VENOM PROTEOMICS, TOXICITY AND CROSS-NEUTRALIZATION OF SAMAR COBRA (*NAJA SAMARENSIS*) FROM THE SOUTHERN PHILIPPINES

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ABSTRACT

Snakebite envenoming by *Naja samarensis*, a medically important species endemic to the southern Philippines, results in neuromuscular paralysis and death from respiratory failure. Antivenom is the definitive treatment, but there is currently no species-specific antivenom for *N. samarensis*. Instead, Philippine Cobra Antivenom (PCAV), raised against *Naja philippinensis* (Philippine Cobra endemic to the northern Philippines), is used empirically to treat envenoming by *N. samarensis* in the south. Yet, the composition and toxicity of *N. samarensis* venom, physicochemical properties, and cross-neutralization capacity of PCAV against *N. samarensis* venom remain understudied. Hence, the present study investigated the venom proteome of *N. samarensis* through reverse-phase high-performance liquid chromatography, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and tandem mass spectrometry (LC-MS/MS). The immunological binding activity of PCAV to *N. samarensis* venom and its protein components was examined with indirect enzyme-linked immunosorbent assay (ELISA) and immunoblotting. The toxicity and neutralization of *N. samarensis* venom and its principal lethal component, i.e., short-chain alpha-neurotoxin (SαNTX) using PCAV, were subsequently investigated in mice. The protein composition of PCAV was then examined using size-exclusion chromatography, SDS-PAGE and LC-MS/MS. The study further assessed the immunoreactivity of PCAV, containing antibodies primarily targeting SαNTX, against venoms of various cobra species, phylogenetically related cobra-like snakes (*Hemachatus haemachatus, Aspidelaps scutatus, and Walterinnesia aegyptia*), and alpha-neurotoxins from selected elapid species. The study identified 31 distinct proteoforms from seven toxin families in the venom proteome of *N. samarensis*.

The three-finger toxin family are the most dominant components (comprising ~90% of total venom proteins), with SαNTX being the primary constituent (74.2%). Other proteins identified were snake venom metalloproteinases, phospholipases A_2, cysteine-rich secretory proteins, venom nerve growth factor, L-amino acid oxidase and vespryn. In ELISA, PCAV demonstrated comparable immunoreactivity toward the venoms of *N. samarensis* and *N. philippinensis*, based on its half-maximum effective concentrations of binding (EC_{50}), 1.22 and 1.63 μg/ml, respectively (p>0.05). Immunoblotting showed that PCAV was able to bind strongly to the venom proteins, including toxins with low molecular mass (<10 kDa). This was further supported by PCAV immunorecognition of *N. samarensis* venom fractions containing the lethal SαNTX. *N. samarensis* venom was highly lethal to mice (intravenous median lethal dose, LD_{50}=0.20 μg/g), attributed to the abundant SαNTX (LD_{50}=0.18 μg/g) in its venom. PCAV was able to cross-neutralize the toxicity of *N. samarensis* venom and its SαNTX *in vivo* with moderate efficacy (neutralization potency=0.17 mg/ml and 0.20 mg/ml, respectively). Physicochemical profiling of PCAV revealed that it is a $F(ab')_2$ antivenom product with an immunoglobulin content of ~80%. PCAV cross-reactivity was moderate toward venoms of major cobra species in Asia and short-neurotoxins from selected Asian species. PCAV cross-reactivity toward venoms of African cobras, other phylogenetically related cobra-like species and long neurotoxins were generally low. The finding implies that *N. samarensis* and *N. philippinensis* SαNTX have unique antigenicity distinct from other cobra species, including those of Asian lineage with SαNTX-dominant venom phenotype. Together, the findings shed light on the composition and toxicity of *N samarensis* venom and provide insights into the use of PCAV in the treatment of *N. samarensis* envenoming.

Keywords: Southern Philippine Cobra, Spitting cobra, Venomics, Alpha-neurotoxins, Immunoreactivity

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ABSTRAK

Envenomasi ular *Naja samarensis*, spesis penting dari segi perubatan endemik di selatan Filipina boleh mengakibatkan kelumpuhan neuromuskular and kematian akibat daripada kegagalan sistem pernafasan. Antivenom (anti bisa ular) merupakan satu-satunya rawatan bagi pembisaan ular, namun pada masa kini tiada antivenom yang specifik untuk envenomasi *N. samarensis*. Sebaliknya, Philippine Cobra Antivenom (PCAV) yang dibuat dengan bisa dari *Naja philippinensis* (ular tedung endemik di Filipina) digunakan secara empirik untuk merawat envenomasi *N. samarensis* di selatan. Namun, komposisi dan ketoksikan *N. samarensis*, sifat fizikokimia dan kapasiti peneutralan PCAV terhadap *N. samarensis* masih belum dikaji. Maka, kajian ini bertujuan untuk menyelidik komposisi proteome *N. samarensis* melalui kromatografi cecair prestasi tinggi fasa terbalik (RP-HPLC), sodium dodecyl sulfat-polyacrylamide gel elecktroforesis (SDS-PAGE) dan spektometri jisim (LC-MS/MS). Asai imunoserapan terangkai enzim (ELISA) oleh PCAV terhadap bisa ular *N. samarensis* and komponen protein dikaji dengan kaedah ELISA dan pemblotan western. Ujian toksisiti dan peneutralisasian bisa ular *N. samarensis* and komponen maut utamanya, iaitu alpha-neurotoxin pendek (SαNTX) mengunakan PCAV di kaji dalam tikus. Komposisi protein PCAV kemudiannya dikaji mengunakan kaedah kromatografi pengecualian saiz, SDS-PAGE dan LC-MS/MS. Kerberkesanan immunoreaktiviti PCAV yang mengandungi antibodi menyasarkan SαNTX terhadap variasi spesis ular tedung dan 'ular seperti ular tedung' yang terhubung secara filogenetik (*Hemachatus haemachatus, Aspidelaps scutatus,* dan *Walterinnesia aegyptia*) dan neurotoksin alpha dari spesis elapid terpilih turut dikaji. Hasil kajian mengenal pasti 31 proteoform berbeza daipada tujuh keluarga

toksin dalam proteome *N. samarensis*. Toksin tiga-jari (3FTx) merupakan kumpulan toksin paling utama (merangkumi ~90% daripada keseluruhan jumlah protein), dan didominasi oleh SαNTX (74.2%). Lain-lain protein yang dikenalpasti ialah, snake venom metalloproteinases, phospholipases A_2, cysteine-rich secretory proteins, venom nerve growth factor, L-amino acid oxidase dan vespryn. Dari hasil kajian ELISA, PCAV menunjukkan kesan immunoreaktivti yang setanding terhadap bisa ular dari *N. samarensis* dan *N. philippinensis* berdasarkan dos efektif median, ED_{50}, 1.22 dan 1.63 µg/ml (p>0.05). Kajian pemblotan western menunjukkan bahawa PCAV mampu mengikat kuat terhadap protein, termasuk toksin yang mempunya jisim molekul rendah (<10 kDa). Ini disokong dengan keberkesanan PCAV dalam mengenalpasti kandungan SαNTX dalam pecahan bisa ular *N. samarensis*. Kajian kemautan menunjukkan *N. samarensis* sangat toksik (intravena dos maut median LD_{50}=0.20 µg/g) yang boleh dikaitkan dengan peratusan SαNTX (LD_{50}=0.18 µg/g) dalam bisa ularnya. PCAV mampu meneutralkan toksiti bisa ular *N. samarensis* dan SαNTX-nya *in vivo* dengan keberkesanan yang sederhana (potensi peneutralan, 0.17-0.20 mg/ml). Profil fizikokimia PCAV menunjukkan bahawa PCAV adalah produk antivenom $F(ab')_2$ dengan kandungan imunoglobulin sebanyak ~80%. Keberkesanan PCAV terhadap bisa ular dari spesis ular tedung di Asia dan neurotoksin pendek daripada spesis Asiatik terpilih adalah sederhana. Secara umumnya, keberkesanan PCAV terhadap bisa ular tedung dari Afrika, spesis lain yang berhubung secara filogenetik dan neurotoksin panjang adalah rendah. Kajian menunjukkan bahawa SαNTX *N. samarensis* dan *N. philippinensis* mempunyai antigenesiti unik yang berbeza dari spesis ular tedung lain termasuk dari keturunan Asia yang didominasi fenotip SαNTX. Pada kesimpulannya, hasil kajian ini mencirikan komposisi dan sifat toksik bisa ular *N. samarensis* dan keberkesanan pengunaan PCAV untuk rawatan envenomasi *N. samarensis*.

Kata kunci: Ular Tedung Filipina Selatan, Ular tedung meludah, Venomik, Neurotoksin alpha, Kereaktifan imun

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS AND ABBREVIATIONS

3FTx	:	Three-finger toxin
3FTxs	:	Three-finger toxins
3FTx-CTX	:	Cytotoxin subtype of three-finger toxin
3FTx-NTX	:	Neurotoxin subtype of three-finger toxin neurotoxins
5′NUC	:	5′ nucleotidase
αNTX	:	Alpha-neurotoxins
αNTXs	:	Alpha-neurotoxins
µg	:	Microgram
µg/g	:	Microgram per gram
µl	:	Microliter
°C	:	Degrees in Celcius
ABC	:	Ammonium bicarbonate
AchE	:	Acetylcholinesterase
ACN	:	Acetonitrile
A. scutatus	:	*Aspidelaps scutatus*
abs	:	Absorbance
ABSmax	:	Maximal absorbance
APS	:	Ammonium persulfate
AUC	:	Area under curve
BCA	:	Bicinchoninic acid
BPP	:	Bradykinin-potentiating peptides
BSA	:	Bovine serum albumin
C. rhodostoma	:	*Calloselasma rhodostoma*
CIOMS	:	Council for International Organization of Medical Science

C.I.	:	Confidence interval
CRiSP	:	Cysteine-rich secretory protein
CTX	:	Cytotoxin/cytotoxin-like homolog
CTXs	:	Cytotoxins/cytotoxin-like homologs
CVF	:	Cobra venom factor
D. vestigiata	:	*Demansia vestigiata*
Dis	:	Disintegrin
D. coronoides	:	*Drysdalia coronoides*
DTT	:	Dithiothreitol
EC_{50}	:	Half maximal effective concentration
ED_{50}	:	Median effective dose
ELISA	:	Enzyme-linked immunosorbent assay
ER50	:	Median effective ratio
Etc.	:	Et cetera
$F(ab')_2$	:	Fragment antigen binding
FDR	:	False discovery rate
Fc	:	Fragment crystallizable region
FA	:	Formic acid
g	:	Gram
h	:	Hour
H. haemachatus	:	*Hemachatus haemachatus*
H. curtus	:	*Hydrophis curtus*
HRP	:	Horseradish peroxidase
HYA	:	Hyaluronidases
IACUC	:	Institutional Animal Care and Use Committee
ICR strain mice	:	Institute of Cancer Research strain mice

IgG	:	Immunoglobulin G
i.v.	:	Intravenous
IAA	:	Iodoacetamide
K_{av}	:	Partition coefficient
kDa	:	Kilodalton
KSPI	:	Kunitz-serine protease inhibitor
LαNTX	:	Long-chain alpha-neurotoxin
LAAO	:	L-amino acid oxidase
L. colubrina	:	*Laticauda colubrina*
LC-MS/MS	:	Tandem mass spectrometry
LD_{50}	:	Median lethal dose
LipA	:	Lysosomal lipase A
logMW	:	Log molecular weight
MALDI	:	Matrix-assisted laser desorption ionization
MEGA	:	Molecular Evolutionary Genetics Analysis
mg/g	:	Milligram/gram
min	:	Minute
ml	:	Milliliter
mM	:	Millimolar
MS/MS	:	Tandem mass spectrometry
MSI	:	Mean spectral intensity
MTLP	:	Muscarinic toxin-like protein
MUSCLE	:	Multiple Sequence Comparison by Log-Expectation

mV	:	Millivolt
MW	:	Molecular weight
n-P	:	Normalized potency
nAChR	:	Nicotinic acetylcholine receptor
N. annulifera	:	*Naja annulifera*
N. atra	:	*Naja atra*
N. haje	:	*Naja haje*
N. kaouthia	:	*Naja kaouthia*
N. karachiensis	:	*Naja karachiensis*
N. katiensis	:	*Naja katiensis*
N. melanoleuca	:	*Naja melanoleuca*
N. mossambica	:	*Naja mossambica*
N. naja	:	*Naja naja*
N. nigricincta nigricincta	:	*Naja nigricincta nigricincta*
N. nigricollis	:	*Naja nigricollis*
N. nivea	:	*Naja nivea*
N. nubiae	:	*Naja nubiae*
N. oxiana	:	*Naja oxiana*
N. pallida	:	*Naja pallida*
N. philippinensis	:	*Naja philippinensis*
N. samarensis	:	*Naja samarensis*
N. senegalensis	:	*Naja senegalensis*
N. siamensis	:	*Naja siamensis*
N. sputatrix	:	*Naja sputatrix*
N. sumatrana	:	*Naja sumatrana*