Lethal toxic Dose (i.p LD_{50}), total protein contents and comparative hemolytic potential of (^{99m}Tc labeled & non-labeled) *Naja naja karachiensis* venom

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Abstract: Recent recognition about snake bite envenomation on June, 2017 as neglected tropical disease under category-A by World Health Organization advocated again its undeniable importance. Present circumstances reasoned to work on a neglected subspecies of *Naja naja*, *i.e.*, *Naja naja karachiensis* (*N. n. karachensis*) has been documented for frequent deaths in Pakistan. In this study median lethal toxic dose (LD_{50}) was determined intraperitoneally in Swiss albino mice and was found to be 2.0µg/g (2.0mg/kg) equal in potency to *Naja pallida* (red spitting African cobra). Total protein contents (188±0.011µg / 200µg of dry weight) were high enough (94%) to represent an arsenal of proteins. Furthermore, ^{99m}Tc was labeled 99.9% with venom and didn't find to alter hemolytic activity of venom in dose dependent manner at 125µg/ml ($p \wp 0.5$), 250 µg/ml ($p \wp 0.1$) and 500 µg/ml ($p \wp 0.1$) when compared with its crude form. Present work will pave the way for proteomics study in effective production of antidote against specific species of snakes as dare demand of it has been felt since long period of time in Pakistan.

Keywords: Intraperitoneal LD_{50}, *Naja naja karachiensis*, protein contents, hemolytic activity.

INTRODUCTION

The World Health Organization (WHO) once again recognized on June 9th 2017, the burden of snake bite envenomation and enlisted in Neglected Tropical Diseases (NTD) under category A (Chippaux, 2017). It is owing to the brachychronic toxic event along with devastating hazardous effects for both tropical and developing countries disproportionately for the poor particularly in rural areas. Perhaps snake bite envenomation has become a nightmare for the inhabitants of Northern Africa, Latin America, Middle East and other Southern Asian countries like Pakistan and India (Santhosh *et al*., 2013). Numerous annual deaths, physical handicaps and sequela has been reasoned to strengthen the research on toxic snake's venom. *N. n. karachiensis* a neglected sub-species of *Naja naja* among one of the deadly venomous snakes has been reported from southern Punjab province of Pakistan. It has been reported to cause little hemorrhage, pain, necrosis, infected gums, coagulopathies, hematuria, renal damage, hepatic injury and cardio toxicity in the victims post envenomation (Asad *et al*., 2014a; Asad *et al*., 2014b). Pakistani *Naja naja* has been described recently in detail for its proteome picture to highlight its various components, while inorganic (metallic & non-metallic) constituents were reported first of all by Asad *et al.*, in 2017 using ICP-OES analytical technique (Wong *et al*., 2017; Asad *et al*., 2017). Nevertheless, impact of lethal potency (LD_{50}) to compare the actual toxic properties of various venoms can't be overlooked thus subjected for this work (Oukkache *et al*., 2014).

Since several decades radio tracers have been used extensively as a diagnostic tool in the field of nuclear medicine after tagging with various radiopharmaceuticals ($\wp$80%). Like other compounds (labeled with radio isotopes) snake venoms have been placed on top priorities due to their frequent awful consequences. Technetium-99m is one of the prominent isotopes (140eV, 6h $t_{½}$) has been used to map out *in vivo* distribution (bioavailability) and to evaluate several features including kinetic study (Pujatti *et al*., 2005; Asad *et al*., 2015). Pharmacokinetic

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parameters and standard serum therapy protocol professed radio labeling must not alter any toxic property of a venom (Shirmardi *et al*., 2010; Murugesan *et al*., 1999). Therefore, in this work an effort was made to evaluate hemolytic potential of crude and radio labeled venom to assure labeling didn't affect any biological activity to depth insights unrevealed mechanism of toxicity and to assist in future specific anti-venom production as dare need of it has been realized in Pakistan (Pujatti *et al*., 2005; Chippaux, 2017; Ali *et al*., 2013).

MATERIAL AND METHODS

^{99m}Mo/ ^{99m}Tc generator and other chemicals

Molybdenum-technetium (^{99m}Mo/^{99m}Tc) generator was provided by Isotope production unit, PINSTECH, Islamabad, Pakistan. Rest of all the chemicals were purchased from Sigma-Aldrich unless and otherwise specified.

Snake venom collection

Pakistani cobra snakes (*N. n. karachiensis*) were gathered from Cholistan desert (Punjab, Pakistan). Snake venom was collected by compressing the glands below their eyes and mixed with 100ml deionized water. Subsequently, it was subjected to the centrifugation (10,000 rpm) for a period of 1h and ultimately lyophilized. It was reconstituted in saline before use (Asad *et al*., 2013; Asad *et al*., 2014c).

Laboratory animals

Male Swiss albino mice (20±5g) were gifted by Dr. Shahzad Hussain (Veterinarian, UVAS, Lahore, Pakistan). All animals were acclimatized in the laboratory conditions by providing their respective standards for chow, water and light.

Ethical clearance

Experimental work was conducted by following the *Helsinki* declaration, after getting permission (institutional animal ethical committee & volunteers consent for blood donation) with reference to the letter # (admin/432/27/4/2013/MINAR/Multan).

Intraperitonial lethal toxic dose of Pakistani cobra venom (LD_{50})

Intraperitoneal Lethal Toxic Dose (LD_{50}) about 50% population of *N. n. karachiensis* venom was calculated by following the procedures adopted by Meier and Theakston (1986). Additional guidelines were also considered as followed by Aird and Kaiser (1985). LD_{50} is the minimum amount of cobra venom responsible for 50% deaths in an unlimited time in mice.

Briefly, three different doses (D) of venom (5, 10 & 25 µg/g, 0.2ml) were given to the Swiss albino mice via intraperitoneal (i.p) route. For each single dose two mice were used and mean survival time (T) was determined, however, average time was considered between injection and death interval in minutes. A curve between D and D/T was constructed and lethal toxic dose was calculated where regression line intersected the y axis of the plot (Aird and Kaiser, 1985; Meier and Theakston, 1986; Roy, 2011).

Protein concentration determination

Protein concentration in the *N. n. karachiensis* venom was determined via Micro BCA protein kit (#23235) method supplied by Thermo Scientific™. Bovine serum albumin (BSA) was used in concentration ranges from 0.5 to 200 µg/ml to construct standard curve. Briefly, equal volume (0.15ml, 37°C) of working reagents was incubated with venom for 120min in 96 well plate. Subsequently, at 25°C absorbance (562nm) was recorded via Tecan infinite M 200pro micro plate reader equipped with Magellan 7 software. Protein concentration in venom sample (0.2 mg/0.15ml) was estimated with the help of standard curve of BSA (Brown *et al*., 1989; Smith *et al*., 1985; Kessler and Fanestil 1986; Wiechelman *et al*., 1988).

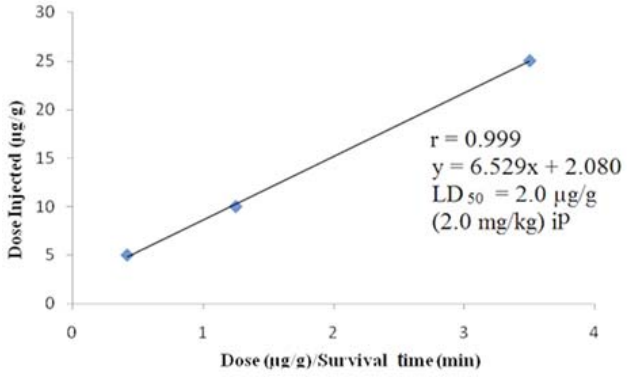

Fig. 1: LD_{50} determination (*N. n. karachiensis* venom).

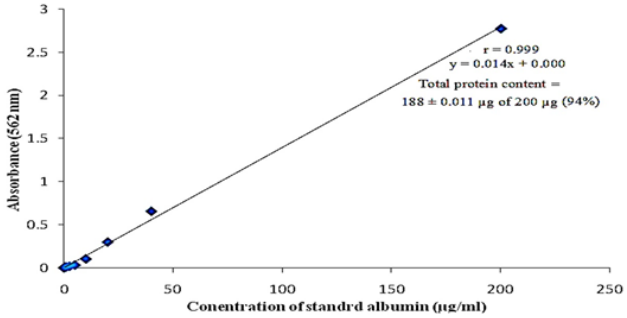

Fig. 2: Protein concentration determination in *N. n. karachiensis* venom via bovine serum albumin (BSA) standard curve (BCA method).

Radio labeling of venom and percentage yield

Freshly eluted technetium (Na $^{99m}TcO_4$) from ^{99}Mo/^{99m}Tc generator was used to label with *N. n. karachiensis* venom. Acidic solution of stannous chloride dihydrate (100µg) and venom (2mg/ml) was mixed with 55.5 MBq

Table 1: Labeling percentage yield of cobra venom with ^{99m}Tc

Strip #	Counts/30 sec	Percentage activity
1.	440	0.232
2.	185220	97.7
3.	499	0.263
4.	451	0.238
5.	460	0.242
6.	500	0.263
7.	1400	0.738
8.	200	0.105
9.	190	0.100
10.	110	0.058
Total	189470	99.98

Table 2: A comparison of hemolytic tendency between unlabeled and ^{99m}Tc labeled Pakistani cobra (*N. n. karachiensis*) venom.

Observation	Evaluated samples							
	^{99m}Tc-labeled venom (µg/ml)			unlabeled venom (µg/ml)			Hyposaline (0.25%)	Saline (0.9%)
	125	250	500	125	250	500		
Absorbance (Mean ± SEM)	0.075 ± 0.0005	0.082 ± 0.0008	0.106 ± 0.0005	0.075 ± 0.000	0.085 ± 0.001	0.107 ± 0.0005	0.201 ± 0.001	0.052 ± 0.0003
Hemolytic percentage	50.3	55.0	71.1	50.3	57.0	71.8	100	0
Comments	p ℘ 0.5	p ℘ 0.1	p ℘ 0.1	Select to compare	Select to compare	Select to compare	Positive control	Negative Control

(1.5 mCi) radioactivity (^{99m}Tc). Before incubation (5-10 min) pH of the final mixture was adjusted to 6.5 and percentage labeling was confirmed by using chromatographic (TLC) technique. Small volume of labeled venom was applied at the end of a paper strip (1.5 cm× 10 cm) in a small vial having acetone as mobile phase. After development of chromatogram it was dried and cut into 10 segmented. Radioactivity was determined for each and every segment using NaI well type gamma counter (Cap-Ria 16 gamma counter) (Asad *et al*., 2015).

Biological activity (hemolytic activity) of radio labeled and crude venom

Hemolytic activity was assayed to confirm radio labeling procedure didn't alter toxic properties of the venom by adopting the procedure documented by Asad *et al.,* (2014) (Asad *et al*., 2014c; Pujatti *et al*., 2005).

Briefly blood was collected from healthy Rhesus positive volunteers to obtain packed cells volume. Equal volume of 1% human red blood corpuscles (HRBC) were incubated with various concentration of technetium labeled and non-labeled Pakistani cobra venom (0.1, 0.2 & 0.5 mg/ml) in presence of a suitable buffer (PBS, 0.1M, pH 7.4) for a period of half an hour at 37ºC. After centrifugation (1000 rpm for 3 min) absorbance at 540 nm was measured, however, deionized water (+ ve) and saline (-ve) was used as control. Hemolytic activity was determined by using the formula given below.

Percentage of hemolysis = Absorbance of the treated sample / Absorbance of the control × 100.

STATISTICAL ANALYSIS

All numerical values presented here are the mean ± SEM of three replicates and they were calculated by the conventional statistical method. Student *t*-test was applied to compare the results with the standard, while, probability was set at p℘0.1.

RESULTS

The most reliable model to evaluate *N. n. karachiensis* median lethal dose/toxicity (LD_{50}) value was evaluated in mice. LD_{50} was assessed via i.p route and found to be 2.0 µg/g (2.0 mg/kg) after extrapolation of regression line (y =6.529x + 2.080 & r=0.999) when intersected the ordinate scale as shown in fig. 1. Moreover, a straight line equation (y=0.014x+0.000) and standard curve for BSA was obtained with +ve correlation coefficient (r=0.999) as shown in fig. 2. Total protein contents were found to be 188±0.011µg of 200µg of dry weight (1g). Overall 94% protein contents were observed in Pakistani cobra (*N. n. karachiensis*) venom. Cobra venom was labeled first time successfully up to 99.9% with ^{99m}Tc as per detail shown in table 1. Furthermore, it was proved ^{99m}Tc didn't alter hemolytic activity even in dose dependent manner at 125 µg/ml (p ℘ 0.5), 250µg/ml (p ℘ 0.1) and 500 µg/ml of

venom ($p \wp 0.1$) when compared with its un-labeled (crude venom) form as shown in table 2.

DISCUSSION

Prompt immunotherapeutic and symptomatic management is mandatory particularly in serious events caused by deadly venomous Elapidae snakes envenomation. Fight against various Naja species (*N. n. karachiensis*) is a priority public health matter mainly rely on scarce epidemiological data attributed for limited and inappropriate immunogenic mixture (antisera) production (Oukkache *et al*., 2014; Ali *et al*., 2013). As part of the combat, LD_{50} is a crucial step for an appropriate assessment of snake venom toxicity and to choice and establish antisera batch having relevant antidotal capacity. LD_{50} values differ considerably with diversity in geographical locations, individual handling technique(s), targeted animals and their number along with route of venom administration (highest value with subcutaneous route while lowest value with intracerebroventricular route) (Oukkache *et al*., 2014). Indeed cobra venom is a discrete decoction of proteins and its lethal effect attributed to the presence of phospholipase A_2 (PLA_2), alkaline phosphatase (ALP), phosphodiesterase (PDE), 5'-nucleotidase (5'-NA) and three finger toxins (3FTX) predominantly (Wong *et al*., 2017). Intraperitoneal LD_{50} for this venom was observed to be 2.0µg/g (2.0mg/kg). Interestingly, it was found equal to the red spitting African cobra *i.e., Naja pallida* (LD_{50} =2.0mg/kg, i.p). However, it was slightly potent when compared to the *N. n. naja* (i.p LD_{50} =2.8mg/kg) while *Ophiophagus hannah* was revealed more potent (i.p LD_{50} =1.644 mg/kg) to it (Asad *et al*., 2016). It was recorded less potent when compared to the other Naja species *viz.*, *Naja haje* (i.p LD_{50} =0.185mg/kg), *N. n. atra* (i.p LD_{50} =0.62mg/kg), *Naja nigricollis* (i.p LD_{50}=0.44 mg/kg), *Naja melanoleuca* (i.p LD_{50} =0.324 mg/kg), *Naja kaouthia* (i.p LD_{50} =0.225mg/kg) and *Naja nivea* (i.p LD_{50}=0.4 mg/kg) (Asad *et al*., 2016). Before this study i.m. LD_{50} (1.2 mg/Kg) of *N. n. karachiensis* venom was the only documented value, therefore, must be determined for remaining routes of injection (Riaz *et al*., 2015). Protein content (188±0.011µg/200µg of dry weight) for *N. n. karachiensis* venom was found 94% as high enough as reported earlier for colubrid venoms. Indeed snake venom is usually abundant in proteinous content (70% to 90%), therefore, Pakistani cobra venom was professed a goldmine of proteins (Asad *et al*., 2016). On a more practical level, proteomics study about *N. n. karachiensis* venom will be valuable in highlighting intricate phenomenon of envenomation which might be effective in rapid production of specific antidote against *N. n. karachiensis* venom whose dire need has been reported previously in literature (Ali *et al*., 2013). Radio labeling (direct labeling of toxin) has been considered very promising to disclose the mysterious of envenomation and to perform several biological activities along with pharmacokinetics of venom. It is owing to the ease in application with technetium-99m without modifications (blocking or deblocking) in functional groups of venom (toxins) (Asad *et al*., 2015). This study reports for the first time 99.9 % tagging of *N. n. karachiensis* venom with ^{99m}Tc as reported previously for *Scorpaena plumier, Mesobuthus eupeus, N. n. karachiensis* (97.7%) and Crotalus venoms (Pujatti *et al*., 2005; Asad *et al*., 2015; Shirmardi *et al*., 2010). Furthermore, ^{99m}Tc labeled venom was found to pose hemolytic activity similar to the unlabeled venom ($p \wp 0.1$) strongly deduced that labeling procedure didn't mask any toxic of property of *N. n. karachiensis* venom (table 2). Destabilization of HRBCs membranes owing to the PLA_2 via its complex with cell membrane lipids led to the hydrolysis of phospholipids along with liberation of lysophospholipids and free fatty acids. Hyposaline induced hemolysis was observed due to the transient resealing fissures in the HRBCs during swelling process of the cells (Asad *et al*., $2014_{(C)}$). On more practical scale present study will help to expose the mysterious of toxicity and to open several avenues to understand the mechanism of envenomation along with effective production of specific antidote in future.

CONCLUSION

Snake bite envenomation is one of the serious health related issues led to high mortality and morbidity all over the world. *Naja naja karachiensis* is one of the venomous snakes attributed for plenty of deaths in Southern Punjab Pakistan. Its i.p. LD_{50} was found to be 2.0 µg/g (2.0 mg/kg). Total protein contents were recorded 188 ± 0.011 µg / 200 µg of dry weight (94%). Venom was labeled 99.9% with ^{99m}Tc and labeling procedure was not proved to alter toxic biological properties (hemolytic activity). This study will help in effective production of anti-venom against local species of snakes in Pakistan to neutralize envenomation.

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REFERENCES

Aird SD and Kaiser I (1985). Toxicity assays. *Toxicon.*, **23**: 11-13.

Ali SA, Yang DC, Jackson TNW, Undeim EAB, Koludarov I, Wood K, Jones A, Hodgson WC, McCarthy S, Ruder T and Fry BG (2013). Venom proteomic characterization and relative anti- venom

neutralizatio of two medically important Pakistani elapid snakes (*Bungarus sindanus* and *Naja naja*). *J. Proteomics*, **89**: 15-23.

Asad MHHB, Jaeger AK and Hussian I (2017). ICP-OES analysis of *Naja naja karachiensis* venom: Inorganic ions for turning on and off enzymatic actions. *Curr. Sci*, **112**(3): 458-460.

Asad MHHB (2016). Biochemical and radio labeling studies of venom *Naja naja karchiensis* with its neutralization with medicinal plants of Pakistan. PhD thesis submitted at Department of Pharmacy, COMSATS Institute of Information Technology, Abbottabad, Pakistan. pp.30-200.

Asad MHHB, Durr-e-Sabih, Ahmad I, Choudhry BA, Murtaza G and Hussian I (2015). Biodistribution and kinetic studies of technetium-99m labeled *Naja naja karachiensis* venom via gamma scintigraphic and SPECT images. *Pak. J. Pharm. Sci.*, **28**(4): 1233-1238.

Asad MHHB, Murtaza G, Ubaid M, Durr-e-Sabih, Sajjad A, Mehmood R, Mehmood Q, Ansari MM, Karim S, Mehmood Z and Hussain I (2014a). *Naja naja karachiensis* envenomation: Biochemical parameters for cardiac, liver and renal damage along with their neutralization by medicinal plants. *Biomed Res Int.,* Art id **970540**: 1-13.

Asad MHHB, Durr-e-Sabih, Choudary BA, Asad AF, Murtaza G and Hussain I (2014b). Compensatory effects of medicinal plants of Pakistan upon prolongation of coagulation assays induced by *Naja naja karachiensis* bite. *Curr. Sci.*, **106**: 870-873.

Asad MHHB, Durr-e-Sabih, Chaudhary BA, Ahamd I, Hussain MS, Izhar N, Akaml N, Shahzad AH and Hussain I (2014c). Anti-hemolytic (anti-venom) activity of Pakistani medicinal plants upon *Naja naja karachiensis* venom induced hemolysis. *J. Anim. Plant Sci.*, **24**: 1701-1708.

Asad MHHB, Razi MT, Durr-e-Sabih, Saqib QN, Nasim MJ, Murtaza G and Hussain I (2013). Anti-venom potential of Pakistani medicinal plants: Inhibition of anticoagulation activity of *Naja naja karachiensis* toxin. *Curr. Sci.*, **105**: 1419-1424.

Brown RE, Jarvis KL and Hyland KJ (1989). Protein measurement using bicinchoninic acid: elimination of interfering substances. *Anal. Biochem*, **180**: 136-139.

Chippaux JP (2017). Snake bite envenomation turns again into a neglected tropical disease. *J. Venom. Anim. Toxins incl. Trop. Dis.*, **23**: 38.

Kessler R and Fanestil D (1986). Interference by lipids in the determination of protein using bicinchoninic acid. *Anal. Biochem.*, **159**: 138-42.

Meier J and Theakston RDG (1986). Approximate LD_{50} determination of snake venoms using eight to ten experimental animals. *Toxicon*, **24**: 395-410.

Murugesan S, Radha Krishna Murthy K, Noronha OPD and Samuel AM (1999). Tc-99m scorpion venom: Labelling, biodistribution and scintiimaging. *J. Venom. Anim. Toxins.*, **5**: 35-46.

Oukkache N, Jaoudi REI, Ghalim N, Chgoury F, Bouhaouala B, Mdaghri NEI and Sabatier JM (2014). Evaluation of the lethal potency of scorpion and snake venoms and comparision between intraperitoneal and intravenous injection routes. *Toxins*, **6**: 1873-1881.

Pujatti PB, Simal CJR and Santos RGD (2005). Preparation of *Crotalus* venom radiolabeled with technetium-99m as a tool for bio distribution study. *Braz. Arch Biol. Technol.*, **48**: 9-12.

Riaz Z, Zaman MQ, Ullah Z, Habib-ur-Rehman, Yousaf MS, Rabbani I, Khan MI, Hussian MS, Tahir S and Majeed KA (2015). Bio-physiological effects of LD_{50} of crude venom of black Pakistani cobra (*Naja naja karachiensis*) in mice. *J. Anim. Plant. Sci.*, **25**(5): 1344-1348.

Roy A (2011). Structural and functional characterization of Haditoxin, a novel neurotoxin isolated from the venom of *Ophiophagus hannah* (king cobra). PhD thesis submitted at Department of Biological Sciences, National University of Singapore, Singapore, pp.4-50.

Santhosh MS, Hemshekhar M, Sunitha K, Thushara RM, Jnaneshwari S, Kemparaju K and Girish KS (2013). Snake venom induced local toxicities: Plant secondary metabolites as an auxiliary therapy. *Mini. Rev. Med. Chem.*, **13**: 106-123.

Shirmardi SP, Gandomkar M, Shamsaei M, Mirakabadi AZ, Maragheh MG, Shafiei M and Vahidfar N (2010). Preparation and bio distribution study of a 99mTc labeled toxic fraction of Iranian *Mesobuthus eupeus* scorpion venom. *Iran J. Nucl. Med.*, **18**: 37-44.

Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ and Klenk DC (1985). Measurement of protein using bicinchoninic acid. *Anal. Biochem.*, **150**: 76-85.

Wiechelman KJ, Braun RD and Fitzpatrick JD (1988). Investigation of the bicinchoninic acid protein assay: Identification of the groups responsible for color formation. *Anal. Biochem,* **175**: 231-237.

Wong KY, Tan CHT, Tan KY, Naeem QH and Tan NH (2017). Elucidating the biogeographical variation of the venom of *Naja naja* (spectacled cobra) from Pakistan through a venom-decomplexing proteomic study. *J. Proteomics*, ttps://doi.org/10.1016/j.jport.2017.12.012.