laboratory.²⁵ Venom or toxin with a challenge dose of $5 \times \text{LD}_{50}$ in 50 μL saline was preincubated at 37°C for 30 minutes with various concentrations of antivenom to give a total volume of 200 μL . The mixture was then injected intravenously into the mice ($N = 4$ per dose). The mice were allowed free access to food and water *ad libitum*, and the ratio of survival was recorded at 24 hours postinjection. Neutralizing capacity was expressed as ED₅₀ defined as the amount of reconstituted antivenom that gives 50% survival in the venom-challenged animals. If 200 μL of reconstituted antivenom failed to give full protection for the mice, a lower challenge dose ($2.5 \times$ or $1.5 \times \text{LD}_{50}$) was used. Neutralization capacity was also expressed in term of “neutralization potency” (P, the amount of venom that is completely neutralized by a unit volume of antivenom) calculated according to Morais and others.²⁶ The neutralization potency

is a more direct indicator of antivenom neutralizing capacity and is theoretically unaffected by the number of LD₅₀ in the challenge dose.

In vivo challenge-rescue experiment in mice. To confirm the *in vivo* protective capacity of the VPAV against *N. naja* venom, a challenge-rescue experiment was conducted. ICR mice (20–25 g, $N = 4$) were subcutaneously inoculated with five times subcutaneous median lethal dose ($5 \times \text{SC LD}_{50}$) of *N. naja* venom and the neurological signs and symptoms were monitored.²⁷ In a separate experiment, 200 μL of the antivenom was intravenously administered to envenomed mice (20–25 g, $N = 4$, inoculated subcutaneously with $5 \times \text{LD}_{50}$ of the venom) upon the onset of posterior limb paralysis. All mice were observed for 24 hours for signs of deterioration or recovery. They were allowed free access to food and water *ad libitum* throughout the study period.

RESULTS

Isolation of the major toxins from the venom of Pakistani *N. naja* venom. Using Resource S cation-exchange chromatography, the Pakistani *N. naja* venom was fractionated into 11 peaks, of which the five major peaks constituted 80% of the total area of chromatogram (Figure 1). These major peaks were assigned as Fractions 1, 2, 3, 4, and 5 and were further fractionated by RP-HPLC individually over 180 minutes (Figure 2). Fraction 1 that contained acidic proteins was resolved into two peaks, F1a and F1b, successively eluted between 75 and 80 minutes. F1a and F1b shared approximately equal abundance. Fraction 2 was resolved into one main peak F2a (comprising > 90% profile area of the RP-HPLC) at 80 minutes and several minor peaks throughout the course of elution. Fractions 3 and 4 on the cation-exchange chromatogram appeared to contain overlapping proteins. They showed similar elution pattern on RP-HPLC. The major peaks identified include peaks F3a and F3b from Fraction 3, and F4a and F4b from Fraction 4, respectively (Figure 2). Of note, F3a and F3b shared similar elution times as with F4a and F4b, that is, 50 minutes and 75 minutes, respectively. Fraction 5 yielded one major peak, F5a eluted at 100 minutes; in addition to a few minor ones (< 5% profile area). The purity

FIGURE 1. Cation-exchange high-performance liquid chromatography of *Naja naja* (Pakistan) venom. Lyophilized venom (5 mg) was subjected to Resource S (1 mL column) liquid chromatography. A multistep linear gradient of 0.8 M sodium chloride (NaCl) in 20 mM 2-(N-morpholino)ethanesulfonic acid, pH 6.0 was used for elution of the venom proteins (NaCl gradient: 0–30% from 5 to 40 minutes followed by 30–100% from 40 to 55 minutes).

FIGURE 2. Purification of *Naja naja* (Pakistan) venom toxins. C_{18} reverse-phase high-performance liquid chromatography (RP-HPLC) of the five major fractions collected from cation-exchange liquid chromatography of the whole venom: (A) Fraction 1; (B) Fraction 2; (C) Fraction 3; (D) Fraction 4; (E) Fraction 5. The column was preequilibrated with 0.1% trifluoroacetic acid (TFA) in water as Eluent A and eluted with 0.1% TFA in acetonitrile as Eluent B using the following gradient: 5% B for 10 minutes, 5–15% B over 20 minutes, 15–45% B over 120 minutes, and 45–70% B over 20 minutes. (F) Sodium dodecyl sulfate polyacrylamide gel electrophoresis of the *N. naja* (Pakistan) venom toxins purified from the RP-HPLC.

of these major RP-HPLC fractions, labeled F1a–F5a, were verified on reducing SDS-PAGE to reveal a single homogeneous band each (7–14 kDa), except for F2 where an additional faint band at 17 kDa was observed, indicating the presence of small amount of contaminant. The purified proteins were identified and validated using MALDI-TOF-TOF after in gel tryptic digestion. All proteins were identified at significant protein scores, with sequence coverage between 36% and 93% mapped to homologous isoforms of *Naja* species available in the database (Table 1). In brief, F1a and F1b are both acidic PLA_2 . F2a, F3b, and F4b are long α -neurotoxins (LNTX), whereas F3a and F4a are both short α -neurotoxins (SNTX). From the elution time as well as matched peptides obtained from MS, we deduce that F3a is identical to F4a, and F3b is identical to F4b. F5a, on the other hand, is a cytotoxin (CTX) homologue.

Together, these six main toxins account for about 80% of the total abundance of venom proteins. The two acidic PLA_2 , termed in this study as Nn-P PLA_2 -1 and PLA_2 -2, respectively, accounts for 5.0% and 7.3% of the total venom protein abundance, respectively. The short postsynaptic NTX, labeled as Nn-P SNTX-1, accounts for 8.0%, whereas the two long postsynaptic NTX, labeled as Nn-P LNTX-1 and Nn-P LNTX-2 account for 6.1% and 22.2% of the total abundance, respectively. The CTX, Nn-P CTX-1, accounts for 31.3% of the total abundance.

Neutralization of the Pakistani *N. naja* venom by antivenoms.

The intravenous and subcutaneous LD_{50} values for Pakistani *N. naja* in mice were 0.22 μ g/g and 0.60 μ g/g, respectively.

The three antivenoms tested, that is, VPAV, Taiwan neuro bivalent antivenom (NBAV) and NKMAV neuro bivalent antivenom neutralized the lethal effect of the venom at varying degree: NKMAV was apparently more potent with a potency value of 1.11 mg/mL, 2- to 3-fold higher than that of VPAV and NBAV. Table 2 shows the details of neutralization results for the three antivenoms used against the venom.

Major toxins of Pakistani *N. naja* venom: biological and lethal activities, neutralization by antivenoms.

The lethal activity of the purified toxins was tested in mice via intravenous route and the results were shown in Table 3. The SNTX (Nn-P SNTX) has the lowest LD_{50} (the most lethal) value of 0.07 μ g/g, whereas the two LNTXs, Nn-P LNTX-1 and Nn-P LNTX-2, have LD_{50} values of 0.18 and 0.11 μ g/g, respectively. The cytotoxin (Nn-P CTX-1) demonstrated a much higher LD_{50} value of 1.6 μ g/g. In contrast, both the acidic PLA_2 s (Nn-P PLA_2 -1 and Nn-p PLA_2 -2) were not lethal in mice even at 2.0 μ g/g, a dose that is 10 times the LD_{50} of the crude venom. Mice injected with the PLA_2 s did not show remarkable altered behavior or signs of toxicity. The two PLA_2 s, nonetheless, exhibit strong phospholipase A activity (1,822 μ mol of fatty acid released/min/mg and 1,223 μ mol of fatty acid released/min/mg, respectively).

The capability of the three antivenoms to neutralize the purified major lethal toxins was examined in mice (Table 3). VPAV, NKMAV, and TBAV neutralized each toxin at different degree of effectiveness. In general, the neutralization potency was consistently low (potency values of 0.03–0.04 mg/mL)

TABLE 1
Identification of the toxins purified from *Naja naja* (Pakistan) venom by MALDI TOF-TOF and their respective protein abundances

Protein fractions	Protein/protein families	MH ⁺	Peptide sequence	Accession no. (species)	Protein score*	% Venom protein abundance
F1a	Acidic phospholipase A ₂	812.4211	NLYQFKN	P15445 (<i>N. naja</i>)	89	5
		1842.6768	RSWWDFADYGCYCGRG			
		1188.5328	RGGSGTPVDDLDR			
		1157.5311	KISGCWPYFKT			
		1697.6881	KTYSYEC SQGLTCKG			
F1b	Acidic phospholipase A ₂	1684.5903	KGDNNACAASVCDCLRL	P15445 (<i>N. naja</i>)	120	7.3
		812.4546	NLYQFKN			
		1842.7264	RSWWDFADYGCYCGRG			
		1188.5758	RGGSGTPVDDLDR			
		1157.5706	KISGCWPYFKT			
F2a	Long neurotoxin	1697.7422	KTYSYEC SQGLTCKG	P01391 (<i>Naja kaouthia</i>)	145	6.1
		1684.6503	KGDNNACAASVCDCLRL			
		1391.6636	RVDLGCAATCPTVKT			
F3a	Short neurotoxin	2370.0068	KTGVDIQCSTDNCNPFPTKR	P01427 (<i>Naja oxiana</i>)	104	7.1
		1754.8026	LECHNQSSQPPTTKT			
		1014.4738	KKWWS DHRG			
F3b	Long neurotoxin	1316.6473	KVKPGVNLNCCRT	P25668 (<i>N. naja</i>)	116	18.7
		1181.5924	RCFITPDITSKD			
		1301.5483	KTWCDGFCIRG			
F4a	Short neurotoxin	1575.7821	KRVDLGCAATCPTVRT	P01427 (<i>N. oxiana</i>)	98	0.9
		1014.4817	KKWWS DHRG			
		1316.6479	KVKPGVNLNCCRT			
F4b	Long neurotoxin	1181.6136	RCFITPDITSKD	P25668 (<i>N. naja</i>)	199	3.5
		1301.5679	KTWCDAFCSIRG			
		1575.8035	KTGVDIQCSTDNCNPFPTRK			
F5a	Cytotoxin	817.5400	KLIPLAYKT	P01447 (<i>N. naja</i>)	155	31.3
		872.4276	KMYMVS NKT			
		1104.5548	KRGCIDVCPKN			
		1771.8478	KNSLV LKYECCNTDRC			
		1117.4327	KYECCNTDRCN			

MALDI TOF = matrix-assisted laser desorption/ionization time-of-flight.

*Protein score > 67 is significant ($P < 0.05$).

against Nn-P SNTX-1 (SNTX) for all the three antivenoms. Against Nn-P LNTX-2 (the abundant isoform of long NTX), the potency was highest for NKMAV (potency = 0.50 mg/mL), followed by VPAV (potency = 0.29 mg/mL) and NBAV (potency = 0.18 mg/mL). On the other hand, VPAV neutralized the CTX, Nn-P CTX-1 much more effectively (potency = 0.40 mg/mL) compared with NKMAV (potency = 0.29 mg/mL) and NBAV (potency = 0.21 mg/mL).

In vivo antivenom rescue for experimentally envenomed mice. In experimental envenomation, mice developed posterior limb paralysis within 40 minutes following subcutaneous envenomation. This was accompanied by increasing difficulty in movement and breathing. Complete paralysis and death ensued approximately 120 minutes postenvenomation. In mice injected with VPAV intravenously upon the onset of posterior limb paralysis, neurotoxic death was prevented. The reversibility of neurotoxicity was, however, not immediate, and the mice recovered slowly over the next few hours. In average, the mice were able to move freely and feed

spontaneously approximately 9–10 hours after antivenom rescue (Table 4).

DISCUSSION

Venoms of Asiatic and African cobras (*Naja* sp.) comprise of two important toxin families: three-finger toxins (3FTx) and PLA₂.^{28,29} Within the 3FTx family, there are at least two toxin types with different tissue targets and toxic activities, that is, the SNTX and LNTXs, which block postsynaptic nicotinic receptors, and CTX (also known as cardiotoxins), which exhibit cytolytic activity. Earlier, compositional variations of cobra venoms have been reported and attributed to factors such as differences in the biogeographical distribution of the species. This is a phenomenon relevant to *N. naja*, a species extensively distributes throughout the Indian and Indo-Pakistan subcontinents.^{5,30} It is intriguing to note that the Pakistani *N. naja* venom has a low LD₅₀ (0.22 µg/g), a value that reflects high lethality of the venom and is comparable to

TABLE 2
Neutralization of *Naja naja* (Pakistan) venom by cobra antivenoms

Venom	VINS polyvalent antivenom (India)				<i>Naja kaouthia</i> monovalent antivenom (Thailand)			Neuro bivalent antivenom (Taiwan)		
	IV LD ₅₀ (µg/g)	ED ₅₀ (µL/challenge dose)	ED ₅₀ (mg/mL)	Potency (mg/mL)	ED ₅₀ (µL/challenge dose)	ED ₅₀ (mg/mL)	Potency (mg/mL)	ED ₅₀ (µL/challenge dose)	ED ₅₀ (mg/mL)	Potency (mg/mL)
<i>N. naja</i> (Pakistan)	0.22 (0.12–0.40)	32.77/ 5.0 LD ₅₀	0.77 (0.69–0.85)	0.61	18.00/ 5.0 LD ₅₀	1.39 (1.25–1.55)	1.11	75.00/ 5.0 LD ₅₀	0.34 (0.32–0.35)	0.27

ED₅₀ = dose at which 50% of mice survived; IV = intravenous; LD₅₀ = median lethal dose.

TABLE 3
Neutralization of *Naja naja* (Pakistan) venom toxins by cobra antivenoms

Toxin	VPAV			NKMAV			NBAV		
	IV LD ₅₀ (µg/g)	ED ₅₀ (µL/challenge dose)	ED ₅₀ (mg/mL)	Potency (mg/mL)	ED ₅₀ (µL/challenge dose)	ED ₅₀ (mg/mL)	Potency (mg/mL)	ED ₅₀ (µL/challenge dose)	Potency (mg/mL)
F1a	≥ 2.0	ND	—	—	ND	ND	—	—	—
F1b	≥ 2.0	ND	—	—	ND	ND	—	—	—
F2a	0.18 (0.15–0.21)	16.20/2.5 LD ₅₀	0.63 (0.45–0.87)	0.38	ND	ND	—	—	—
F3a	0.07 (0.06–0.08)	85.00/2.5 LD ₅₀	0.047 (0.045–0.05)	0.028	70.00/2.5 LD ₅₀	0.058 (0.052–0.064)	0.035	58.37/2.5 LD ₅₀	0.041
F3b	0.11 (0.10–0.12)	14.13/2.5 LD ₅₀	0.48 (0.33–0.60)	0.29	7.59/2.5 LD ₅₀	0.83 (0.58–1.20)	0.50	20.67/2.5 LD ₅₀	0.18
F5a	1.566 (1.23–1.92)	44.00/1.5 LD ₅₀	1.23 (1.18–1.28)	0.41	62.00/1.5 LD ₅₀	0.87 (0.82–0.93)	0.29	85.00/1.5 LD ₅₀	0.21

CTX = cytotoxin; ED₅₀ = dose at which 50% of mice survived; LD₅₀ = median lethal dose; NBAV = neuro bivalent antivenom (Taiwan); ND = not determined; NKMAV = *Naja kaouthia* monovalent antivenom (Thailand); NTX = neurotoxin; PLA₂ = phospholipase A₂; VPAV = VINS polyvalent antivenom (India).

TABLE 4

Onset of early posterior limb paralysis in envenomed mice (20–25 g, *N* = 4) and the time to recovery achieved with the injection of VINS polyvalent antivenom (IV 200 µL)

Time (minutes) postvenomation	Onset of posterior limb paralysis	Partial recovery (weak, slow limb movement)	Complete recovery (able to move and feed freely)
25	1	—	—
35	2, 3, 4	—	—
55	—	—	—
75	—	—	—
95	—	—	—
240	—	1	—
280	—	3, 4	—
300	—	2	—
540	—	—	1
600	—	—	2, 3, 4

IV = intravenous. Numbers 1–4 represent the mice under observation.

that of the Thai monocled cobra (*N. kaouthia*, 0.20 µg/g)² and *Naja philippinensis* (0.18 µg/g).³¹ Surprisingly, the venoms of *N. naja* from India and Sri Lanka were noted to be less neurotoxic with higher LD₅₀ values,³¹ approximately 5–10 times that of the Pakistani species revealed in this study. This is supported by the venom profiling where α-NTXs (with LD₅₀ values of 0.07–0.18 µg/g) made up nearly 40% abundance of the Pakistani *N. naja* venom, comparable to that found in the Thai monocled cobra. On the other hand, the venoms from the Indian and Sri Lankan species were known to contain much lesser α-NTXs (3–6%)¹¹ while being dominated by CTXs (higher LD₅₀ value > 1 µg/g). This phenomenon has great implication on the management practice of *N. naja* envenomation in South Asia, as imported antivenoms used in Sri Lanka, Bangladesh, Pakistan, and Nepal basically are sourced from India where most of the products are raised against snakes from a single source in Tamil Nadu (southeastern India). This poses a question on the suitability of the use of Indian antivenom in Pakistan when venom variations between the two regions are found to be remarkable. In Pakistan, the two main types of antivenoms available for treatment of snakebite include a local product, which is a liquid polyvalent antivenom against the Big Four (from Pakistan) manufactured by the National Institutes of Health Islamabad (Pakistan), and VPAV, an Indian product that is used widely in areas where the local liquid antivenom is not available. Earlier, Ali and others,¹² showed that the other Indian antivenom, Bharat polyvalent antivenom (also produced using the Big Four venoms from India) was totally ineffective in neutralizing the neurotoxic effect of Pakistani *N. naja* venom. This might be partly due to the inferior quality of the said antivenom, for instance, in terms of its lower protein (antibody) content and potency, as shown in another report.³¹ Results from the current study on the moderate efficacy of VPAV indicate that there is similar toxin antigenicity in both the venoms from Indian and Pakistani species, despite the variation noted in the relative abundance of their NTXs. As we extended the cross-neutralization study involving heterologous antivenoms indicated for *N. kaouthia* (NKMAV) and *N. atra* (NBAV), it is evident that NKMAV, with its relatively higher potency (twice that of VPAV), is perhaps the antivenom with the right formulation to neutralize venom that has high NTXs content, in particular the LNTX subtypes that are abundant in the venoms of Thai *N. kaouthia* and Pakistani *N. naja*. The Taiwanese product, NBAV, was the least effective in this

study although *N. atra* (Taiwan) venom is known to contain a substantial amount of SNTXs (but not LNTXs).¹⁸ This implies the possibility that antivenom produced against predominantly SNTXs would have limitation in neutralizing venom with abundant LNTXs. This was further elucidated in the neutralization study of individual toxins.

The findings on the toxicity of individual toxin verified that both the short and LNTXs are the principal lethal components in the Pakistani *N. naja* venom. The two acidic PLA₂s, however, do not possess significant lethal activity upon envenoming despite their high catalytic (enzymatic) activities, consistent with a recent report on the Indian *N. naja* venom.³² The IV LD₅₀ values of the two NTX subtypes are in agreement with findings for other elapid species.^{19,25} Indeed, these are highly potent toxins that are responsible for the rapid onset of neuromuscular paralysis and death in most elapid envenomations, via the blockade of postsynaptic nicotinic cholinergic receptors.³³ Neutralization of these toxins is therefore essential to halt or reverse the venom-induced neurotoxicity. NKMAV was able to neutralize LNTX most potently among the three antivenoms, presumably due to the high content of LNTX in the immunogen (Thai *N. kaouthia* venom) used in its production. In contrast, LNTXs were less abundant in the venoms of Indian *N. naja* and Taiwanese *N. atra*. Of note, all three antivenoms show very weak neutralization against the SNTX. Ironically, this was seen with NBAV, which was thought to be able to perform better on SNTX, as one of its immunogens, *N. atra* venom contains a higher share of SNTXs.¹⁸ The findings are consistent with previous studies that revealed weak neutralization capacity of antivenoms toward SNTXs.^{19,25} The findings also reflect the difficulty in raising immunoglobulins with sufficiently good reactivity toward SNTXs of elapid species. This could be due to the small molecular size and short peptide sequence of SNTX that limit its immunogenicity in host animals.

The isolated CTX/cardiotoxin has a much higher IV LD₅₀ value (generally > 1.0 µg/g). Considering the whole venom has a rather low LD₅₀ value and a relatively smaller amount of CTX, it is possible that the toxin plays a relatively minor role in the lethality of the venom. In addition, the bioavailability of CTX when injected intramuscularly is lower compared with that of NTXs.³⁴ The CTX, however, is involved in cell death and tissue damages, and thus is likely to contribute to the crippling necrotic activity associated with cobra bites. Of the three antivenoms tested, the highest efficacy shown by VPAV in neutralizing CTX is consistent with the composition of Indian *N. naja* venom (the immunogen) that contains predominantly CTXs.¹¹ Both the Thai *N. kaouthia* and Taiwanese *N. atra* venoms contain lower abundance of CTXs compare with venom of the Indian *N. naja*.^{2,18}

Although NKMAV appears to be the most effective in neutralizing the venom lethality of Pakistani *N. naja*, it is not practical for the Thai antivenom to be proposed for use in Pakistan, partly because of cost and logistic factors. Where relevant, VPAV was chosen in this study to test for in vivo protection in an experimental envenoming model. The early neurotoxic sign that is, posterior limb paralysis was used to indicate antivenom treatment of "rescue." Mice that antivenom rescue was delayed (up to 30–60 minutes after the onset of posterior limb paralysis) generally did not survive the envenomation (data not shown). The observation high-

lights the importance of early commencement of antivenom therapy—although in real situation, antivenom is only indicated upon the onset of the very first sign indicating systemic toxicity (typically ptosis in cobra bites), since not all snakebites result in systemic envenomation, while antivenom itself is a great risk for anaphylaxis.³⁵ Mice that were rescued in this study, however, took a long time (close to 10 hours) after the antivenom (VPAV) injection to fully recover from paralysis and muscle weakness. This was made in comparison to mice envenomed with *N. kaouthia* venom and rescued with NKMAV in another study (unpublished), where full recovery was observed within 2–4 hours. The observation illustrated that the reversibility of venom-induced neurotoxicity likely involves complex in vivo mechanisms, where toxins absorbed progressively from the inoculation site are to be sequestered and eliminated by circulating antivenom, provided the antivenom is effective and sufficient in amount.³⁶ In addition, based on the VPAV potency of 0.6 mg/mL, assuming that the venom inoculated subcutaneously (60–80 µg per mouse) was fully absorbed, it would require approximately 100–130 µL of the antivenom for neutralization; hence, the VPAV rescue dose given (200 µL) was considered optimal. Nonetheless, the slow progress in recovery indicated that a higher dose or repeated dosing of this antivenom may be required in vivo to hasten complete recovery. This is a major limitation that concerns the use of Indian antivenom for cobra envenoming in Pakistan.

CONCLUSION

The study reveals that the Pakistani *N. naja* venom varies from what is known for the Indian and Sri Lankan species. The venom is distinctly more neurotoxic with a lower LD₅₀ value. The findings also demonstrate the merits and limitations of three commercial antivenoms, including a widely distributed Indian product, in neutralizing the venom with correlations to the composition of principal toxins in the venom. Of note, effective neutralization for Pakistani *N. naja* venom requires an antivenom that has high potency against the LNTXs, an approach that may be achieved by including a venom with abundant LNTXs as immunogen. Meanwhile, further research is needed to look into the limitation on antivenom neutralization against the challenging SNTX. Presumably, in addition to the existing low dose, low volume, multi-site immunization protocol used by some producers,³⁷ cross-linking of venom proteins,³⁸ and enrichment of SNTX in the immunogen mixture may serve to boost the immunogenicity of the toxin, thereby broadening the neutralization spectrum and enhancing the efficacy of cobra antivenoms. It is our hope that the insights provided could be useful for the optimization of antivenom formulation and treatment in the future.

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