TOXINOLOGICAL AND PHARMACOLOGICAL CHARACTERIZATION OF SOUTHEAST ASIAN *NAJA KAOUTHIA* (MONOCLED COBRA) VENOM

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FACULTY OF MEDICINE
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ABSTRACT

Snakebite envenomation is a neglected tropical disease and a serious public health problem in many countries in the tropics and subtropics, including Malaysia and Thailand. Antivenom remains as the only definitive treatment for snakebite envenomation; unfortunately, many nations do not have financial and technical resources to produce their own specific snake antivenom. These nations are relying on imported antivenoms that may not be very effective in treating envenomation by local snake species, due to geographical variations in the venom composition. These differences are medically relevant as they can lead to varied envenoming effects and treatment outcome. The monocled cobra (*Naja kaouthia*) is one of the most common dangerous species widely distributed in Indochina, northern Malayan Peninsula, northeastern India and southern China. The *N. kaouthia* venom from Thailand and Malaysia were previously shown to be substantially different in their median lethal doses (LD_{50}); however, the differences in their venom compositions and pharmacological actions have not been elucidated. This present work profiled the venom proteomes of *N. kaouthia* from three different geographical regions, i.e. Malaysia (NK-M), Thailand (NK-T) and Vietnam (NK-V) using reverse-phase HPLC, SDS-PAGE and tandem mass spectrometry. The venom lethality and mechanism of neuromuscular blockade were also investigated *in vivo* using mice and *in vitro* using chick biventer cervicis nerve-muscle (CBCNM) preparation, while the neutralization of venom-induced toxic effects was assessed using Thai *N. kaouthia* Monovalent Antivenom (NKMAV) and/or Neuro Polyvalent Antivenom (NPAV) produced by Thai Red Cross Society. The venom proteome results revealed remarkable biogeographical variations in all three *N. kaouthia* venoms, with three-finger toxin (3FTx) being the most abundant but also the most varied among three venom samples studied. These venoms also exhibit differences in venom lethality and neuromuscular depressant

activity that reflect the proteomic findings, with NK-T being the most lethal and most neurotoxic. Despite the variation in proteome, Thai-produced antivenoms were capable of neutralizing toxic effects of all three venoms with varying degree of effectiveness. The findings suggest that Thai-produced antivenoms could be used for the treatment of *N. kaouthia* bites in Malaysia and Vietnam. However, the recommended antivenom dosage may be tailored and further optimized. This present work also investigated the toxin-specific neutralization by NKMAV to understand why cobra antivenoms are generally of limited neutralization potency (< 2 mg/ml). The principal toxins of NK-T and Malaysian beaked sea snake (*Hydrophis schistosus*, HS-M) were purified and their neutralization by NKMAV and Australian CSL Sea Snake Antivenom (SSAV) were investigated. The neutralization profiles showed the low efficacy of antivenoms against low molecular mass toxins, particularly against the short neurotoxin (SNTX) of both venoms examined. This indicates that the limiting factor in neutralization potency is the poor ability of antivenoms to neutralize SNTX. Nevertheless, the SSAV was still substantially superior to NKMAV in neutralizing SNTX, presumably because the sea snake venom used as an immunogen in SSAV production contains a large amount of SNTX. The toxin-specific neutralization findings suggest that it is possible to improve the efficacy of cobra antivenom by increasing the amount of SNTX in the immunogen.

ABSTRAK

Pembisaan ular adalah satu penyakit terabai tropika dan merupakan satu ancaman kesihatan awam yang dikongsi bersama antara negara-negara tropika dan subtropika, termasuk negara Malaysia dan Thailand. Sehingga hari ini, antibisa ular (*antivenom*) kekal sebagai rawatan muktamad bagi pembisaan ular, akan tetapi, banyak negara masih tidak berupaya untuk menghasilkan *antivenom* yang khusus (spesifik) untuk kepentingan perubatan di negara mereka kerana kekurangan sumber teknikal dan kewangan. Malangnya, negara-negara ini masih perlu bergantung pada *antivenom* import yang berkemungkinan kurang berkesan terhadap bisa ular tempatan, disebabkan oleh perbezaan komposisi bisa ular akibat daripada variasi biogeografi. Hal ini adalah penting kerana perbezaan komposisi bisa akan mengakibatkan kesan pembisaan dan hasil rawatan yang berlainan pada mangsa pembisaan ular. Ular tedung senduk (*Naja kaouthia*) ialah jenis ular bisa yang berleluasa di kawasan Indochina, utara Semenanjung Tanah Melayu, timur laut India dan selatan China. Sebelum ini, perbezaan yang ketara dalam dos maut median (LD_{50}) bisa *N. kaouthia* dari negara Thailand dan Malaysia telah ditunjukkan. Walau bagaimanapun, perbezaan komposisi bisa dan tindakan farmakologi masih belum dijelaskan. Oleh itu, kajian ini memperkenalkan profil proteomik bisa *N. kaouthia* yang berasal dari tiga rantau Asia Tenggara, iaitu Malaysia (NK-M), Thailand (NK-T) dan Vietnam (NK-V), dengan menggunakan RP-HPLC, SDS-PAGE dan spektrometri jisim. Tambahan pula, kemautan bisa dan mekanisme sekatan otot-saraf juga diselidik secara *in vivo* dengan menggunakan tikus mencit makmal dan secara *in vitro* dengan menggunakan persediaan otot-saraf *chick biventer cervicis* (CBCNM). Selain itu, potensi peneutralan *antivenom* terhadap kesan toksik bisa juga diselidik dengan menggunakan *antivenom* monovalen khusus untuk *N. kaouthia* Thailand (NKMAV), buatan *Thai Red Cross Society*. Hasil kajian proteomic menunjukkan bahawa toksin tiga-jari (3FTx) merupakan antara toksin yang paling

banyak diekspreskan dan menunjukkan kepelbagaian antara tiga sampel bisa *N. kaouthia* yang dikaji. Di samping itu, bisa *N. kaouthia* juga menunjukkan perbezaan kemautan dan aktiviti sekatan otot-saraf yang mencerminkan penemuan proteomik, iaitu NK-T adalah antara yang paling maut dan paling neurotoksik. Walaupun variasi proteomik bisa diperhatikan, *antivenom* buatan Thailand (NKMAV) mampu meneutralkan kesan-kesan toksik tiga bisa ular kajian pada pelbagai darjah keberkesanan. Penemuan ini mencadangkan bahawa *antivenom* buatan Thailand boleh digunakan sebagai rawatan pembisaan *N. kaouthia* di negara Malaysia dan Vietnam. Namun demikian, dos *antivenom* yang dicadang perlu dioptimum dengan sewajarnya. Kajian ini juga dilanjutkan untuk menyiasat kekhususan peneutralan *antivenom* terhadap toksin bisa untuk memahami limitasi *antivenom* ular tedung yang sering difahamkan mempunyai potensi peneutralan yang terhad (< 2 mg/ml). Toksin-toksin utama dari NK-T dan ular laut bermuncung asal Malaysia (*Hydrophis schistosus*, HS-M) ditulenkan dan potensi peneutralan oleh NKMAV dan *Australia CSL Sea Snake Antivenom* (SSAV) disiasat. Profil peneutralan menunjukkan *antivenom* berpotensi rendah terhadap semua toksin berjisim rendah, terutamanya terhadap neurotoksin pendek (SNTX) dalam kedua-dua bisa ular kajian. Hal ini menunjukkan faktor pengehad bagi peneutralan bisa adalah disebabkan kekurangupayaan *antivenom* dalam meneutralkan SNTX. Walau bagaimanapun, hasil kajian menunjukkan SSAV lebih unggul berbanding NKMAV dalam peneutralan SNTX. Hal ini mungkin disebabkan sebahagian besar bisa ular laut yang dijadikan imunogen SSAV terdiri daripada SNTX. Penemuan ini mencadangkan bahawa keberkesanan *antivenom* ular tedung dapat dipertingkatkan dengan penambahan kuantiti SNTX sebagai imunogen dalam penghasilan *antivenom*.

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µg	:	microgram
µl	:	microliter
µm	:	micromolar
3FTx	:	three-finger toxin
5'NUC	:	5'nucleotidase
ACh	:	acetylcholine
ACN	:	acetonitrile
BCA	:	bicinchoninic acid
CBCNM	:	chick biventer cervicis nerve-muscle
CCh	:	carbachol
CRISP	:	cysteine-rich secretory protein
CTL	:	c-type lectin
CTX	:	cytotoxin/cardiotoxin
CVF	:	cobra venom factor
d-TC	:	d-tubocurarine
ED_{100}	:	maximal effective dose (µl)
ED_{50}	:	median effective dose (µl)
ER_{50}	:	median effective ratio (mg/ml)
$F(ab')_2$	:	immunoglobulin fragments $F(ab')_2$
Fab	:	immunoglobulin fragments Fab
g	:	gram
gt	:	gram tension
HS-M	:	*Hydrophis schistosus* of Malaysia
i.v.	:	intravenous

LIST OF SYMBOLS AND ABBREVIATIONS

IgG	:	immunoglobulin G
KCl	:	potassium chloride
kDa	:	kilodalton
KUN	:	Kunitz-type protease inhibitor
LAAO	:	L-amino acid oxidase
LD_{100}	:	maximal lethal dose (µg/g)
LD_{50}	:	median lethal dose (µg/g)
LNTX	:	long-chain neurotoxin
mAChR	:	muscarinic acetylcholine receptor
MALDI-TOF/TOF	:	matrix assisted laser desorption/ionization-time of flight/timeof flight
mg	:	milligram
min	:	minute
ml	:	milliliter
mM	:	millimolar
mm	:	millimeter
MS/MS	:	tandem mass spectrometry
MTLP	:	muscarinic toxin-like protein
nAChR	:	nicotinic acetylcholine receptor
nanoESI	:	nanoelectrospray ionization
NGF	:	nerve growth factor
NK-M	:	*Naja kaouthia* of Malaysia
NKMAV	:	*Naja kaouthia* Monovalent Antivenom
NK-T	:	*Naja kaouthia* of Thailand
NK-V	:	*Naja kaouthia* of Vietnam

LIST OF SYMBOLS AND ABBREVIATIONS

nm	:	nanometer
NMJ	:	neuromuscular junction
NP	:	natriuretic peptide
n-P	:	normalized neutralization potency
NPAV	:	Neuro Polyvalent Antivenom
P	:	neutralization potency
PDE	:	phosphodiesterase
PLA_2	:	phospholipase A_2
RP-HPLC	:	reverse-phase high-performance liquid chromatography
s.c.	:	subcutaneous
SDS-PAGE	:	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	:	standard error of the mean
SNTX	:	short-chain neurotoxin
SSAV	:	Sea Snake Antivenom
SVMP	:	snake venom metalloproteinase
TFA	:	trifluoroacetic acid
TS	:	toxicity score
V	:	volt
v/v	:	volume/volume
VICC	:	venom-induced consumptive coagulopathy
w/v	:	weight/volume
WHO	:	World Health Organization
WNTX	:	weak neurotoxin/toxin

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CHAPTER 1: GENERAL INTRODUCTION

1.1 Snake Venoms and their Biological Impacts

Snake venoms are toxic secretions produced by highly specialized venom glands in venomous snakes and are capable of causing deleterious effects when injected into a recipient organism (Mackessy, 2002b). Venoms are considered evolutionary products across various lineages; they consist of biologically active proteins that mainly recruited and adapted from the gene of proteins involved in key physiological functions, e.g. hemostasis or neurotransmission (Fry, 2005; Fry et al., 2006). The prevailing thought on venom evolution agrees that repeated gene duplication creates redundancy, allowing gene copies to be selectively expressed in the venom gland. The "free copy" of gene subsequently underwent neo-functionalization through positive selection and molecular adaptation at accelerated rates, driven by changes in the ecological niche, diet and predator-prey arms race (Kordiš & Gubenšek, 2000). Besides gene duplications, alternative splicing and alterations of domain structures will also generate novel toxin genes (Casewell et al., 2013). Taken together, the emergence of paralogous groups of multigene families across taxonomic lineages gave rise to multiple isoforms within each major toxin family, resulting in a vast functional diversity of venom proteins (Calvete et al., 2009).

Venoms leave significant impacts on the ecology and humans' lives. Venomous snakes never prey on humans but an unpleasant encounter with humans can result in defensive snakebite causing morbidity and mortality. The immense variety of snake venom proteins, however, forms a fascinating medical paradox when they are developed into novel drugs to treat various ailments.

1.2 Snakebite Envenomation

Snakebite envenomation remains a serious public health threat in many tropical and subtropical countries, affecting vast rural populations particularly in the South Asia, Southeast Asia, and sub-Saharan Africa (Alirol et al., 2010; WHO, 2010a, 2010b). There are approximately 5.5 million snakebite cases annually, resulting in close to 2 million envenomations with approximately 100,000 deaths (Kasturiratne et al., 2008; Mohapatra et al., 2011; Rahman et al., 2010). It causes significant mortality and possibly leads to permanent physical disability even if the victim survives the envenomation. Most of the affected victims have been reported to come from the impoverished population engaged in agricultural activities, herders or fisheries activities. Snakebite envenomation therefore exerts a direct socioeconomic impact to these communities and contributes to the continuity of poverty and inequity (Gutierrez et al., 2013). Indeed, snakebite envenomation has long been known as a disease of poverty (Harrison et al., 2009) and since 2009, it has been on the list of WHO Neglected Tropical Disease (NTDs) (WHO, 2010a) owing to the persistent underestimation of its morbidity and mortality especially from the less developed regions. This unfavorable condition is aggravated further by an inadequate supply of good quality antivenom and limited understanding of venom variability. Through the years, various integrated multifocal approaches have been proposed by toxinologists to effectively combat the global crisis of snakebite envenomation (Gutierrez et al., 2014; Gutierrez et al., 2013; Gutierrez et al., 2010; Williams et al., 2011).

1.2.1 South Asia

Across the world, the highest incidences and mortality rates of snakebite envenomation are reported in South Asia, in countries like India, Pakistan, Sri Lanka, Bhutan and Nepal (Alirol et al., 2010). India appears to suffer this condition most seriously, with the highest number of resultant deaths (35,000-50,000 deaths) reported annually. Meanwhile, severe envenomation cases have also been reported in Pakistan (40,000 cases per year), Sri Lanka (33,000 cases per year) and Nepal (20,000 cases per year) (Alirol et al., 2010; Kasturiratne et al., 2008). Medically important species that causing the most significant envenomation problem in South Asia are often called the "Big Four" which include the common cobra (*Naja naja*), Russell's viper (*Daboia russelii*), saw-scaled vipers (*Echis carinatus*) and Indian krait (*Bungarus caeruleus*). Hump-nosed pit viper, *Hypnale hypnale*, is also important but mainly endemic to the Western Ghat of India and Sri Lanka (de Silva et al., 1993; Harris et al., 2010). Furthermore, the open-style habitation and the practice of sleeping on the floor of local people have contributed to the increased risk of snakebite envenomation (Alirol et al., 2010).

1.2.2 Southeast Asia

In Southeast Asia, snakebite envenomation is an occupational health hazard in many countries in the Indochina (Thailand, Vietnam, Myanmar, Cambodia, and Laos) as well as Malaysia and Indonesia. These tropical countries with dense vegetation, conducive temperature and humidity, and warm coastal waters make an ideal habitat for many terrestrial and aquatic snakes. Among many venomous snakes in this region, elapids (especially cobras, kraits and sea snakes) and viperids (viper and pit vipers) were the leading cause reported in most cases of snakebite envenomation. Of all elapids species, cobras (*Naja* sp.) – classified as a Category I medically dangerous snake by WHO

(WHO, 2010b), appear to be one of the most common biters that are capable of delivering a large amount of deadly venom (note: Category I species are highly venomous snakes that are common or widespread to cause numerous snakebites, and could result in high levels of morbidity, disability or mortality). Cobras are generally adaptable to a wide range of habitats, ranging from natural to anthropogenically modified environments, and they generally distribute widely over Southeast Asia. In general, cobra venoms are neurotoxic and can cause rapid death (Ranawaka et al., 2013). Besides, the venom also contains abundant cytotoxic components that can lead to extensive tissue necrosis and possible crippling deformity (Alirol et al., 2010; Reid, 1964).

1.2.3 Medical Significance of *Naja kaouthia* in Southeast Asia

Monocled cobra (*Naja kaouthia*) is a common non-spitting cobra that widely distributes throughout the Indochina subcontinent, the northern Malayan Peninsula, as well as north-eastern India and southern China (Alirol et al., 2010; Chew et al., 2011; Mohapatra et al., 2011). *N. kaouthia* envenomation could be rapidly lethal and therefore, it is one of the most medically important species. Due to its potent neurotoxicity, *N. kaouthia* venom is capable of causing rapid onset of neuromuscular paralysis (Bernheim et al., 2001; Kulkeaw et al., 2007), in which the delay or inadequate treatment can lead to worsening respiratory failure and death (Stiles, 1993; Wongtongkam et al., 2005). Besides, in most cases of *N. kaouthia* envenomation, extensive tissue necrosis is not uncommon. To date, although species-specific antivenom against *N. kaouthia* is produced by the Thai Red Cross Society, Queen Saovabha Memorial Institute (QSMI, Bangkok), it is however, not manufactured or widely available in many other countries including Malaysia. In addition, variable clinical manifestations and presentations have also been reported in victims envenomed by *N. kaouthia* from different geographical

regions (Khandelwal et al., 2007; Wongtongkam et al., 2005), and thus research are in need to elucidate the variations observed. Recently, preclinical assessments on *N. kaouthia* venoms from Malaysia and Thailand have documented substantial differences of venoms in terms of lethality and neutralization by antivenom (Leong et al., 2012; Leong et al., 2014). These findings imply the occurrence of geographical variations in the toxin composition of venom from *N. kaouthia*, a well-known wide-ranging species. However, to date, the information regarding biogeographical variations of *N. kaouthia* venom (detailing the toxin subtypes and relative abundance) remains lacking.

1.3 Global Challenges in Management of Snakebite Envenomation

The management and control programs for snakebite envenomation are confronted with various challenges (Gutierrez et al., 2014; Gutierrez et al., 2013). For decades, snakebite envenomation in many parts of the world has failed to receive proper attention and support from health authorities, partly due to the lack of systematic epidemiological data. Despite being listed as a neglected tropical disease by WHO, it is ironic that the neglected status of this disease is now aggravated by its removal from the said list in 2015. The neglected status of snakebite envenomation hinders effective communication between countries and hampers international efforts in tackling the challenges faced, one of which being the critical condition of antivenom production and supply. Moreover, the treatment with antivenom therapy is regionally specific and the implementation of same therapy across different snakebite is hardly achieved (WHO, 2010c; Williams et al., 2011). In recent years, many antivenom manufacturing plants ceased production ostensibly for limited market demand and difficulty in making profits. Although antivenom is life-saving and an essential medicine as categorized by WHO, the use is often species- and region-specific (unlike most of the other generic medicines), while its market is very much contained within the poor rural populations. Also, the lack of

antivenom supply in rural areas (where most snakebites occur) causes many victims needing to travel a long distance to the nearest health center for antivenom. The problem could lead to secondary issues as victims will go to traditional healers prior to acquiring appropriate treatment, and this could cause substantial delay in obtaining proper medical treatment. Apart from this, the efficacy and safety of antivenom products constitute the most important issues to be addressed.

1.3.1 Challenges Faced in Use of Regional Antivenom

To date, antivenom remains as the only validated etiological treatment for snakebite envenomation. The quality of antivenom relies on the efficacy or potency, and the spectrum of coverage for venom neutralization. As an immunoglobulin derivative, antivenom works by binding to venom protein antigens and forming immunocomplexes which are void of toxic activities. The efficacy of antivenom in this regard is mainly governed by two factors: (1) the formulation of immunogen used in the production of antivenom; (2) the antigenicity of venom proteins, which can vary even within a species. Efficacious antivenom is generally a cornerstone to effective treatment for snakebite cases. The issue of antivenom production is highly relevant in the region of Southeast Asia where many countries do not have financial and technical resources to produce sufficient antivenom for use in the country. At the moment, many developing nations, including Malaysia still depend on antivenom imported from foreign countries to meet local needs. However, the problem with using antivenom from non-domestic manufacturers is the use of immunogen (venoms) from species that are non-native to the importing countries, and thus the effectiveness of these antivenoms in the importing nation must be rigorously assessed (Gutierrez et al., 2014; Warrell, 2008). In fact, clinical reports indicated that the treatment outcome of using imported antivenom can vary greatly depending on the geographical area (Alirol et al., 2010; Shashidharamurthy

& Kemparaju, 2007). The implication is also relevant for the case of cobra (*Naja*) envenomation in Southeast Asia, where the knowledge of possible geographical variations in the regional venom is lacking (Furtado et al., 2003; Salazar et al., 2008). Besides, the principal toxins in cobra venom e.g. neurotoxins have also shown to exhibit low antigenicity, deserving further investigation to improve the efficacy of antivenom neutralization (Leong et al., 2015).

1.4 Recent Approach toward the Optimization of Antivenom Production

Recent breakthroughs in -omics technologies, especially in proteomic research, enabled scientists to unravel the detail of compositional variations of snake venoms (Calvete et al., 2009). In-depth information about venom composition and immunological properties of the toxins helps to elucidate the variations of the toxic effects of venoms and the discrepancies of treatment response to antivenom (Khandelwal et al., 2007; Ronan-Bentle et al., 2004; Wongtongkam et al., 2005). This information is important to provide clearer insights into the production of antivenom with improved efficacy and wider geographical coverage. An integrated approach that incorporates venom proteomic study, toxin antigenic epitope mapping, cross-reactivity assessment and functional neutralization study, either *in vitro* or *in viv*o, could be applied in the future studies to improve the production of antivenom (Calvete, 2014; Fox & Serrano, 2008a; Warrell et al., 2013). It is hoped that an integrated study will be able to bridge the knowledge gaps concerning snakebites envenomation, particularly the *N. kaouthia* envenomation that being an important medical issue in Southeast Asia. Essentially, the study is hoped to unveil comprehensively the intraspecific variations in the proteomes, mechanisms and immunoneutralization of the venom of *N. kaouthia*.

1.5 Research Questions and Hypotheses

i. The *N. kaouthia* venoms from different geographical regions were previously shown to be different in lethality, clinical manifestation of envenomation and response to antivenom – What is the main cause of these discrepancies?

Hypothesis: This is due to remarkable geographical variations of the venoms, characterized by differences in the expression of key venom toxins.

ii. In Southeast Asia, many countries depend on imported cobra antivenom supply from non-domestic manufacturers (typically from Thailand) that use immunogen (cobra venoms) from species that are non-native to the importing countries – How effective is the Thai-produced *N. kaouthia* antivenom in neutralizing venoms of *N. kaouthia* from other regions e.g. Malaysia and Vietnam?

Hypothesis: The Thai antivenoms (monovalent and polyvalent) are effective in neutralizing the *N. kaouthia* venoms from Malaysia and Vietnam with varying degree of efficacy.

iii. How do venom variations affect the mechanistic action of neurotoxicity induced by the *N. kaouthia* venoms?

Hypothesis: The differences in the expression of key toxins will mediate the neurotoxic activity differently, via the pre/postsynaptic blockade and/or myotoxic effect.

iv. Cobra antivenom generally possesses limited neutralization potency – What are the limiting factors and how do these contribute to the low neutralization potency of elapid antivenom?

Hypothesis: The toxins with small molecular mass have lesser immunogenic sites to stimulate the production of high titer antibodies during horse immunization. Cobra venom which key toxins are of small molecular sizes tends to have low neutralization by antivenom.

1.6 Objectives

The present study was carried out to answer the research questions and to validate the hypotheses made. Upon the completion of the study, it is hoped that the following objectives will be achieved:

i. To profile and characterize the variations in venom proteomes of *N. kaouthia* venoms from three different Southeast Asia regions (Malaysia, Thailand and Vietnam; NK-M, NK-T and NK-V) (**Chapter 4**).

ii. To examine the impact of the venom variations on the lethal effect and antivenom neutralization of the *N. kaouthia* venoms (NK-M, NK-T and NK-V) from three different Southeast Asian regions (**Chapter 4**).

iii. To elucidate the differences in the mechanistic action of neurotoxicity induced by the three *N. kaouthia* venoms (NK-M, NK-T and NK-V) from different Southeast Asia regions (**Chapter 5**).

iv. To investigate the limitation of commercial antivenoms in neutralizing specific key toxins purified from *N. kaouthia* venom (**Chapter 6**).

CHAPTER 2: LITERATURE REVIEW

2.1 Classification of Snakes

Snakes are reptiles of the suborder Serpentes (Clade: Ophidia) that are widespread throughout the world: living snakes can be found on every continent except Antartica, and on most smaller land masses. Some large islands do not have native snakes, e.g. Ireland, Iceland, Greenland and islands of New Zealand, as well as many small islands of the Atlantic and central Pacific, although some islands (including New Zealand) may be infrequently visited by some aquatic serpents that come ashore. By 2015, approximately over 3500 snake species have been recognized and categorized into more than 20 families (www.reptile-database.org). Of these, the superfamily Colubroidea (advanced snake) comprises the majority of the snake species and it represents one of the most conspicuous and well-known radiations of terrestrial vertebrates. The morphologically and ecologically diverse advanced snake of the Colubroidea has been classified into seven families inferred from a large scale likelihood-based analysis (Pyron et al., 2011): these are Lamprophiidae, Xenodermatidae, Pareatidae, Homalopsidae, Colubridae, Elapidae and Viperidae (Figure 2.1). Among these families, the Colubridae includes a mix of mildly-venomous species, while both Elapidae and Viperidae families comprise typically of venomous species.

2.2 Venomous Snakes

2.2.1 Viperids

The Viperidae family consists of approximately 331 species that belong to 34 genera in 4 subfamilies: Crotalinae (pit vipers), Viperinae (true vipers), Azemiopinae (Fea's viper) and Causinae (night adders) (Wüster et al., 2008). Viperids can be found in

almost all continents of the world, including America, Africa, Europe and Asia (McDiarmid et al., 1999). Unlike elapids, almost all viperids possess keeled scales, short tails, and a triangle-shaped head which is distinct from the neck. Moreover, they are solenoglyphous (refer to **Section 2.3**), with a pair of long, hollow and hinged fangs that enable them to freely extend and fold during the biting. In addition, the longer fangs allow a deeper penetration into the dermal and/or even the underlying muscle layer of prey or victims. The components of viperid venoms are generally (though not exclusively) hemotoxic and can lead to severe hemorrhage and coagulopathy. Among viperids, Crotalinae and Viperinae are two largest subfamilies. Members of Crotalinae are also known as "pit vipers" with the presence of heat-sensing organs located between the eye and nostril. These sensing pits function to detect the movement of prey and predators through thermal (infrared) radiation emitted from a body (Krochmal et al., 2004). On the other hand, members of Viperinae that are commonly known as "true vipers", do not have such heat-sensing organs.

2.2.2 Elapids

The Elapidae family consists of 61 genera with more than 300 recognized snake species and is distributed worldwide, mostly in tropical and subtropical regions, but never found in the Europe continents. The family covers snake species on land and sea, as well as those living in brackish water, including Elapinae (cobras), Bungarinae (kraits), Micrurinae (coral snakes), Acanthophiinae (Australian elapids), Hydrophiinae (sea snakes) and Laticaudidae (sea kraits). Similar to viperids, the phylogeny of Elapidae family is yet to be universally recognized, and more molecular evidence is needed for a standardized classification. These elapids are similar in morphology: they have long and slender bodies with smooth scales, and a head that is usually covered with large shields and not always distinct from the neck. In general, they are

proteroglyphous (refer to **Section 2.3**), with enlarged and fixed tubular fangs located in maxilla bone. These fangs are normally only a fraction of an inch long (even for a 2-meter long king cobra); and in envenomation, fangs usually sink merely into the subcutaneous tissue. In Asia and Africa, "true cobras" of genus *Naja* are the most diverse and widely distributed elapids, and most of them are medically important. Other genera closely related to *Naja* cobras, and are sometimes referred to as "cobras" include *Aspidelaps* (coral cobras), *Boulengerina* (water cobras, recently proposed to be synonymized with *Naja*), *Hemachatus* (ringhals), *Ophiophagus* (king cobra), *Pseudonaja* (brown snake) and *Walterinnesia* (dessert black snake). *Naja* cobras are widespread throughout Africa, Southwest Asia, South Asia and Southeast Asia, including Southern China and the island of Taiwan (O'Shea, 2011). Two decades ago, the taxonomy of Asiatic cobras has undergone systematic revision from older nomenclatures that were used to be in a state of confusion (Wüster, 1996; Wüster & Thorpe, 1991). The systematic revision left an impact on many Southeast Asian cobras including the species in this study, *Naja kaouthia.* The envenomation by *Naja* sp. is potentially lethal, as they are capable of delivering a large amount of highly lethal venom that usually leads to rapid onset of neuromuscular paralysis, where death can ensue in a few hours due to respiratory failure (WHO, 2010a; Wongtongkam et al., 2005).

2.2.3 Classification of Asiatic Cobras (Genus *Naja*)

Asiatic cobras were one of the ill-defined snake populations and were long regarded as a single species, *Naja naja*, the Indian spectacled cobra described by Carl Linnaeus in 1758. The word *Naja* is a Sanskrit word for snake, nāgá. Over the years, the Asiatic cobras had been named inconsistently at variant level in different regions, causing the confusion of its systematics. In addition to the variable morphology among cobras, the

inadequate understanding of the biological variation of their venom composition also complicates the knowledge of venom toxinology and the clinical management of snakebite envenomation, in particular when the venom composition is a crucial factor in antivenom production and treatment (Warrell, 1986). Throughout the years, many attempts have been carried out to revise the systematics of cobras; one of the latest was published in 1996 based on the combination of multivariate analysis of morphological characters and mitochondrial DNA sequence (Wüster, 1996). This latest revision is widely accepted and has resulted in the splitting of one formerly of single cobra species with binomial nomenclature (*Naja naja*) into at least 10 different species, including the medically significant species in the Southeast Asian region such as *Naja kaouthia*, *Naja siamensis*, *Naja sputatrix* and *Naja sumatrana* (Table 2.1).

The monocled cobra was commonly designated as *Naja naja kaouthia* (Thai/Siamese cobra) or *Naja naja siamensis* in the previous toxinological literature. The interchangeable use of the two former nomenclatures was mainly due to the practice where *N. naja siamensis* and *N. naja kaouthia* both were commonly used to denote cobras from Thailand. In fact, there are marked differences among these Thai cobras, and two distinct species have been recognized in the 1990's: *Naja siamensis* and *Naja kaouthia* (Wüster & Thorpe, 1994; Wüster et al., 1995). The two cobras were widely distributed in Thailand and parts of the Indochina. Interestingly, *N. siamensis* is a spitting cobra species, while *N. kaouthia* is a non-spitter.

On the other hand, the spitting cobra widely distributed in the Malayan Peninsula (including the southern Thailand), Sumatera, Java and Borneo had previously been known by various names such as *N. naja sumatrana* (Sumatra), *N. naja miolepis* (Borneo and Palawan) and *N. naja sputatrix* (Malayan Peninsula, Bangka and Belitung) in different regions. They have subsequently been recognized as two different species: *N. sumatrana* that is distributed in the Malayan Peninsula, Sumatra and Borneo, and *N.*

sputatrix confined to Java (Wüster, 1996; Wüster & Thorpe, 1989). Unfortunately, the earlier literature on snake venom study from this region did not make clear distinction between the two species. Many reports had been citing *N. naja sputatrix* in the olden days; without recording the source of the snakes, it is almost impossible nowadays to tell whether the venom studied belonged to which species. This is particularly confusing when the venom was obtained through supplier who did not know better the original source of venom or the snake.

In general, a standard classification of Asiatic cobras is crucial, as the locality information is important, particularly in the case where the venom composition vary considerably even within the same species. The recent major taxonomical revision by Wüster (Wüster, 1996) has provided great impact on a better understanding of the Asiatic cobra venoms today without confusion over the authenticity of the species identity (Table 2.1) and relates the current state of knowledge of the systematics of the Asiatic cobras to the nomenclature which has been used in old literature.

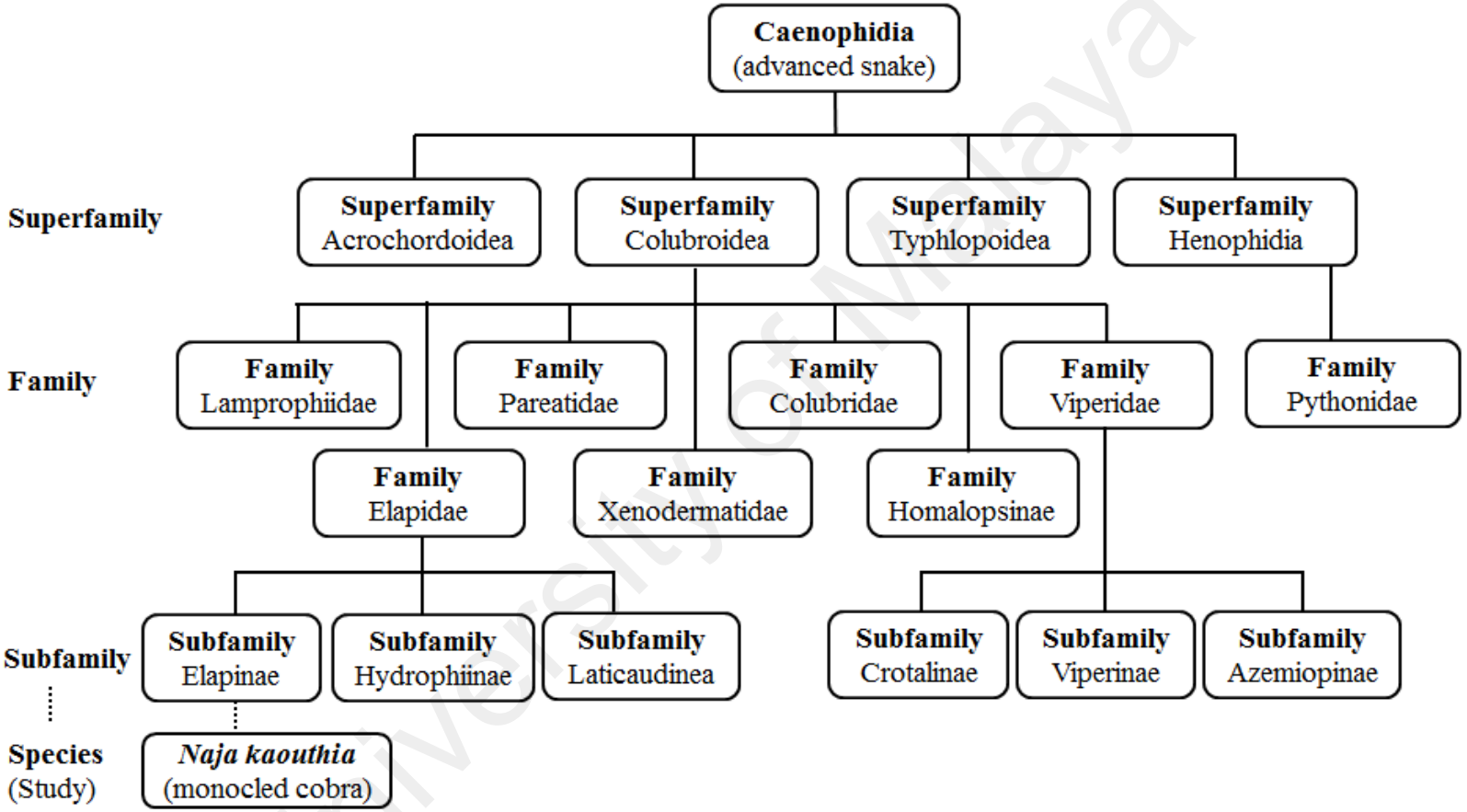


Figure 2.1 Classification of snakes[1].

[1] The classification was summarized and drawn according Pyron et al. (2011). The phylogeny of advanced snakes (Colubroidea), with discovery of a new subfamily and comparison of support methods for likelihood trees. *Mol Phylogent Evol*, 58, 329-342.

Table 2.1: The latest scientific nomenclatures of several important Asiatic cobras with their older nomenclatures commonly reported in the earlier literature. (adapted from Wüster, 1996)

Current species (scientific names)	Nomenclatures used in the earlier literature and populations for which used
Naja kaouthia (monocled cobra)	*N. naja kaouthia* (common), *N. naja siamensis* (common in the toxinological literature), *N. naja sputatrix* (Vietnam, rare), *N. naja leucodira* (Reid, 1964), *N. kaouthia suphanensis* (yellow form from central Thailand, rare)
Naja siamensis (Indochinese spitting cobra)	*N. naja kaouthia* (Thailand, Cambodia, Vietnam, through confusion), *N. naja sputatrix* (Thailand), *N. naja isanensis*, *N. naja atra* (Thailand), *N. atra* (Thailand), *N. sputatrix atra* (rare, Thailand), *N. sputatrix isanensis*, *Naja isanensis*
Naja sputatrix (southern Indonesian spitting cobra)	*N. naja sputatrix*
Naja sumatrana (Equatorial spitting cobra)	*N. naja sumatrana* (Sumatra), *N. naja sp*uratrix (common, Malayan Peninsula, Bangka, Belitung), *N. naja miolepis* (Borneo), *N. naja leucodira* (Malayan Peninsula, Sumatra), *N. naja kaouthia* (yellow form from northern Malaysia, Java - Lingenhöle & Trutnau, 1989)
Naja atra (Chinese cobra)	*N. naja atra* (common), *N. sputatrix afra* (China, northern Vietnam - Lingenhöle & Trutnau, 1989)
Naja naja (Indian spectacled cobra)	*N. naja naja* (common), *N. naja oxiana* (patternless specimens from northern India), *N. naja indusi* (NW India, northern Pakistan, rare), *N. naja karachiensis* (black form from southern Pakistan), *N. naja polyocellata* (Sri Lanka, rare), *N. naja caeca* (patternless specimens from northern India-rare)
Naja oxiana (Central Asian cobra)	*N. naja oxiana*, *N. naja caeca* (rare)
Naja philipinensis (northern Philippine cobra)	*N. naja philippinensis*
Naja sagittifera (Andaman cobra)	*N. naja kaouthia*, *N. naja sagittifba*
Naja samarensis (southeastern Philippine cobra)	*N. naja samarensis*

2.3 Venom Delivering System

Snake bites for two purposes: predation (for food) and/or self-defence. Unfortunate encounters with human where they are trodden upon or mishandled typically result in defensive bites, which lead to envenomation and the associated morbidity and mortality. Venomous snakes are equipped with a highly specialized apparatus capable of injecting lethal venoms into prey or victims (Jackson, 2003). Differing from non-venomous aglyphous snakes such as pythons and boas, which teeth are large and recurved in shape but without fangs, the venomous snake possesses different types of dentition: (1) Viperidae has shortened, movable maxilla, and a pair of long, hinged tubular front fangs (solenoglyphous); (2) Elapidae has fixed and tubular but rather short front fangs (proteroglyphous); (3) Colubridae may have enlarged or grooved posteriorly located fangs (opisthoglyphous) (Figure 2.2). These dentitions have independently evolved to become an apparatus that is effective in delivering venom during a strike (Vonk et al., 2008). In additions, the presence of grooves at varying depth and degree of closure in these modified fangs contribute to different geometric and hydrodynamics of venom during snakebite envenomation (Young et al., 2011). The solenoglyphous dentition displayed by viperids is generally thought to be the most sophisticated and the most derived, although both Viperidae (solenoglyphs) and Elapidae (proteroglyphs) were thought to have evolved independently from a common ancestor (Fry et al., 2009). All these dentition structures are associated with a Duvernoy's or venom gland located toward the rear of the upper jaw. During biting, penetration of fangs into the prey's tissue is accompanied by contraction of muscle tissues around the venom gland. Thus, it generates a contraction force that facilitates the venom out of the lumen through the duct and into the canal of tubular fang, creating an effective venom delivery into the wound to cause diverse pathophysiological actions (Weinstein et al., 2009).

2.3.1 Venom Delivering in Cobra (Genus *Naja*)

Medically significant cobra species (genus *Naja*) in Asia can be generally grouped into spitting (e.g. *Naja siamensis*, *Naja sputatrix*, *Naja sumatrana*, *Naja philipinensis*) or non-spitting (e.g. *Naja kaouthia*, *Naja atra*, *Naja naja*) type (WHO, 2010a). There are at least 15 cobra species capable of spitting venom up to a distance of 8 feet away, with more than 90% precision in hitting the target. The venom-spitting behavior of cobra is suggested to be an adaptation of long-distance weaponry for cobra as a primary defensive mechanism to repel the aggressor (specifically, the primate). The venom is typically sprayed into the eyes and can cause venom ophthalmia, resulting in severe ocular inflammation, conjunctivitis and permanent blindness if left untreated (Chu et al., 2010).

It has been established that the spitting mechanism of cobras is highly associated with the change in the morphology of snake fangs, as well as the musculature of snake's head (Figure 2.3). The fangs of non-spitting cobras contain grooves that are completely closed, forming a hollow tube along the front edge with the absence of ridges; while spitting cobras contain ridges at the basal of discharged orifice. The presence of smaller discharged orifice in spitting cobras plays a key role in enabling the ejection of venom to proceed far forward and upward at high speed through the exit orifice and reach the target at full speed (Berthe et al., 2009).

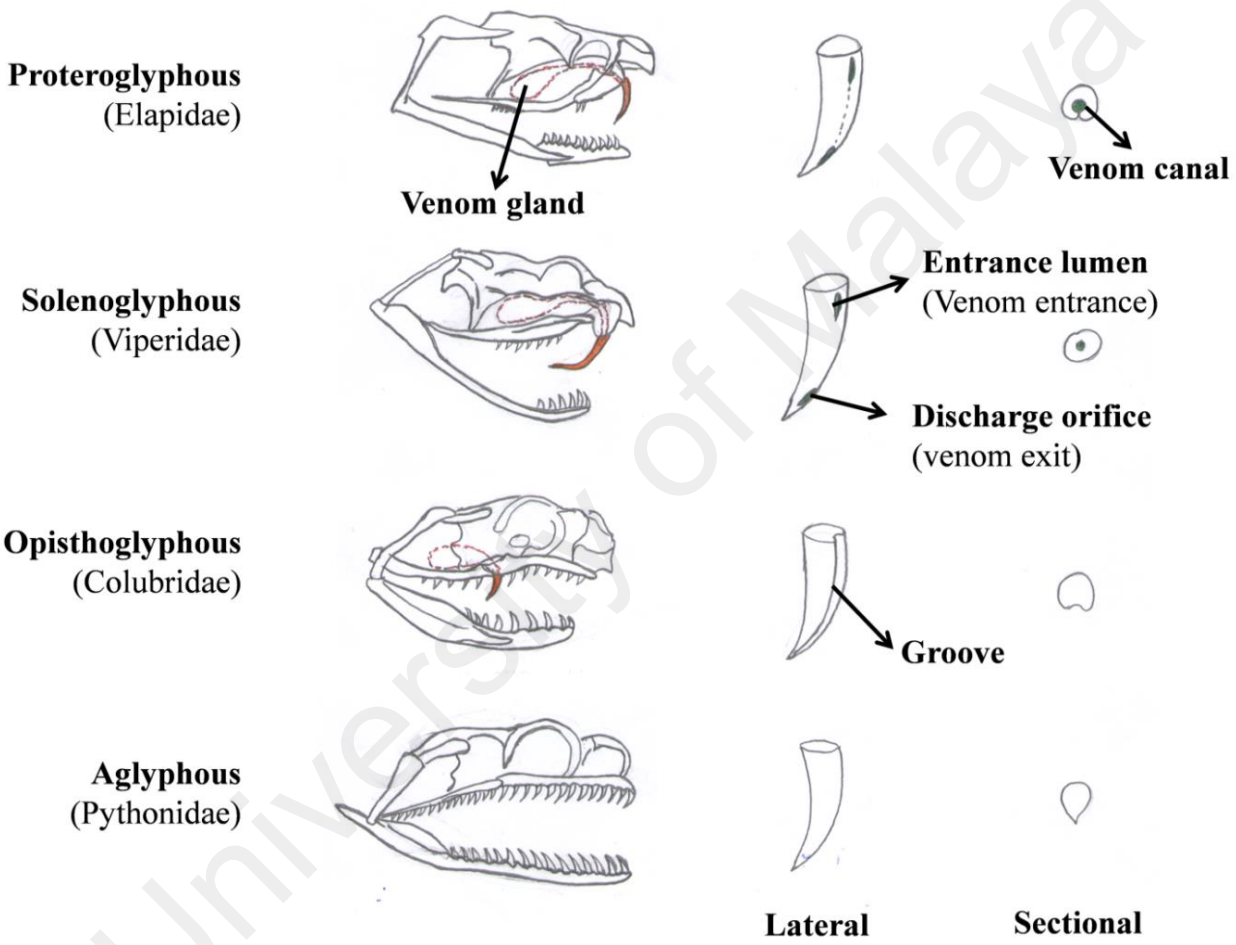


Figure 2.2 Types of dentition in different advanced snakes (proteroglyphous, solenoglyphous, opisthoglyphous and aglyphous). (1) The skull and fangs (colored in red) of different types of snakes; (2) The lateral view of snake fangs; (3) The sectional view of snake fangs. The image was produced by drawing with modification from Cundall (Cundall, 1983).

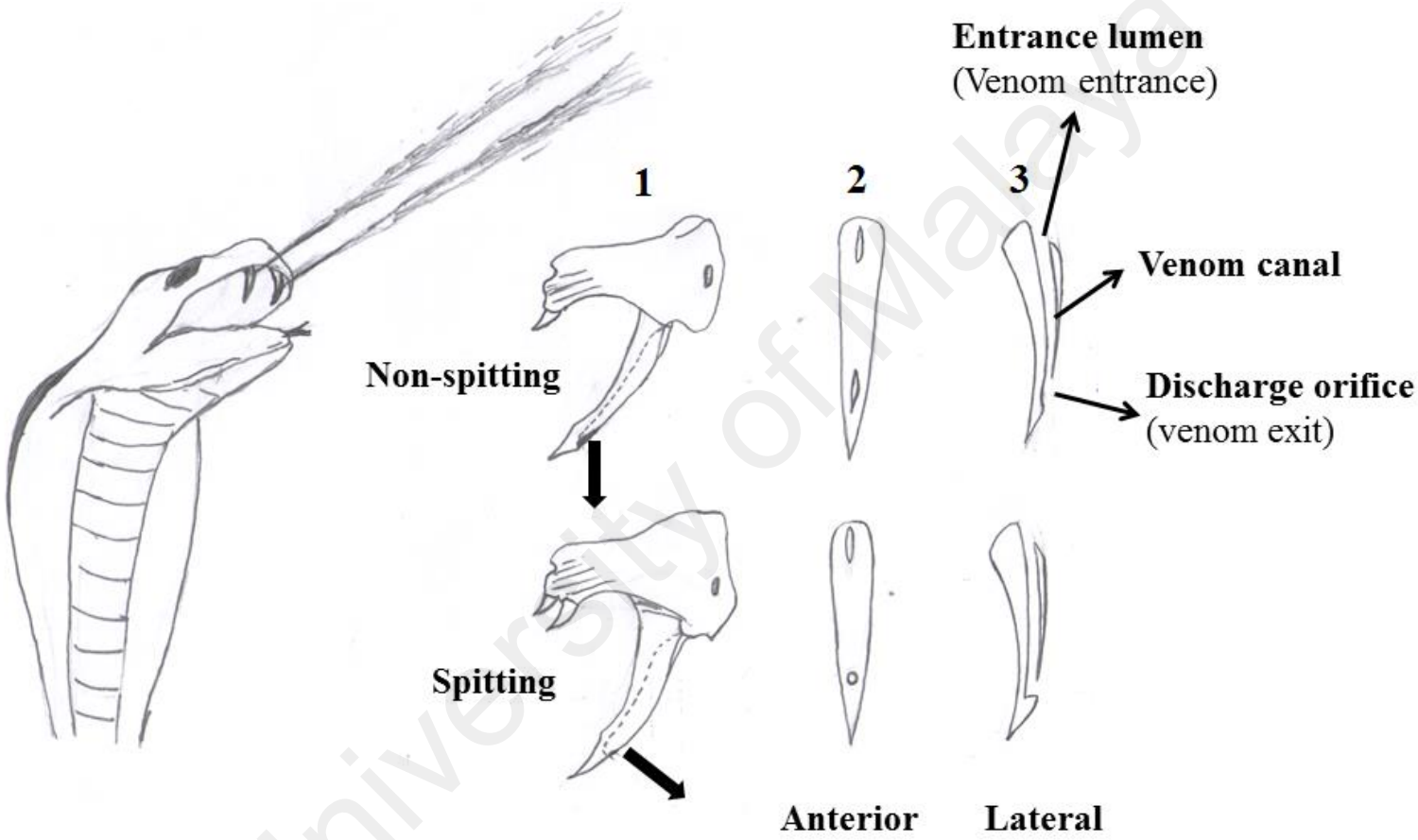


Figure 2.3 The fang's structure of the spitting and non-spitting cobras. (1) The venom flow of cobra fangs; (2) The anterior view of cobra fangs; (3) The lateral view of cobra fangs. The image was produced by drawing with modification from Bogert (Bogert, 1943).

2.4 Snake Venom

2.4.1 "Venom" and "Poison"

The terms "snake venom" and "snake poison" are commonly used interchangeably by the public disregarding the true nature of venom or poison. Similarly, "snake poisoning" is frequently used by media and even professionals from the medical community disregarding the pathophysiology and mechanism of the disease. Venom and poison are differentiated by the nature of compounds and their modes or routes of delivery into a recipient organism (Mackessy, 2002b). Venom is a biological secretion made up of various bioactive compounds produced in a specialized gland. Accompanied by a special suite of behavior, venom is introduced into the prey through a highly specialized venom-delivering system such as stings, spines and fangs; giving rise to deleterious effects in the recipient (Mackessy, 2009). On the other hand, poison can be natural or synthetic entity and the toxicity can be introduced through inhalation, ingestion or skin contact, either intentionally or accidently. Needless to say, there are also snakes which are truly poisonous as they harbor toxic compounds in the body (such as those in the nuchal gland of keelback snakes) and the ingestion (by some population) can cause poisoning.

2.4.2 Venom Components

Snake venom is a cocktail of secretory proteins and peptides that typically constitute 90-95% of the venom dry weight. In addition, it also contains a small amount of carbohydrates, metal ions, amines, nucleosides, lipids and free amino acids. Venom proteins and peptides are mostly pharmacologically active compounds; termed "toxins", where they are responsible for various toxic effects observed in envenomation. Snake venoms developed through a series of evolutionary events and they represent largely the

trophic adaptive trait of advanced snakes. Molecular adaptation accompanied with protein neofunctionalization led to the emergence of various toxins to suit the ecological niche (Mackessy, 2009). When injected, snake venom toxins are capable of interfering with the normal physiology, especially the neurological and hemostatic functions that are needed in sustaining the prey animal's life (Barlow et al., 2009). Biochemically, these toxic proteins can be classified into non-enzymatic toxins (e.g. three-finger toxin, platelet aggregation factor, serine protease inhibitor, C-type lectin and lectin-like protein (collectively called snaclec), natriuretic peptide, nerve growth factor), or enzymatic toxins (e.g. phospholipase A_2, metalloproteinase, disintegrin, phosphodiesterase, acetylcholinesterase, L-amino acid oxidase, serine protease) (Fox & Serrano, 2009; Gopalakrishnakone et al., 1997; Kang et al., 2011). Nonetheless, the toxic activity of some "enzymatic" toxins may not be directly related to its catalytic activities, for instance, neurotoxic and myotoxic phospholipase A_2.

2.4.3 The Non-spitting Cobra, *Naja kaouthia* (Monocled Cobra)

Monocled cobra (*Naja kaouthia*) is a common species of genus *Naja*, and the word *kaouthia* is derived from Bengali term "keauthia" meaning "monocle", which describes "O"-shaped mark on the hood of the snake (Mohapatra et al., 2011). Earlier, the monocled cobra was described under different scientific names (*Naja naja siamensis* or *Naja naja kaouthia*) and the taxonomy of this species was in a constant state of confusion until clarified about 2 decades ago (Wüster, 1996). This species adapts well to a range of habitats from natural to anthropogenically impacted environments, contributing to the wide distribution of its population throughout many parts of Asia. The envenomation by *N. kaouthia* is highly lethal as the neurotoxic venom is capable of causing rapid neuromuscular paralysis in victims (WHO, 2010a; Wongtongkam et al., 2005). The delay or inadequate treatment may fail to reverse the neurotoxicity and

hence, death ensues. Moreover, envenomation by *N. kaouthia* is usually accompanied by extensive tissue necrosis that often results in crippling disability in surviving victims (Wongtongkam et al., 2005).

2.4.3.1 Toxinological Studies on *Naja kaouthia* (Monocled Cobra) Venom

Since the 1970's, a number of studies had reported the isolation and characterization of different toxins from what was known differently as Thai/Siamese cobra, *Naja naja kaouthia*, or *Naja kaouthia*, from various locales (specified and unspecified). The toxin that had been characterized, included long-chain neurotoxins (Karlsson & Eaker, 1972), short-chain neurotoxins (Cheng et al., 2002; Meng et al., 2002), weak neurotoxins (Utkin et al., 2001a; Utkin et al., 2001b), cytotoxins/cardiotoxins (Joubert & Taljaard, 1980a), and phospholipase A_2s (Joubert & Taljaard, 1980b; Mukherjee, 2007). These studies were mainly focused on selected toxins of interest; whereas the first proteomic study on *N. kaouthia* venom was only reported in 2007 by using 2D-gel electrophoresis and mass spectrometry (Kulkeaw et al., 2007). From the study, six toxin families were identified from *N. kaouthia* venom sourced from Thailand: namely three-finger toxin (3FTx), phospholipase A_2 (PLA_2), cobra venom factor (CVF), snake venom metalloproteinase (SVMP), nerve growth factor (NGF) and cysteine-rich secretory protein (CRISP). Recently, another 2D-gel electrophoretic study reported the venom proteome of *N. kaouthia* venom sourced from Malaysia. The author concluded that only five toxin families (3FTx, PLA_2, CVF, SVMP and ohanin/vespryn) were detected in the venom (Vejayan et al., 2014). These studies consistently reported the presence of 3FTx in *N. kaouthia* venoms, a well-established fact that was supported by several *in vitro* studies using chick biventer cervicis nerve-muscle (CBCNM) preparation and/or rodent phrenic nerve hemidiaphragm (Barfaraz & Harvey, 1994; Harvey et al., 1994). However, with recent advances in proteomic technologies, it is apparent that there is knowledge

gap in the characterization of *N. kaouthia* venom, especially with regards to the detail of toxin subtypes and their relative compositions, as well as the mechanism of pathophysiological actions of venom and antivenom neutralization.

A recent study on the assessment of antivenom potency has demonstrated distinct differences in venom lethality and antivenom neutralization profiles of *N. kaouthia* sourced from Malaysia and Thailand (Leong et al., 2012; Leong et al., 2014). The study implied the existence of geographical variations in toxin composition of this widely occurring species. This presumably to be the reason behind the discrepancies of clinical manifestations and treatment outcome of *N. kaouthia* envenomation in different geographical locations (Khandelwal et al., 2007; Ronan-Bentle et al., 2004; Wongtongkam et al., 2005). Thus, there is a need to bridge the knowledge gap to obtain a better understanding of the biogeographical and functional variations of *N. kaouthia* venom in the region.

2.4.4 Clinical Manifestations of Snakebite Envenomation

Bites by venomous snakes can result in envenomation syndrome with various clinical presentations of toxicity: local toxicity (tissue inflammation and necrosis) or systemic effect when a substantial amount of toxins are absorbed and distributed into the body (Gutiérrez, 2016; WHO, 2010a). Among these, neurotoxicity and hemotoxicity are the most commonly reported manifestations. “Neurotoxicity” is usually associated with the bite by elapid snakes such as cobras, kraits, coral snakes and sea snakes, while “hemotoxic bites” are generally inflicted by viperids. However, many snake venoms could elicit a combination of variable pathophysiological effects, including neurotoxicity, myonecrosis, hemorrhage, coagulopathy, renal failure and intravascular hemolysis due to the presence of multiple types of toxin in snake venoms (Mackessy, 2009). Examples of snake venom which can cause a constellation of mixed syndromes

include Russell's vipers (neurotoxicity, myotoxicity, nephrotoxicity, hemotoxicity and cytotoxicity) (Tan et al., 2015c) and Australian tiger snake (*Notechis scutatus*) (neurotoxicity, hemotoxicity) (Cullimore et al., 2013; Lewis, 1994).

2.4.4.1 Neurotoxicity

Neurotoxicity has been well-recognized as the key feature for elapid envenoming and is usually associated with peripheral neuromuscular weakness as a consequence of the defect in signal transmission of the neuromuscular junction (NMJ). The main consequence of the venom-induced neurotoxicity is the paralysis of respiratory muscles (intercostal muscle and diaphragm), leading to respiratory failure in victims and subsequent mortality if left untreated. These venoms generally exert neurotoxic effects through pre-synaptic and/or post-synaptic blockade of the neuromuscular junction of skeletal muscle.

(a) ***Post-synaptic Neurotoxin***

Cobra venom neurotoxins are mainly of post-synaptic neurotoxins (alpha-neurotoxins, and the less abundant weak neurotoxins and muscarinic toxins-like proteins). These neurotoxins belong to the three-finger toxin (3FTx) family, which is comprised of non-enzymatic polypeptides with 60-74 amino acid residues. They fold in a similar pattern with three β-stranded loops extending from a central core, containing four conserved disulfide bridges, forming a structure with three protruded fingers (Hegde et al., 2009). In general, these short polypeptides are non-depolarizing competitive inhibitors of neuromuscular cholinergic transmission, and act by rapidly blocking the post-synaptic nicotinic acetylcholine receptor (nAChR). The envenomation typically presents as descending flaccid paralysis with ptosis being the first detected symptom. This is followed by paralysis of small facial muscles accompanied by the loss

of speech and dysphagia (Ranawaka et al., 2013). Confusion also sets in rapidly, and subsequently brain death as the compromise of respiratory function could cause the brain to suffer from hypoxia.

Among the post-synaptic neurotoxins, alpha-neurotoxin is the most common cholinergic antagonist at nAChR (Servent & Menez, 2001). It is classified based on the peptide length into long-chain (LNTX, 66-74 amino acid residues) and short-chain (SNTX, 60-62 amino acid residues) neurotoxin. Both alpha-neurotoxins (LNTX and SNTX) exhibit a varying degree of receptor binding reversibility (Barber et al., 2013). Other than that, weak neurotoxins (WNTX) and muscarinic toxin-like proteins (MTLP) are also cobra venom neurotoxins. The WNTX possesses much weaker binding affinity to nAChR as compared to alpha-neurotoxins (Poh et al., 2002), whereas the MTLP binds to muscarinic acetylcholine receptor (mAChR) and may be involved in autonomic disturbances (Kukhtina et al., 2000). Other than this, another post-synaptic neurotoxin, the kappa-neurotoxin that was previously reported in the multi-banded krait (*Bungarus multicinctus*) venom exhibit similar cysteine arrangement as with LNTX but binds specifically to neuronal nAChR subtype (Grant & Chiappinelli, 1985).

(b) ***Pre-synaptic Neurotoxin***

Pre-synaptic neurotoxins (beta-neurotoxins) are typically phospholipases A_2 (PLA_2s) and/or PLA_2 complexes that are usually present in venoms of kraits and Australian elapids. Unlike the post-synaptic neurotoxin, these proteins can bind to motor nerve terminals, depleting the synaptic acetylcholine (ACh) vesicle and thus intersecting the cholinergic transmission at the neuromuscular junction (Harris & Scott-Davey, 2013; Hegde et al., 2009; Ranawaka et al., 2013). A typical example is the beta-bungarotoxin from krait venoms (Dixon & Harris, 1999; Prasarnpun et al., 2004). It is well established that the destruction of nerve terminal by pre-synaptic neurotoxins is

irreversible, and hence the antivenom treatment often fails to reverse the neurotoxic effect if treatment is started late. The paralysis caused by pre-synaptic neurotoxins can be prolonged up to several days until new neurotransmitters and nerve terminals are regenerated and normal neuromuscular transmission is restored (Ranawaka et al., 2013; WHO, 2010a; Wongtongkam et al., 2005). Clinically, during the critical period of paralysis, patients could not survive without intensive supportive treatment (including assisted ventilation); this being a serious concern especially for those in rural areas where healthcare services may be suboptimal.

2.4.4.2 Cytotoxicity

Snakebite envenomation usually involves cytotoxicity to varying degree, which typically characterized by dermal necrosis of local tissue around the bite site (Muller et al., 2012). Clinically, venom-induced cytotoxicity may manifest as local tissues necrosis, systemic myonecrosis, nephrotoxicity, hemolysis, cardiotoxicity etc. (Alirol et al., 2010; WHO, 2010a), through the direct or indirect mechanism of cell necrosis and apoptotic-inducing effect (Brook et al., 1987; Omran et al., 2004; Wang et al., 2005). Among various cytotoxic venom components, cytotoxin (CTX) is the most investigated. Structurally, CTXs belong to the three-finger toxin (3FTx) family and they exhibit distinct and diverse pharmacological activities as compared to other 3FTxs. In general, CTXs are basic proteins with hydrophobic three-finger loops that enable these proteins to insert into the anionic phospholipid membrane to cause membrane damage and to initiate a series of intracellular event that can lead to organelle injuries (Dubovskii et al., 2001).

Other than CTX, venom components like L-amino acid oxidase (LAAO), phospholipase A_2 (PLA_2), snake venom metalloproteinases (SVMP), disintegrins, Kunitz-type serine protease inhibitor and snake venom C-type lectin are also cytotoxic

components (Fox, 2013; Gasanov et al., 2014; Gopalakrishnakone et al., 1997; Tan & Fung, 2009). These cytotoxic components may act synergistically to potentiate cytotoxic effect of venom and may facilitate tissue digestion of the prey. In human, the cytotoxic and necrotic effects around the bite wound can be extensive and the development of non-healing wound is common in patients who survived the snakebite envenomation. It is known that these local tissue-damaging effects are usually not reversed by antivenom as the extravenous tissue at the wound site is hardly accessible to the antivenom (WHO, 2010b). This situation can be further complicated by various secondary infections that could lead to gangrene and necessitate amputation. Thus, the wound needs to be handled with extensive care to reduce the risk of developing into a crippling disability.

2.4.4.3 Venom-induced Cytotoxic Complications

Cytotoxicity of venom can cause systematic cell damage and eventually lead to myonecrosis, nephrotoxicity or cardiotoxicity. Myonecrosis is usually a result of myotoxicity, where tissue destruction has spread into the underlying skeletal muscles. Systemic myotoxicity manifested as rhabdomyolysis (and the resultant myoglobinuria) is seen in envenoming by certain species of both elapids and viperids, including sea snakes, some Asiatic kraits, Australian elapids and viperids like Russell's viper and American rattlesnakes, mainly due to the action of myotoxic PLA_2 (Alirol et al., 2010; Gopalakrishnakone et al., 1997; Phillips et al., 1988). Myonecrosis represents a tissue-specific cytotoxic activity of snake venom and is generally caused by two types of snake venom toxins: phospholipase A_2s (Damico et al., 2008) and small basic peptides called myotoxins, e.g. crotamine (Hayashi et al., 2008). The myotoxic effect can lead to other risks of cardiac and renal complications, where myoglobinuria could obstruct the renal tubule and cause acute kidney failure (nephrotoxicity) (Gopalakrishnakone et al., 1997),

while the hyperkalemia may predispose the patient to cardiac arrhythmias. Furthermore, the venom of some spitting elapids contains abundant cytotoxic components that can cause extensive conjunctivitis and corneal epithelial erosion in venom ophthalmia (Chu et al., 2010).

2.4.4.4 Hemotoxicity

Hemotoxic effects of snakebite envenomation are typically caused by viperid or crotalid bites and can result in severe complications, such as coagulopathy (Berling & Isbister, 2015; Isbister, 2009; Kini et al., 2001) and hemorrhage (Fox & Serrano, 2009). Similar to systemic myotoxicity that causes rhabdomyolysis, the hemotoxic effect can lead to intravascular hemolysis, and the resultant hemoglobinuria can potentially cause obstructive tubulopathy and acute kidney injury (nephrotoxicity) (Sitprija, 2006). Notably, snake venom metalloproteinases (SVMP) and snake venom serine protease (SVSP) are also the toxins that can contribute significantly to venom-induced hemotoxicity. SVMPs can be generally classified into PI–PIV subtypes and they exhibit diverse toxic actions (Fox & Serrano, 2005), including hemorrhage, prothrombin activation and fibrinogenolysis (Fox & Serrano, 2009; Gutierrez et al., 2005). On the other hand, SVSPs are capable of inducing defibrinogenation that can lead to venom-induced consumptive coagulopathy (VICC). Both coagulopathy and hemorrhagic syndromes will lead to bleeding and hypovolemic shock, and the hypoperfusion of vital organs such as kidneys will further complicate the management (Mackessy, 2010; Sitprija, 2006).

2.5 Variations in Snake Venom Composition

The composition of snake venom is consequent of evolution and molecular adaptation to suit the specific ecological niche. Venom variations can be considered at several levels: interfamily, intergenus, interspecies and intraspecies (Chippaux et al., 1991). For instance, elapid venoms usually consist mainly of low molecular mass neurotoxins that can cause respiratory paralysis. On the other hand, viperid venoms consist mostly of enzymes that can cause coagulopathy and hemorrhage. Although the venom from closely related snake species generally shares similar venom composition and toxinological activity, yet for some species, the venom composition can vary remarkably, even within congeneric (interspecies) or intraspecific species, as a result of differences in their ecological niche and the consequent genetic adaptation (Mackessy, 2009). The implication of this phenomenon is medically relevant, as diverse toxin composition can lead to varied envenoming effects and treatment outcome (Glenn et al., 1983).

2.5.1 Factors Causing Venom Variations

Intraspecific venom variations as a result of geographical factor have been well-documented. For examples, Indian cobra (*Naja naja*) from different regions of Indian continents (Shashidharamurthy et al., 2002), king cobra (*Ophiophagus hannah*) from two different Southeast Asia localities (Tan et al., 2015a) and Chinese cobra (*N. atra*) from the east and the west of Taiwan Island (Huang et al., 2015) have been reported to exhibit geographical variations. Previous studies also showed that ontogeny and diet differences can influence the composition of venom within the same species, as in the Amazonian pit viper fer-de-lance, *Bothrops atrox* (Guércio et al., 2006). Apart from this, venom paedomorphosis had also been recorded in *Crotalus oreganus concolor* as a result of the ontogenetic shift in diet (Mackessy et al., 2003). Another factor of venom

variations includes sex of the snake (e.g. in *Calloselasma rhodostoma*) (Daltry et al., 1996). These findings suggest that the variation of venom composition induced by the environmental factors can be multi-causal and differ from species to species. Thus, a thorough understanding of the pathogenesis of snakebite requires knowledge on the venom variability. This knowledge can contribute to the improvement of snakebite management, selection of appropriate antivenom as well as the development of effective antivenom for paraspecific use.

2.6 Snake Antivenom

"Antivenomous sera", the prototypic form of today's snake antivenom was first developed by Albert Calmette (Calmette, 1896). The antivenom is essentially immunoglobulins or their derivatives, sourced from animals (horses, sheep, camels) that have been hyperimmunized with repeated sublethal doses of snake venom. Antivenom acts by forming immunocomplexes with toxins, thereby rendering the toxin inactive biologically. For more than a century, snake antivenom remains as the only definite and etiological treatment for snakebite envenomation, where it is applied to confer an artificially acquired passive immunity in patients. The treatment of many other centennial diseases such as diphtheria and tetanus has undergone a tremendous revolution, advancing from the use of biologics (therapeutic antibodies) to preventive medicine where vaccination (prophylaxis) is practiced. Although attempts had been made for vaccination against snakebites envenomation, the outcome has never been satisfactory and this strategy has been long aborted (Chippaux, 2006).

2.6.1 Antivenom: Product Formulation and Pharmacokinetics

There are three main types of antivenom: whole antibody molecules (IgG); Fab immunoglobulin fragments and $F(ab)_2$ immunoglobulin fragments. The whole antibody product consists of the entire immunoglobulin G (IgG) molecule, whereas antibody fragments are derived by digesting the whole IgG into Fab or $F(ab')_2$ using proteolytic enzymes papain and pepsin, respectively (WHO, 2010c). The molecular mass of Fab and $F(ab')_2$ are approximately 50 kDa and 100 kDa respectively, whereas the whole IgG product is much larger in molecular mass (150 kDa). These differences greatly affected the distribution of antivenom in the tissues and the rate of its elimination from the patient, as well as its efficacy (Gutierrez et al., 2003; Seifert & Boyer, 2001). In general, $F(ab')_2$ and IgG have a longer half-life (> 60 hours) to sustain the neutralization effect with less likelihood of venom rebound phenomenon which is seen more commonly with Fab antivenom (half-life approximately 10 hours, predisposing to venom-antivenom mismatch) (Seifert et al., 1997). However, Fab has a bigger volume of distribution and a shorter half-life, which may be favorable for deep tissue penetration to interact with toxins that have been deposited in the tissue. Upon clearance, IgG and $F(ab')_2$ as well as the immunocomplex formed are generally large (> 100 kDa) and are usually eliminated through phagocytosis; whereas Fab and some of its immunocomplexes can be eliminated through kidney filtration. However, the elimination process of Fab and its immunocomplexes may lead to kidney injury as it could obstruct the renal tubule. On the other hand, IgG is prone to cause hypersensitivity, and the removal of Fc fragment from IgG (producing Fab or $F(ab')_2$) greatly reduces the allergenic property of antivenom.

2.6.2 Monovalent and Polyvalent Antivenoms

Based on the immunization protocol and the target species for neutralization, antivenom products can be broadly classified into two types: “monovalent” or mono-specific when the antivenom is raised against a single venom, while “polyvalent” or poly-specific if the antivenom is produced against several types of venom (WHO, 2010c). The monovalent antivenom is usually used if the biting species is identified, whereas polyvalent antivenom is used when biting species cannot be ascertained, or when specific monovalent antivenom is not available. The pros and cons of using polyvalent antivenom vis-à-vis monovalent antivenom have been extensively debated (WHO, 2010c). Although many approaches have been attempted to produce highly effective antivenom with minimal risk of antivenom-induced adverse effects, the fact remains that for elapids, most of the commercially available antivenom is still with moderate efficacy at best. Due to the low to moderate potency, a very large dose of antivenom is usually required for treatment of a severe envenomation by elapids. In particular for cobra bites in this region, where an initial dose of 10 vials of the antivenom is often prescribed, and may need to be followed up with additional doses (10 vials), in which the administration of high dose of antivenom increases the risk of hypersensitive reactions (Malasit et al., 1986).

2.7 Proteomics

2.7.1 Proteomics Studies of Snake Venom

Snake venom is a toxin “cocktail” comprised of a variety of proteins that exhibit diverse pharmacological actions. Modern proteomic technology and bioinformatics have made it possible to study the global expression of venom protein composition, even for those venom proteins that exist in a very low amount (Matthiesen & Mutenda,

2007; Poon & Mathura, 2009). This approach, known also as "venomics" to the toxinologist, offers great potential for a clear understanding of the pathogenesis of envenomation as well as drug discovery and improvement of antivenom production (Calvete et al., 2009; Gutiérrez et al., 2009). In addition, many recent breakthroughs in high-throughput -omics technologies, not only in the area of proteomics using high-resolution mass spectrometry but also in transcriptomics of venom gland and genomics of the snake species using next generation sequencing technology, allows access to unprecedented detailed information that is revolutionizing venom research (Baldwin, 2004).

2.7.2 Separation of Venom Components

Snake venom comprised of various proteins that can be similar or diverse in physical properties. Thus, many proteomics approaches rely on the pre-separation of a target protein that offers a better resolution as compared to gel-free methods (Aebersold & Mann, 2003; Shevchenko et al., 2007). Generally, the pre-separation can be achieved by using liquid chromatography (according to ionic charges, hydrophobicity or molecular mass) and sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), or 2-dimensional gel-electrophoresis (according to isoelectric point and molecular mass) (Pitt, 2009; Russell & Buess, 1970; Tan & Tan, 1988b). The peptide produced from the digestion of proteins interest by proteolytic cleavage (usually trypsin) will then be subjected to protein identification using mass spectrometry. However, venom proteins can also be subjected directly to mass spectrometry (without pre-separation techniques and/or protein digestion).

2.7.3 Mass Spectrometry - Protein Identification

2.7.3.1 Proteins/Peptides Ionization Methods

Mass spectrometry has been established as an extremely powerful tool for protein identification even for complex mixtures. The advance in instrumentations especially the nano-detection by mass spectrometry and bioinformatic tools have enabled the protein characterization easily achieved with the use of a very small amount of samples. The protein or peptide (after digestion) introduced into a mass analyzer will first be ionized by either one of the two common ionization methods: matrix-assisted laser desorption/ionization (MALDI) or electrospray ionization (ESI), where both are "soft-ionization" techniques (Yates et al., 2009) (Figure 2.4).

MALDI is a three-steps technique where the sample is initially fixed with a suitable matrix, and the ionization of samples is carried out using a laser beam to trigger the ablation and desorption of samples and matrix materials (Karas & Krüger, 2003). In an electric field, sample's ions are accelerated according to their mass and electrical charges, where the drift path allows further separation and leads to differences in "time of flight" (TOF) of the desorbed particles. With these approaches, the exact mass of the polypeptides can be calculated by using MALDI-TOF.

In ESI, samples are dissolved in a solvent and injected into a microcapillary with a high voltage applied to create an aerosol of charged droplets of the sample (Ho et al., 2003). These droplets are sprayed through the compartment with diminishing pressure, which will result in the formation of gas-phase multiple-charged analyte ions, and subsequently detect using mass spectrometry. Unlike MALDI, the use of ESI has overcome the propensity of the molecules to fragment where it induces very little fragmentation when ionized. This approach is more advantageous as molecular ions are always observed.

2.7.3.2 Mass Spectrometry Approaches – "Top-Down" and "Bottom-Up"

Following ionization, two approaches are widely applied in the proteomic analysis: "top-down" and "bottom-up" (Chait, 2006). The "bottom-up" strategy is more commonly used in the modern mass spectrometry-based proteomic approach to detect the presence of certain protein(s) in a mixture, which is also known as "shotgun proteomics". This strategy characterizes the protein by assembling the peptide fragment produced through proteolytic cleavages of biological samples (usually by trypsin or chymotrypsin) (Figure 2.5). On the other hand, "top-down" mass spectrometry is a more direct approach that has recently emerged as an alternative to the "bottom-up" strategy (Kellie et al., 2010). This strategy is more advantageous as it can detect the native molecular mass of intact protein using the mass spectrometry (i.e., without prior protease digestion). As protein detection is performed on the native form of protein, the mass information retained are capable of providing a more superior characterization of the entire protein, including the information on post-translational modification.

Tandem mass spectrometry (MS/MS) analysis involves a series of events where precursor ions are created at the initial stage and subjected to fragmentation for the second stage of analysis. Given MS/MS spectra for the peptide fragments will be subjected to filter process and MS/MS spectra above a certain confidence level will be considered for subsequent peptide searching. These MS/MS spectra obtained are matched to the mass of peptide fragments that produced through external enzymatic *in silico* digestion of public/customized protein database by search engines such as MASCOT or SEQUEST using different computational search algorithms. The search engine will then annotate the analyzed sample with protein identity that gives a series of matches according to the similarity in peptide masses/sequences.

It should be noted that the common approach of protein identification relies on the protein database to match to the experimental MS/MS spectra, and thus insufficient of a complete protein database could cause several drawbacks and difficulties in protein identification. This limitation could be partly overcome by recent breakthroughs in high-throughput technologies in the transcriptomic study of snake venom gland and the genomic study of snake species that could greatly contribute to the expansion of data depository dramatically (Tan et al., 2015a; Tan et al., 2015c; Vonk et al., 2013). These studies play an important role to fill up the gaps, as more sequences deposited in the database will help in the development of "venomic" research.

Apart from this, novel proteomics approaches are also introduced to identify the "unknown/unidentified" protein with the use of protein *de novo* sequencing. Generally, the *de novo* sequencing can be achieved by either one of the two different methods: Edman degradation or tandem mass spectrometry. The former (Edman degradation, used for determination of N-terminal sequences) is infrequently used currently as the technique is inadequate for a global analysis of protein and is very time-consuming (Niall, 1973). On the other hand, tandem mass spectrometry can identify the "unknown/unidentified" protein using *de novo* sequencing software package (PEAKS). The software is able to extract amino acid information without the use of protein database where the entire amino acid sequences will be given according to the confidence scores generated from mass spectrometry (Ma et al., 2003). Apart from this, a more robust technique of protein *de novo* sequencing has also been recently introduced into the field, where protein identification could be achieved through the combined analysis using both "bottom-up" and "top-down" strategies with a new algorithm, TBNovo (Liu et al., 2014). The advancement in technologies and bioinformatic tools certainly will lead to another major break-through in "venomic" research.

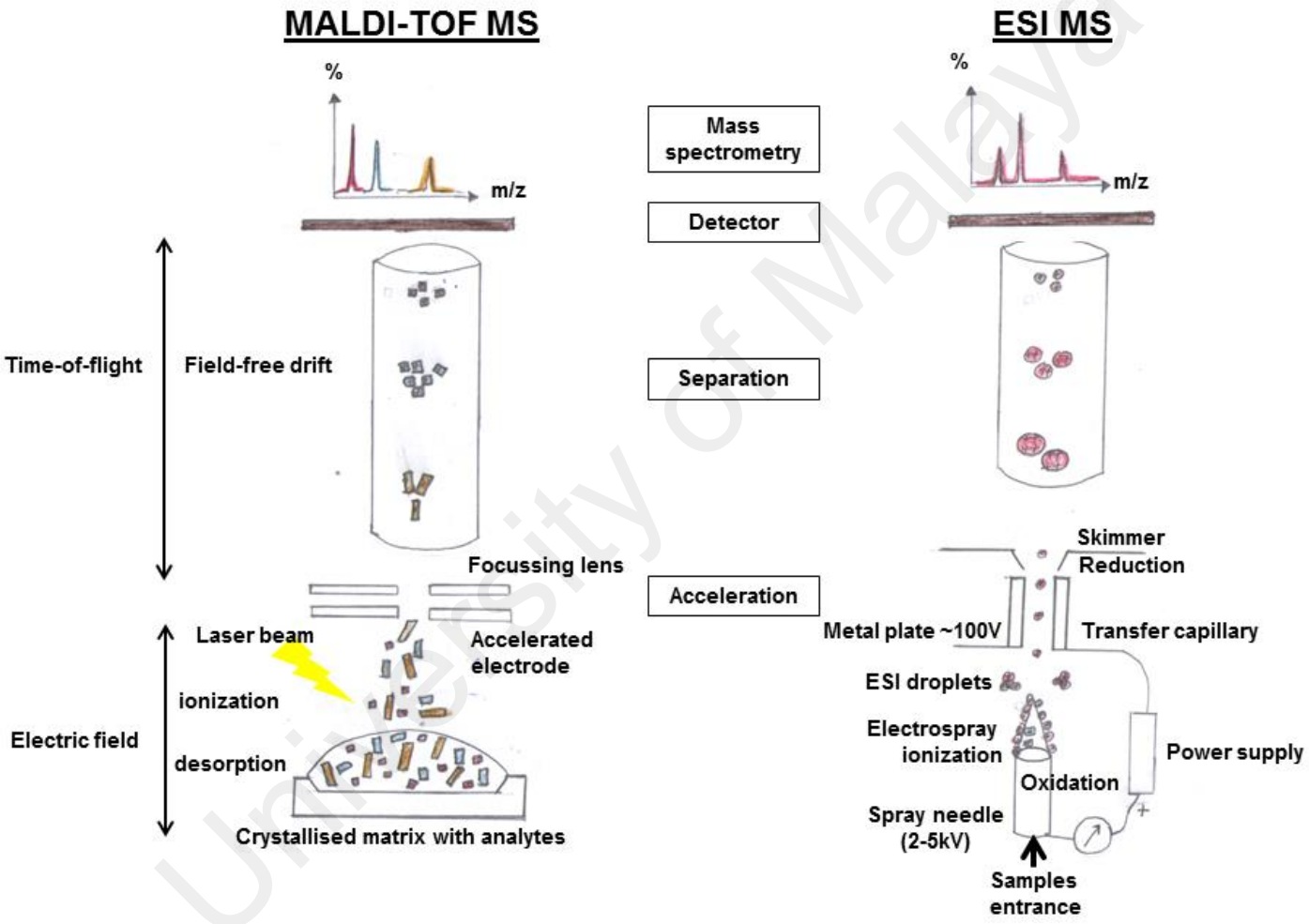


Figure 2.4 Principle of matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) and electrospray ionization (ESI) mass spectrometry protein identification. The image was produced by drawing with modification from Lavigne et al. (Lavigne et al., 2013).

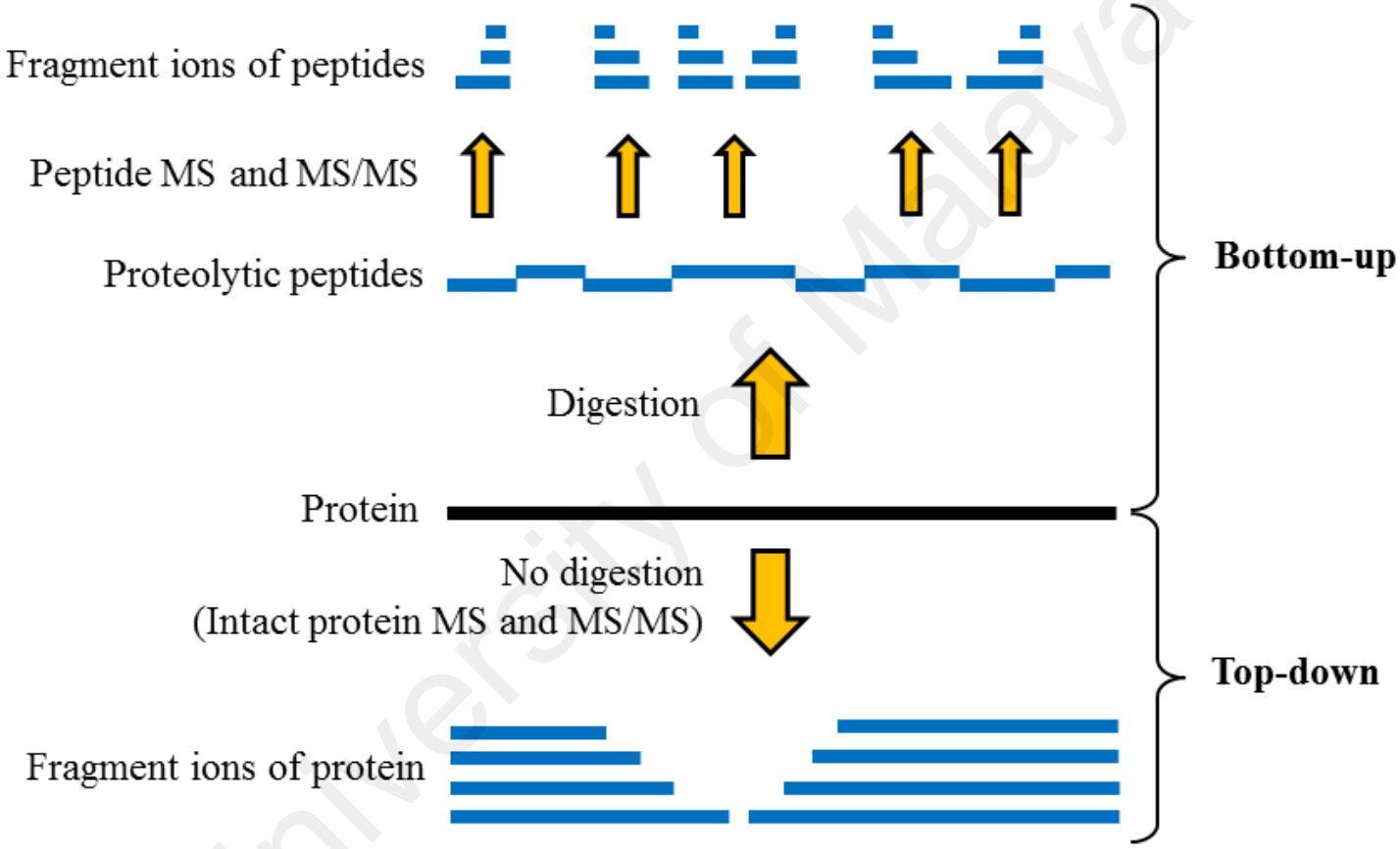


Figure 2.5 "Bottom-up" and "top-down" approach for protein characterization and identification. The "bottom-up" strategy utilized protease to cleave the intact protein into peptides prior to subjected to the tandem mass spectrometry and identification was done by matching the *in silico* digested protein database. The "top-down" strategy uses intact proteins that will be directly analyzed in the mass spectrometry (no protease digestion) and the precursor will be matched to the protein database.

2.8 Toxinological Characterization of Snake Venom

In snakebite envenomation, complex syndromes and clinical presentations are commonly reported in victims. Different snake venoms give rise to different clinical syndromes e.g. neurotoxic, haemotoxic, nephrotoxic, myotoxic and/or cytotoxic effects. However, in many cases, the snakebite envenomation is usually predominated by certain toxic effect although more than one clinical effects can be observed in an envenomation (Mackessy, 2009). The tissue/organ-specific toxicity terminology is therefore only loosely used to denote the main toxic effect expected for the venom of a particular species. With this knowledge, appropriate monitoring and expectant management can be arranged to tackle the likely pathological outcome. These include the measures such as intubation facility for victims suffering from neurotoxic bite, volume replacement protocol for hemotoxic bite, and renal replacement therapy (dialysis) for the bite that may lead to nephrotoxicity.

Clinical study of snakebite envenomation is indispensable and useful for the correlation with fundamental science research. However, the clinical findings occasionally may show inconsistency or wide variability due to human's factors; for instance, the manipulation of the bite wound prior to admission to hospital, and the delay of assessment due to late presentation. Clearly, clinical data alone is inadequate to provide a comprehensive interpretation of the pathophysiological action of venom and its sequelae. Thus, laboratory characterization of toxic effects of snake venom and envenoming treatment is relevant and useful. The use of relevant and appropriate models in toxinological studies can provide clues to the comprehensive elucidation of the pathophysiology of snakebite envenomation, specific to the type of venom studied. Some essential aspects in toxinological characterization of snake venom are summarized in Table 2.2 and discussed in following sections.

2.8.1 Biochemical and Enzymatic Studies

Enzymatic characterization has been widely used as a fundamental profiling method of snake venoms. The enzymatic activity shown by the snake venom reflects the presence of a particular component in the venom, and the level of the activity can be quantitatively measured and compared among different snake venoms (Tan, 1991). Currently, various assays for common snake venom enzymes are well established and are often used prior to in-depth venomic study; these include assays for protease, phosphodiesterase, L-amino acid oxidase, alkaline phosphomonoesterase, 5' nucleotidase, hyaluronidase, phospholipase A_2 and acetylcholinesterase (Table 2.2). Besides, the capability of snake venom in causing coagulopathy and fibrinogenolysis can also be examined enzymatically, and these are especially important in characterizing the viperid or crotalid venoms with prominent hemotoxic effect (Theakston & Reid, 1983).

2.8.2 *In vitro* Characterization (Cell Culture and Isolated Tissue)

2.8.2.1 Toxicity Assessment - Cell Culture

Other than enzymatic assays, toxic effects of snake venom can also be assessed using different cell lines. The use of various types of cell lines represents the different cell target in envenomation. For instance, nicotinic acetylcholine receptors (nAChRs) and cells can be extracted from the *Torpedo* electric organ (Fulpius et al., 1981) and cultured, in which these receptors can be used to examine the binding affinity of neurotoxins (Servent et al., 2000; Servent et al., 1997). In addition, cell lines can also be used to evaluate the cytotoxicity of snake venom (Jamunaa et al., 2012); for instance, myoblast cell culture would be another useful model for myotoxicity screening (Butler et al., 1998) (Table 2.2).

2.8.2.2 Neurotoxic and Myotoxic Studies - Chick Biventer Cervicis Nerve-Muscle (CBCNM)

CBCNM preparation is an isolated tissue from chick consisting of two different types of muscle fibers: focally- and multiply-innervated muscle fibers (Harvey & van Helden, 1981; Toutant et al.). The focally-innervated muscle fibers can mediate the transient "twitch" in response to electrical stimulation whereas the multiply-innervated muscle fibers mediate a more prolonged contraction produced by exogenous agonists of nicotinic receptor. The presence of these two muscle fibers in CBCNM was shown to be more advantageous as compared to rat or mouse phrenic nerve hemidiaphragm (only focally-innervated muscle fibers) and is a simpler and more robust model (Harvey et al., 1994) (Table 2.2).

Using CBCNM, types of neurotoxin binding (pre-synaptic, beta-neurotoxins; post-synaptic, alpha-neurotoxins) can be readily distinguished from the contractile response (without electrical stimulation) to exogenous nicotinic agonists (acetylcholine and/or carbachol). Both beta-neurotoxins and alpha-neurotoxins are able to deplete the nerve-evoked twitches in CBCNM in the presence of electrical stimulation. Nevertheless, only alpha-neurotoxins able to abolish the contractile response of CBCNM to exogenous nicotinic agonists (after incubating with venom) in contrast to the beta-neurotoxin, where the tissue remains responsive to the stimulant. Thus, the CBCNM preparation is an excellent screening tool for neurotoxicity assessment of snake venom, as well as in the characterization of neurotoxins (Harvey et al., 1994; Hodgson & Wickramaratna, 2002; Kornhauser et al., 2010).

On the other hand, electrical stimulation can also be applied directly to the muscle's belly of CBCNM to induce muscle-evoked twitches. The reduction in direct twitches reflects the loss of intactness of muscle fibers, as a result of myotoxicity induced by the

snake venom. In the current study, CBCNM was used as a model to evaluate neurotoxicity and myotoxicity of *N. kaouthia* venoms at different experimental settings (**Chapter 5**).

2.8.3 *In vivo* – Whole Animal Study

2.8.3.1 Pharmacokinetic Study

Pharmacokinetics refer to a study of a series of biological processes involving the absorption, distribution, metabolism and excretion of a drug (or compound) when the substance (compound) has been administered to a living organism. Using animals such as rats, rabbits and swines, pharmacokinetic experiments not only can measure the change of venom or toxin concentration in blood over a time course but also the concentration of venom or toxin in the tissue. The venom's pharmacokinetic study is useful as the information derived can improve understanding of the evolution of clinical syndromes and provides insights into how treatment protocols including dosing regimen can be optimized (Table 2.2).

2.8.3.2 Hemorrhagic, Necrotic and *In vivo* Defibrinogenation

Hemotoxic effects are typically caused by viperid or crotalid bites (Berling & Isbister, 2015; Isbister, 2009; Kini et al., 2001); while necrotic effects occur at the local wound are common in snake envenomation. Hemorrhagic and necrotic effects of snake venom could be examined using the dorsal skin of rodent that contains an excellent vascular network beneath the skin (Gutierrez et al., 1985). On the other hand, defibrinogenation caused by snake venom can also be examined by analyzing the blood of experimentally envenomed rodents (Ramos-Cerrillo et al., 2008) (Table 2.2).

2.8.3.3 Lethality

Lethality of snake venom (or toxin) is commonly presented with the parameter median lethal dose (LD_{50}), which is defined as the amount of venom (or toxin) per gram body mass (µg/g) in order to cause death in 50% of the experimental animals (Ramos-Cerrillo et al., 2008). In toxinological characterization of snake venom, the use of *in vivo* model involves a whole biological system and is likely more reflective of true envenomation process, thus providing a more meaningful and realistic information. It has been known that the administration of venom into the experimental model through different routes could reflect different circumstances of envenomation during the assessment of lethality. For instance, administration of venom through subcutaneous or intramuscular routes mimics the actual envenomation by elapids and viperids, respectively. On the other hand, the intravenous administration of venom ensures a full systemic access of venom into the animal, therefore enabling the assessment and interpretation of its systemic toxicity in the light of the amount of venom that fully bioavailable to the animal. This approach would be useful in experiments examining the neutralization of venom by antivenom under controlled titration (Leong et al., 2012; Leong et al., 2014).

Apart from this, the evolution of clinical syndromes can be closely observed with the use of *in vivo* model (usually rodents), especially for the development of neurotoxic syndrome that is well reflected in rodents experimentally envenomed with elapid venoms. Lethality is an established outcome of neurotoxic envenomation in rodents, and neutralization of the lethal effect (neurotoxicity) by antivenom has been regarded as the gold standard for antivenom assessment (Warrell et al., 2013; WHO, 2010c).

Table 2.2: Characterization of venom toxicity using *in vitro* and *in vivo* methods.

Methods/Model	Examination	Model	Observation	References
Biochemical and Enzymatic assays	Protease	Casein	*In vitro*: caseinolysis	Kunitz, 1947
	Phosphodiesterase	Substrate mixture	*In vitro*: hydrolysis of substrate	Lo & Chen, 1966
	Alkaline phosphomonoesterase	Substrate mixture	*In vitro*: hydrolysis of substrate	Lo & Chen, 1966
	L-amino acid oxidase	Substrate mixture	*In vitro*: oxidization of substrate	Decker, 1977
	5' nucleotidase	Substrate mixture	*In vitro*: release of phosphate	Heppel & Hilmore, 1955
	Hyaluronidase	Substrate mixture	*In vitro*: hydrolysis of substrate	Dorfman, 1955
	Phospholipase A_2	Egg yolk	*In vitro*: phospholipid degradation	Tan & Tan, 1988a
	Acetylcholinesterase	Substrate mixture	*In vitro*: hydrolysis of substrate	Ellman et al., 1961
	Procoagulant	Human plasma	*In vitro*: time of coagulation formed	Theakston & Reid, 1983
	Fibrinogen clotting	Fibrinogen solution	*In vitro*: fibrinogen clotting	Tan & Ponnudurai, 1996
Cell culture	Neurotoxicity	Nicotinic receptor	*In vitro*: binding affinity	Servent et al., 1997
	Cytotoxicity	Normal/cancer cell lines	*In vitro*: cell cytolysis	Jamunaa et al., 2012
	Myotoxicity	Myoblast	*In vitro*: cell cytolysis	Butler et al., 1998)
Isolated tissue	Neurotoxicity	Chick biventer cervicis	*In vitro*: inhibition of nerve-evoked twitches	Harvey et al., 1994
		Rat phrenic nerve hemidiaphragm	*In vitro*: inhibition of nerve-evoked twitches	Harvey et al., 1994
	Myotoxicity	Chick biventer cervicis	*In vitro*: inhibition of nerve-evoked twitches	Harvey et al., 1994
		Rat phrenic nerve hemidiaphragm	*In vitro*: inhibition of nerve-evoked twitches	Harvey et al., 1994
	Nephrotoxicity	Renal proximal tubules	*In vitro*: cell injury was determined by release of lactate dehydrogenase	de Castro et al., 2004
Whole animal	Lethality	Mice	*In vivo*: median lethal dose as indicator	Ramos-Cerrillo et al., 2008
	Haemorrhagic	Mice – dorsal skin	*In vivo*: increase in hemorrhage effect	Gutierrez et al., 1985
	Necrotic	Mice – dorsal skin	*In vivo*: increase in necrotic effect	Gutierrez et al., 1985
	In vivo defibrinogenation	Mice – blood	*In vivo*: dose of blood unable to coagulate	Ramos-Cerrillo et al., 2008
	Pharmacokinetics	Rabbit	*In vivo*: snake venom and toxins pharmacokinetics	Yap et al., 2014b

2.9 Antivenom Neutralization of Venom Toxic Effects

In snakebite envenomation, antivenom therapy is the only established effective treatment (WHO, 2010b). The quality of antivenom, however, varies widely between products (WHO, 2010c). This is of great concern for countries that rely on imported antivenom produced in other countries, as the efficacy of the imported antivenom against local snake venoms is often questionable but not rigorously assessed. Robust and rigorous preclinical assessment of antivenom is indispensable to ensure that only appropriate, effective and safe antivenom would be used for life-saving. Several methods (*in vitro* or *in vivo*) have been practiced in preclinical assessment of antivenom. These are briefly discussed in following sections.

2.9.1 "Antivenomics"

Recent advances in venomics have led to the development of "antivenomics" which help to shed light on the immunocomplexing capability of antivenom (Calvete et al., 2011). Briefly, the venom-antivenom mixture (in solution) is passed through an immunoaffinity column prepared from the protein G to retain the immunocomplex formed. The eluted fraction (that is not retained by affinity column) which contains "unbound/unneutralized" venom proteins is then subjected to reverse-phase HPLC and proteomic analysis to identify the "unbound/unneutralized" venom proteins. However, this approach is based on the immunological binding and does not demonstrate the functional aspect of "neutralization". For instance, the immunocomplex formed may remain active, or continuous absorption of the venom may exceed the amount of antivenom available. The removal of these immunocomplexes by affinity column also does not reflect the actual reversal mechanism of antivenom in a biological system, and thus, it is not entirely appropriate to use antivenomic findings to assess the capacity of

the antivenom to neutralize “toxicity”. Nevertheless, the assay is a good screening tool for assessing the immunological reactivity and binding capacity of antivenom to toxins.

2.9.2 *In vitro* and *In vivo* Neutralization

In view of the limitation of “test-tube” model in reflecting the actual mechanism of antivenom neutralization, more rigorous pre-clinical assessments using rodent model or isolated tissue are necessary. Pre-incubation method, where a challenge dose of venom or toxin is preincubated with serial doses of antivenom, is most commonly practiced. Immunocomplexation is expected to take place under controlled titration, followed by testing the lethal effect of the mixture in whole animals (Leong et al., 2012; Leong et al., 2014; Tan et al., 2011).

An alternative method, the challenge-rescue approach has the benefit of mimicking snakebite envenomation (Lomonte et al., 2009), and it takes into consideration the role of the pharmacokinetics of venom toxins and antivenom in their *in vivo* interaction. This *in vivo* assay will also be able to detect recurrence of toxicity in treated animals, which could be due to venom rebound phenomenon (where venom half-life exceeds that of antivenom) or *in vivo* reversibility of immunocomplexation (where toxins dissociate from the antivenom binding). Often, functional and mechanistic studies such as neurotoxicity assessment using isolated tissues (CBCNM in this study) will also be investigated to further validate the result. Typically, the efficacy of the antivenom is quantitated using parameters such as median effective dose (ED_{50}), median effective ratio (ER_{50}), neutralization potency (P) etc. as shown in Table 2.3. (Finney, 1952; Leong et al., 2012; Morais et al., 2010).

Another approach involving neutralization of major venom toxins has been used to investigate antivenom neutralization of venom (Leong et al., 2015; Leong et al., 2012).

This approach is termed as “toxin-specific neutralization”. As an overall, these *in vitro* and *in vivo* studies contribute significantly to the understanding of the “strength and limitation” of an antivenom in neutralizing specific venom and toxin. This information offers valuable insights into the potency and the species coverage of an antivenom and how it can be further optimized.

Table 2.3: Antivenom neutralization of the venom toxic effects induced by snake venom using *in vitro* and *in vivo* methods.

Toxic effect	Model	Parameter	Methodology	Effective measurement	References
Lethality	Mice	LD_{50}	Preincubation with antivenom for 30 min, followed by *i.v.*, *i.p.* or *subcut* injection.	ED_{50} and P, survival ratio of 50%	Ramos-Cerrillo et al., 2008; Tan et al., 2015d
			Venom injected through *subcut.* or *i.m.*, followed by *i.v.* administration of antivenom		Leong et al., 2014; Tan et al., 2015d
Procoagulant	Mice	MCD	Preincubation with antivenom for 30 min, followed by plasma clotting test	ED, prolonged of clotting time	Theakston & Reid, 1983
Hemorrhagic	Mice	MHD	Preincubation with antivenom for 30 min, followed by *i.d.* injection	ED_{50}, hemorrhagic site reduced by 50%	Gutierrez et al., 1985
Necrotic	Mice	MND	Preincubation with antivenom for 30 min, followed by *i.d.* injection	ED_{50}, necrotic site reduced by 50%	Gutierrez et al., 1985
In vivo defibrinogenation	Mice	MDD	Preincubation with antivenom for 30 min, followed by *i.p.* injection	Minimal dose of clot formation	Ramos-Cerrillo et al., 2008
Neurotoxicity	Tissue	1-5 µg/ml, based on t_{90} value	CBCNM preincubated with antivenom, 10 min prior addition of venom	Reversal (%) of muscle twitches inhibition	Barfaraz & Harvey, 1994; Harvey et al., 1994
Neurotoxicity	Tissue	1-5 µg/ml, based on t_{90} value	CBCNM preincubated with venom, followed by addition of antivenom	Reversal (%) of muscle twitches inhibition	Barfaraz & Harvey, 1994; Harvey et al., 1994
Myotoxicity	Mice	Predetermined dose	Preincubation with antivenom for 30 min followed by *i.m* .injection.	Decrease level of plasma creatine kinase (CK)	Fernandes et al., 2011
	Tissue		CBCNM preincubated with venom, followed by addition of antivenom	Reversal (%) of muscle twitches inhibition	Ramasamy et al., 2004
Nephrotoxicity	Tissue	250 µg/ml	Isolated renal proximal tubules preincubated with venom, followed by addition of antivenom	Measuring the level of lactate dehydrogenase (LDH)	de Castro et al., 2004

LD_{50}: median lethal dose; MCD: minimum coagulant dose; MHD: minimum hemorrhagic dose; MND: minimum necrotic dose; MDD: minimum defibrinogenating dose; t_{90}: time to establish 90% inhibition; ED_{50}: median effective dose; P: neutralization potency; *i.v.*: intravenous; *i.m.*: intramuscular; *i.d.*: intradermal; *i.p.*: intraperitoneal; *subcut.*: subcutaneous; CBCNM: chick biventer cervicis nerve muscle

CHAPTER 3: GENERAL METHODS AND MATERIALS

3.1 Materials

3.1.1 Animals and Ethics Clearance

Albino mice (ICR strain, 20-30 g) used in the present study were supplied by the Animal Experimental Unit, Faculty of Medicine, University of Malaya. Male chicks (4-10 days old) used in the pharmacological study were obtained from a local farm. The animals were handled according to the guideline given by the Council for International Organizations of Medical Sciences (CIOMS) on animal experimentation (Howard-Jones, 1985). All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Malaya, Kuala Lumpur (Ethical clearance letter No. 2013-06-07/MOL/R/FSY and 2014-09-11/PHAR/R/TCH).

3.1.2 Euthanasia

Albino mice (ICR strain, 20-30 g) that survived in the toxicity and neutralization experiments were euthanized using carbon dioxide (CO_2) inhalation (Supplier: The Linde Group, Malaysia), as adapted from IACUC Guideline to provide rapid, painless, stress-free death. Chicks were euthanized using isoflurane (supplied by Animal Experimental Unit, Faculty of Medicine, University of Malaya) prior to biventer cervicis tissue harvesting.

3.1.3 Snake Venoms

The venom of Malaysian *Naja kaouthia* (NK-M) was collected from specimens (n = 5-10) in the northern region of the Malayan Peninsula. Venom specimens from Thailand (NK-T) and Vietnam (NK-V) were pooled samples from adult snakes (n = 5-10) and were gifts from Professor Kavi Ratanabanangkoon of the Chulabhorn Graduate Institute, Bangkok, Thailand. All milked venoms were lyophilized and stored at -20 °C until use.

3.1.4 Snake Antivenoms

Three commercial antivenoms were used in the present study (Table 3.1). Two of these are products of Queen Saovabha Memorial Institute (QSMI), Thai Red Cross Society from Bangkok, Thailand: (a) ***N. kaouthia*** **Monovalent Antivenom** (**NKMAV**; also known as Cobra antivenin; batch: 0080210; expiry date: Aug 9th, 2015), purified $F(ab')_2$ derived from the plasma of horses hyperimmunized specifically against the venom of *N. kaouthia* (Thai monocled cobra); (b) **Neuro Polyvalent Antivenom** (**NPAV**; batch: 0020208; expiry date: Oct 5th, 2014), purified $F(ab')_2$ obtained from the plasma of horses hyperimmunized against a mixture of four venoms from *N. kaouthia* (Thai monocled cobra), *Ophiophagus hannah* (king cobra), *Bungarus candidus* (Malayan krait) and *Bungarus fasciatus* (banded krait), all of Thai origin. The antivenoms were supplied in lyophilized form and reconstituted according to the attached product leaflet: 10 ml of normal saline was added to one vial of the lyophilized antivenom.

Another antivenom product is **Australian CSL Sea Snake Antivenom** (SSAV, produced by CSL Limited (recently operates under the brand Seqirus; batch: 0549-08201; expiry date: April 2015). SSAV contains purified $F(ab')_2$ derived from horses immunized with venoms of common beaked sea snake, *Hydrophis schistosus* (formerly

known as *Enhydrina schistosa*) and Australian common tiger snake (*Notechis scutatus*). SSAV used in this study was packaged as 25 ml liquid in an ampoule containing 1000 units of neutralizing capacity against the targeted venom of *H. schistosus* (Each ampoule is standardized to neutralize 10 mg of *H. schistosus* venom, equal to one unit neutralize 0.01 mg of *H. schistosus* venom).

3.1.5 Chemicals and Consumables

3.1.5.1 Liquid Chromatography Columns and Chemicals

Reverse-phase HPLC column LiChroCART® 250-4 LiChrospher® WP 300 was purchased from Merck (USA). Resource® S cation-exchange column (1 ml) was supplied by GE Healthcare (Sweden). HPLC grade acetonitrile was purchased from Thermo Scientific™ Pierce™ (USA). Trifluoroacetic acid (TFA) and 2-(N-morpholino)-ethanesulfonic acid (MES) were obtained from Sigma-Aldrich (USA).

3.1.5.2 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Acrylamide, N-N'-methylene bisacrylamide, Tris-HCl, ammonium persulfate (APS), sodium dodecyl sulfate (SDS), tetramethylethylenediamine (TEMED), beta-mercaptoethanol, bromophenol blue, Coomassie Blue R-250 were procured from Sigma-Aldrich (USA). Spectra™ Multicolor Broad Range Protein Ladder (10 to 260 kDa/ 10 to 170 kDa) was purchased from Thermo Scientific™ Pierce™ (USA). Methanol (Friendemann Schmidt Chemicals, Germany) and acetic acid (J. T. Baker, USA) were supplied by the respective manufacturers.

3.1.5.3 Protein Digestion and Extraction

Ammonium bicarbonate, dithiothreitol (DTT) and iodoacetamide (IAA) were purchased from Sigma-Aldrich (USA). Mass spectrometry grade trypsin protease was purchased from Thermo Scientific™ Pierce™ (USA). Millipore ZipTip® C_{18} Pipette Tips were supplied by Merck (USA).

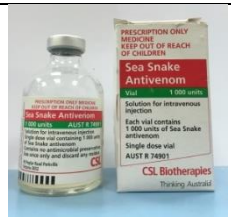
3.1.5.4 Chick Biventer Cervicis Nerve-Muscle (CBCNM) Preparation

Acetylcholine chloride (ACh) and carbachol (CCh) were purchased from Sigma-Aldrich (USA). D-tubocurarine (d-TC) was of pharmaceutical grade and supplied by (Asta-Werke AG, Germany). Sodium chloride (NaCl), potassium chloride (KCl), magnesium sulfate ($MgSO_4$), monopotassium phosphate (KH_2PO_4), calcium chloride ($CaCl_2$), sodium bicarbonate ($NaHCO_3$) and glucose used to prepare physiological salt solution were purchased from Merck (USA).

3.1.5.5 Protein Purification and Concentration Determination

Vivaspin® centrifugal concentrator (2,000 MWCO) was obtained from Sartorius (Germany), while Pierce Bicinchoninic acid (BCA) Protein Assay Kit was from Thermo Scientific™ Pierce™ (USA).

Table 3.1: Information of three antivenoms used in the present study[2].

Antivenom	Venoms used in immunization	Antivenom efficacy (mg/ml) (according to manufacturer)	Manufacturer	Product
Naja kaouthia Monovalent Antivenom (NKMAV)	*Naja kaouthia* (Thai monocled cobra)	0.6 mg	Queen Saovabha Memorial Institute (QSMI), Thai Red Cross Society (Bangkok, Thailand)	
Neuro Polyvalent Antivenom (NPAV)	*Naja kaouthia* (Thai monocled cobra) *Ophiophagus hannah* (King cobra) *Bungarus candidus* (Malayan krait) *Bungarus fasciatus* (Banded krait)	0.6 mg 0.8 mg 0.4 mg 0.6 mg	Queen Saovabha Memorial Institute (QSMI), Thai Red Cross Society (Bangkok, Thailand)	
Australian Sea Snake Antivenom (SSAV)	*Hydrophis schistosus* (formerly *Enhydrina schistosa*) (beaked sea snake) *Notechis scutatus* (Australian tiger snake)	Each ampoule containing 1000 units of neutralizing capacity against *H. schistosus* venom (10 mg)	Australian CSL Limited (presently known as Seqirus Limited)	

[2] The information stated at above was obtained from the product information sheet according to product manufacturers.

3.2 General Methods

3.2.1 Protein Concentration Determination

Protein concentration was determined by using Pierce Bicinchoninic acid (BCA) protein assay kit. The BCA kit works with a similar concept as Lowry protein determination method which using colorimetric detection that indicates the reaction of peptide bonds with copper ions.

3.2.2 High-performance Liquid Chromatography (HPLC)

3.2.2.1 C_{18} Reverse-phase HPLC

Reverse-phase HPLC was conducted using LiChroCART® 250-4 LiChrospher® WP 300 RP-18 (5 µm) HPLC column (Merck, USA) and a Shimadzu LC-20AD HPLC system (Japan) (Figure 3.1). Samples were fractionated according to hydrophobicity, where less hydrophobic proteins were first eluted. The elution protocol of venom samples was adapted from the method described by Correa-Netto et al. (Correa-Netto et al., 2011) to achieve an optimum fractionation. The sample was eluted at 1 ml/min with a linear gradient of 0.1% trifluoroacetic acid (TFA) in water (Solvent A) and 0.1% TFA in 100% acetonitrile (ACN) (Solvent B) (0-5% B for 10 min, followed by 5-15% B over 20 min, 15-45% B over 120 min and 45-70% B over 20 min) (Table 3.2). The protein fractions were detected by measuring UV absorbance at 215 nm.

3.2.2.2 Resource Q Cation-exchange Chromatography

Cation-exchange chromatography was conducted by using Resource® S cation-exchange column (GE Healthcare, Sweden) and Shimadzu LC-20AD HPLC system (Japan) (Figure 3.1). The samples were fractionated according to ionic charges, where

negatively charged acidic proteins were first eluted. The elution protocol was set according to Leong et al. (Leong et al., 2015) and the Resource® S column was pre-equilibrated with 20 mM 2-(N-morpholino)-ethanesulfonic acid (MES), pH 6.0 as eluent A. The elution of proteins was achieved with 0.8 M sodium chloride (NaCl) in 20 mM MES, pH 6.0 as eluent B, using a linear gradient flow of 0-30% B from 5 to 40 min followed by 30-100% B from 40-55 min (Table 3.2). Similarly, the flow rate was set at 1 ml/min, and the protein fractions were detected by measuring UV absorbance at 280 nm.

Table 3.2: Elution protocol of reverse-phase HPLC and cation-exchange chromatography.

Reverse-phase HPLC (180 min)		**Cation-exchange (55 min)**	
Time (min)	Linear gradient (% Solvent B)	Time (min)	Linear gradient (% Solvent B)
0-10	0-5	0-5	0
10-30	5-15	5-40	0-30
30-150	15-45	40-55	30-100
150-170	45-70	55-65	100
170-180	70-100	-	-
180-185	100	-	-

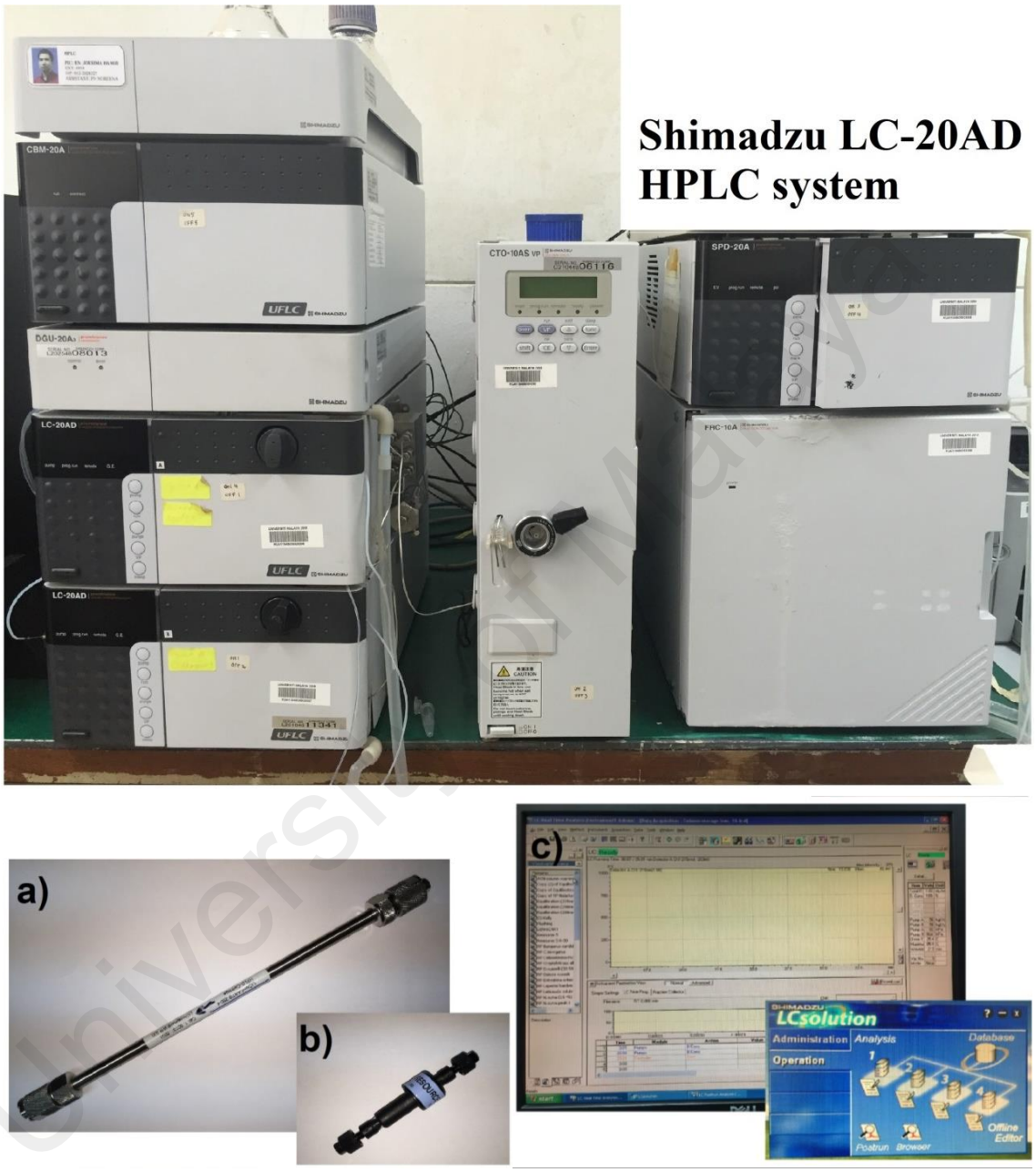


Figure 3.1 Shimadzu LC-20AD HPLC system (Japan). a) LiChroCART® 250-4 LiChrospher® WP 300 RP-18 (5 µm) HPLC column (Merck, USA); b) Resource® S cation-exchange column (GE Healthcare, Sweden); c) Shimadzu LCsolution Software Version 1.23 (Japan).

3.2.3 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The SDS-PAGE was conducted using a mini-PROTEAN® Tetra Cell System (Biorad, USA) according to Laemmli (Laemmli, 1970). SDS-PAGE is a common method for protein separation according to molecular mass by the use of discontinuous polyacrylamide gel as a support medium and SDS as a denaturing agent.

3.2.3.1 Preparation of SDS-Polyacrylamide Gel

Different concentration of acrylamide in SDS-polyacrylamide gel was used depending on the molecular mass of proteins. The present study used 15% separating SDS-polyacrylamide gel for all the separation, and the gel preparation was carried out as follows:

Solution A **:** 29.2% (w/v) acrylamide, 0.8% N'-N-methylene bisacrylamide, in ddH_2O

Solution B **:** 1.5 M Tris-HCl, pH 8.8, containing 0.4% (w/v) sodium dodecyl sulfate

Solution C **:** 10% (w/v) ammonium persulfate (APS), freshly prepared

Solution D **:** 0.5 M Tris-HCl, pH 6.8, containing 0.4% sodium dodecyl sulfate (w/v)

Electrophoresis buffer **:** 0.025 M Tris, 0.192 M glycine, 0.1% (w/v) sodium dodecyl sulfate, pH 8.3

Sample loading buffer **:** 62 mM Tris-HCl, 2.3% (w/v) sodium dodecyl sulfate, 10% (v/v) glycerol, 5% (v/v) beta-mercaptoethanol and 0.005% bromophenol blue, pH 6.8

Staining solution **:** 0.2% (w/v) Coomassie Blue R-250, 40% (v/v) methanol, 10% (v/v) acetic acid in ddH_2O

Destaining solution **:** 5% (v/v) methanol, 7% (v/v) acetic acid in ddH_2O

Table 3.3: Preparation of separating and stacking gel.

Solution	15% separating gel	4% stacking gel
A (ml)	4.5	1.4
B (ml)	3.0	-
C (µl)	100	100
D (ml)	-	2.5
Ultrapure water (ddH_2O) (ml)	1.5	6.1
TEMED (µl)	10	10

* The amount is sufficient for the preparation of 2 gels

In the preparation of separating gel, the solution was mixed according to Table 3.3 and poured into the glass plates. A thin layer of isopropanol was layered on the top of the gel to prevent contact with air during the gel polymerization process and to provide an even distribution of gel surface. Once the gel is polymerized, the isopropanol on the gel surface was washed off using ddH_2O and any excessive fluid was removed. The stacking solution was then added to the top of the polymerized separating gel, and a dedicated comb was inserted between the glass plate to form sample loading wells. Ammonium persulfate (solution C) and TEMED were added at the last step of the solution preparation to prevent early polymerization.

3.2.3.2 Procedure of SDS-PAGE

Protein samples were mixed with the sample loading buffer in 1:1 volume ratio and subjected to boiling for 10 min to denature the proteins. The boiled samples were left at ambient temperature to cool down. For SDS-PAGE system set up, the chamber was filled with running buffer to the indicated level and connected to power supply. Next, the samples and protein standard (Spectra™ Multicolor Broad Range Protein Ladder) were loaded into the loading wells using a pipette and the electrophoresis separation was conducted at a constant voltage of 90 volts for ~2 h and 30 min. At the end of the electrophoresis, the SDS-polyacrylamide gel was carefully removed from the glass plate and stained using staining solution for 15 min and subsequently destained until the gel background is clear.

3.2.4 Trypsin Digestion of Protein

The present study applied "bottom-up" approach in mass spectrometry analysis, where the proteins of interest were digested using trypsin prior to the analysis by mass spectrometry. The target proteins were either subjected directly to protein digestion to obtain free peptides (gel-free "in-solution" digestion) or adopted the pre-separation by SDS-PAGE which offers a better resolution as compared to gel-free methods (Aebersold & Mann, 2003; Shevchenko et al., 2007). For proteins separation by SDS-PAGE, the proteins in the gel that stained with Coomassie Blue R250 were enzymatically digested, cleaved and extracted from a gel ("in-gel" digestion).

3.2.4.1 In-solution Digestion

For "in-solution" digestion, various reagents and buffers were prepared as following:

Trypsin stock **:** 0.1 µg/µl trypsin in ultrapure water (ddH_2O) with 1 mM HCl

Digestion buffer **:** 50 mM ammonium bicarbonate in ddH_2O

Reducing buffer **:** 100 mM dithiothreitol (DTT) in ddH_2O

Alkylation buffer **:** 100 mM iodoacetamide (IAA) in ddH_2O

Fifteen microliters (µl) of digestion buffer and 1.5 µl of reducing buffer were added to 0.5 ml microcentrifuge tube, followed by addition of 10 µl protein samples. The final volume was adjusted to 27 µl with ddH_2O and incubated at 95 ºC for 5 min. The samples were allowed to cool to ambient temperature, and 3 µl of alkylation buffer was added and was incubated in dark (ambient temperature) for 20 min. Next, 1 µl of trypsin stock was added to the reaction tube and incubated at 37 ºC for 3 hours. Finally, an additional 1 µl of trypsin stock was added to the tubes and incubated overnight at 30 ºC for complete digestion.

3.2.4.2 In-gel Digestion

For “in-gel” digestion, various reagents and buffers were prepared as follow:

Trypsin stock **:** 2 ng/µl trypsin with 1 mM HCl in 50 mM ammonium bicarbonate

Digestion buffer **:** 50 mM/100 mM ammonium bicarbonate in ddH_2O

Reducing buffer **:** 10 mM dithiothreitol (DTT) in 100 mM ammonium bicarbonate

Alkylation buffer **:** 55 mM iodoacetamide (IAA) in 100 mM ammonium bicarbonate

The protein bands of interest were excised using scalpel blade from the Coomassie Blue R250 stained SDS-PAGE gel. The excised gels were cut into small pieces (1 mm x 1 mm) and consecutively washed three times with 100 µl of 50% acetonitrile (ACN) in digestion buffer to destain the gel plugs. The destained gel plugs were subsequently processed for reducing by incubated with 150 µl of reducing buffer for 30 min at 60 ºC and allowed to cool to ambient temperature. Next, the proteins were alkylated by incubating the gel plugs with 150 µl of alkylating buffer for 20 min in dark and subsequently processed for three consecutive washing using 500 µl of 50% ACN in digestion buffer. Then, the gel plugs were dehydrated by incubating with 50 µl of 100% ACN for 15 min with shaking and dried with speed-vac for 10-15 min at ambient temperature. The dried gel plugs were incubated with 75 µl trypsin stock overnight at 37 ºC using a heated block (Techne Dri-block DB-2D, UK).

3.2.5 Extraction and Desalting of Digested Peptides

For "in-solution" proteomic analysis, the digested peptides were presence in the sample solution after overnight digestion. The extraction and desalting of peptides were then carried out concurrently by using the Millipore ZipTip® C_{18} Pipette Tips (herein referred as ZipTip). For "in-gel" digestion, the digested peptides were firstly extracted from the gel plugs that had been incubated overnight, by adding 50% ACN. The extracted liquid was transferred to a new centrifuge tube. The liquid was lyophilized and reconstituted with 10 µl of ddH_2O with 0.1% formic acid (FA), and subsequently processed for peptide extraction and desalting using the following reagents:

Wetting solution **:** 50% acetonitrile (ACN) in ddH_2O

Equilibration/wash solution **:** 0.1% formic acid (FA) in ddH_2O

Elution solution **:** 0.1% formic acid (FA) in 50% acetonitrile (ACN)

The ZipTip was wet by 10 µl wetting solution (no need to be in bold) (repeat 3x) and equilibrated with equilibration solution (repeat 3x). Next, the digested samples were aspirated into ZipTip and dispensed (repeat 10x) to bind the peptides onto the C_{18} beads. The bound peptides were washed with washing solution (repeat 3x) to eliminate the salt content. Lastly, the peptides were eluted out from the C_{18} beads of the ZipTip by aspirating and dispensing in the elution solution (repeat 3x) in a new centrifuge tube. The extracted and desalted peptides were lyophilized and kept until further use.

3.2.6 Protein Identification using Mass Spectrometry

3.2.6.1 Matrix-Assisted Laser Desorption/Ionization-Time of Flight/Time of Flight (MALDI-TOF/TOF)

MALDI-TOF/TOF was performed using AB SCIEX 5800 TOF/TOF™ Plus Analyzer (AB SCIEX, USA) equipped with a neodymium: yttrium-aluminium-garnet laser (laser wavelength was 349 nm) (Figure 3.2). The TOF/TOF calibration mixtures (AB SCIEX, USA) 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 800-4000 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 (S/N) of 100. The raw mass spectra acquired were exported to AB SCIEX ProteinPilot™ Software search against all non-redundant NCBI Serpentes database (taxid: 8570) (updated August 2014). MS peak filter mass range 800-4000 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 (C.I.) above 95% were considered as confident identification.

3.2.6.2 Nanoelectrospray Ionization: Thermo Scientific Orbitrap Fusion Tribrid LC/MS

Nanoelectrospray ionization (nanoESI) was performed using Thermo Scientific™ Pierce™ Orbitrap Fusion™ Tribrid™ with an Easy-nLC™ 1000 Ultra-high pressure LC on a Thermo Scientific™ Pierce™ EASY-Spray™ PepMap C_{18} column (2 µm, 75 µm x 25 cm) (Thermo Scientific, USA) (Figure 3.2). The peptides were separated over 44 min gradient eluted at 300 nl/ml with 0.1% FA in water (Solvent A) and 0.1% FA in 100% ACN (Solvent B) (0-5% B in 5 min, followed by 5-50% B over 30 min and 50-95% B over 2 min). The run was terminated by holding a 95% B for 7 min. MS1 data was acquired on an Orbitrap Fusion mass spectrometry using full scan method according to the following parameters: scan range 300-2000 m/z, orbitrap resolution 240,000; AGC target 200,000; maximum injection time of 50 ms. MS2 data were collected using the following parameters: rapid scan rate, CID collision energy 30%, 2 m/z isolation window, AGC 10000 and maximum injection time of 35 ms. MS2 precursors were selected for a 3 sec cycle. The precursors with an assigned monoisotopic m/z and a charge state of 2-6 were interrogated. The precursors were filtered using a 70 sec dynamic exclusion window. MS/MS spectra were searched using Thermo Scientific™ Pierce™ Proteome Discoverer™ Software Version 1.4 (Thermo Scientific, USA) with SEQUEST® against the non-redundant NCBI Serpentes database (taxid: 8570) (updated August 2014) downloaded from NCBI database (http://www.ncbi.nlm.nih.gov/protein/?term=Serpentes). Precursor and fragment mass tolerances were set to 10 ppm and 0.8 Da respectively and allowing up to two missed cleavages. Carbamidomethylation of cysteine was set as a fixed modification. The high confidence level filter with false discovery rate (FDR) of 1% was applied to the peptides and the protein ID with the highest protein score (> 10) was considered as a confident identification.

3.2.6.3 Nanoelectrospray Ionization: Agilent 6550 Accurate-Mass Q-TOF LC/MS

Samples were loaded in a large capacity chip using a 300 Å, C18, 160 nl enrichment column and 75 µm × 150 mm analytical column (Agilent part No. G4240-62010) with a flow rate of 4 µl/min, using a capillary pump and 0.4 µl/min, using a Nano pump of Agilent 1200 series. The digested peptide eluates were then subjected to nano-electrospray ionization (nanoESI) MS/MS using an Agilent 1200 HPLC-Chip/MS Interface, coupled with Agilent 6550 Accurate-Mass Q-TOF LC/MS system (Agilent Technologies, USA) (Figure 3.2). Injection volume was adjusted to 2 µl per sample, and the mobile phases were 0.1% formic acid in water (A) and 100% acetonitrile with 0.1% formic acid (B). The gradient applied was: 5–50% solution B for 11 min, 50–70% solution B for 4 min, and 70% solution B for 3 min, using Agilent 1200 series nano-flow LC pump. Ion polarity was set to positive ionization mode. The flow rate of drying gas flow rate was 11 L/min and the drying gas temperature was 290 °C. Fragmentor voltage was 175 V and the capillary voltage was set to 1800 V. Spectra were acquired in an MS/MS mode with an MS scan range of 200–3000 m/z and MS/MS scan range of 50–3200 m/z. Precursor charge selection was set as doubly charged state and above with the exclusion of precursors 1221.9906 m/z (z = 1) and 299.2944 (z = 1) set as reference ions. Data was extracted with MH+ mass range between 50-3200 Da, and processed with Agilent Spectrum Mill MS Proteomics Workbench software packages Version B.04.00 (Agilent Technologies, USA). Carbamidomethylation of cysteine was set as a fixed modification. The peptide finger mapping was modified to search specifically against non-redundant NCBI database with taxonomy set to Serpentes (taxid: 8570). Protein identifications were validated with the following filters: protein score > 20, peptides scored > 6 and scored peak intensity (SPI) > 70%. False discovery rate (FDR) was less than 1% for peptides and 0% for protein identification.

Figure 3.2 Mass spectrometry instruments. a) AB SCIEX 5800 TOF/TOF™ Plus Analyzer matrix-assisted laser desorption/ionization-time of flight-time of flight/ time of flight (MALDI-TOF/TOF); b) Thermo Scientific Orbitrap Fusion Tribrid LC/MS; c) Agilent 6550 Accurate-Mass Q-TOF LC/MS. Product images were obtained from the website of each manufacturer (AB SCIEX, Thermo Fisher Scientific and Agilent Technologies, respectively).

3.2.7 Estimation of the Relative Abundance of Protein

The relative abundance of protein in venom fractions was estimated by peak area measurement using Shimadzu LCsolution Software Version 1.23 (Japan). The fraction showing a protein band in SDS-PAGE was directly implemented with the relative abundance obtained from peak area measurement; while the fraction with various protein bands in SDS-PAGE was estimated by densitometry using Thermo Scientific™ myImageAnalysis™ Software (USA). The relative abundance of protein in percentage was then accumulated according to the protein identity and family.

3.2.8 Determination of Venom Lethality - Median Lethal Dose (LD_{50})

The median lethal dose (LD_{50}) was determined by intravenous (*i.v.*, via the caudal vein) or subcutaneous (*s.c.*, under the loose skin over the neck) routes of administration into ICR mice (20-25 g, n = 4). Mice were allowed free access to food and water *ad libitum*. The survival ratio was recorded after 48 hours and LD_{50} was calculated using the Probit analysis method (Finney, 1952).

3.2.9 Antivenom Neutralization

3.2.9.1 *In vitro* Immunocomplexation

In vitro immunocomplexation of venom and antivenom was conducted as described by Ramos-Cerrillo et al. (Ramos-Cerrillo et al., 2008). A challenge dose of 5x LD_{50} of venom dissolved in 50 µl normal saline was preincubated at 37º C for 30 min with various dilutions of reconstituted antivenom in normal saline to give a total volume of 250 µl. The venom-antivenom mixture was subsequently injected intravenously (*i.v.*) into the caudal vein of the mice (20-25 g, n = 4). The mice were allowed free access to food and water *ad libitum*, and the ratio of survival was recorded at 48 hours post-injection. If 200 µl of reconstituted antivenom failed to give full protection for the mice, a lower challenge dose (2.5x or 1.5x LD_{50}) was used. All challenge doses were proven to be above lethal dose 100% (LD_{100}) when injected intravenously into the mice.

3.2.9.2 *In vivo* Challenge-rescue Experiments in Mice

In vivo challenge-rescue experiment was carried out with venom and antivenom injected independently. A dose of 5x LD_{50} of the venom was injected into mice (20-25 g, n = 6) subcutaneously (*s.c.*), followed by intravenous (*i.v.*) injection of 200 µl appropriately diluted reconstituted antivenom, at the early sign of posterior limb paralysis. The mice were allowed free access to food and water *ad libitum*, and the mice were observed for clinical recovery for a period of 48 hours. All challenge doses were proven to be above lethal dose 100% (LD_{100}) when injected subcutaneously into the mice.

3.2.10 Statistical Analysis

3.2.10.1 Median Lethal Dose, Median Effective Dose and Neutralization Potency

In venom/toxin lethality study, the median lethal dose (LD_{50}) of the venom and the median effective dose (ED_{50}) (antivenom dose (µl) at which 50% of mice survived) of antivenom were expressed with 95% confidence interval (C.I.) according to Probit analysis method (Finney, 1952) using Biostat 2009 software (AnalystSoft Inc.).

In antivenom neutralization assays, the median effective ratio (ER_{50}) values were calculated (ER_{50}, defined as the ratio of the amount of venom (mg) to the volume dose of antivenom (ml) at which 50% of mice survived) based on the total amount of venom or toxin injected. The ER_{50} was calculated using the equation below:

$$\mathrm{ER_{50}(mg/ml)} = \frac{\text{Amount of venom } [\mathrm{n} \times \mathrm{LD_{50}(\mu g/g)} \times \text{mice weight (g)}]}{\mathrm{ED_{50}(\mu l)}}$$

Besides, the neutralization capacity was also expressed in term of "neutralization potency" (P, defined as the amount of venom (mg) neutralized completely by one ml antivenom) according to Morais et al. (Morais et al., 2010). The "potency" (P) was calculated using the equation below:

$$\text{Potency, P (mg/ml)} = \frac{\text{Amount of venom } [(\mathrm{n}-1) \times \mathrm{LD_{50}\ (\mu g/g)} \times \text{mice weight (g)}]}{\mathrm{LD_{50}(\mu l)}}$$

The neutralization potency is a more direct indicator of antivenom neutralizing capacity and is theoretically independent of the dosage of challenge dose. For comparative purpose, P values of different antivenoms were normalized by their respective protein amount. The normalized Potency (n-P) was defined as the amount of venom (mg) completely neutralized per unit amount of antivenom protein (g).

The normalization of "neutralization potency" was calculated using the following equation:

$$\text{Normalized Potency, n} - \text{P (mg/g)} = \frac{\text{Potency, P (mg/ml)}}{\text{Protein concentration (mg/ml)}} \times 1000$$

3.2.10.2 Chick Biventer Cervicis

In experiments involving chick biventer cervicis nerve-muscle (CBCNM) preparation, the statistical significance ($p < 0.05$) between the mean value of contracture and twitch tension readings were analyzed by one-way ANOVA (Dunnett's multiple comparison tests) using SPSS Version 16.0 (IBM, USA).

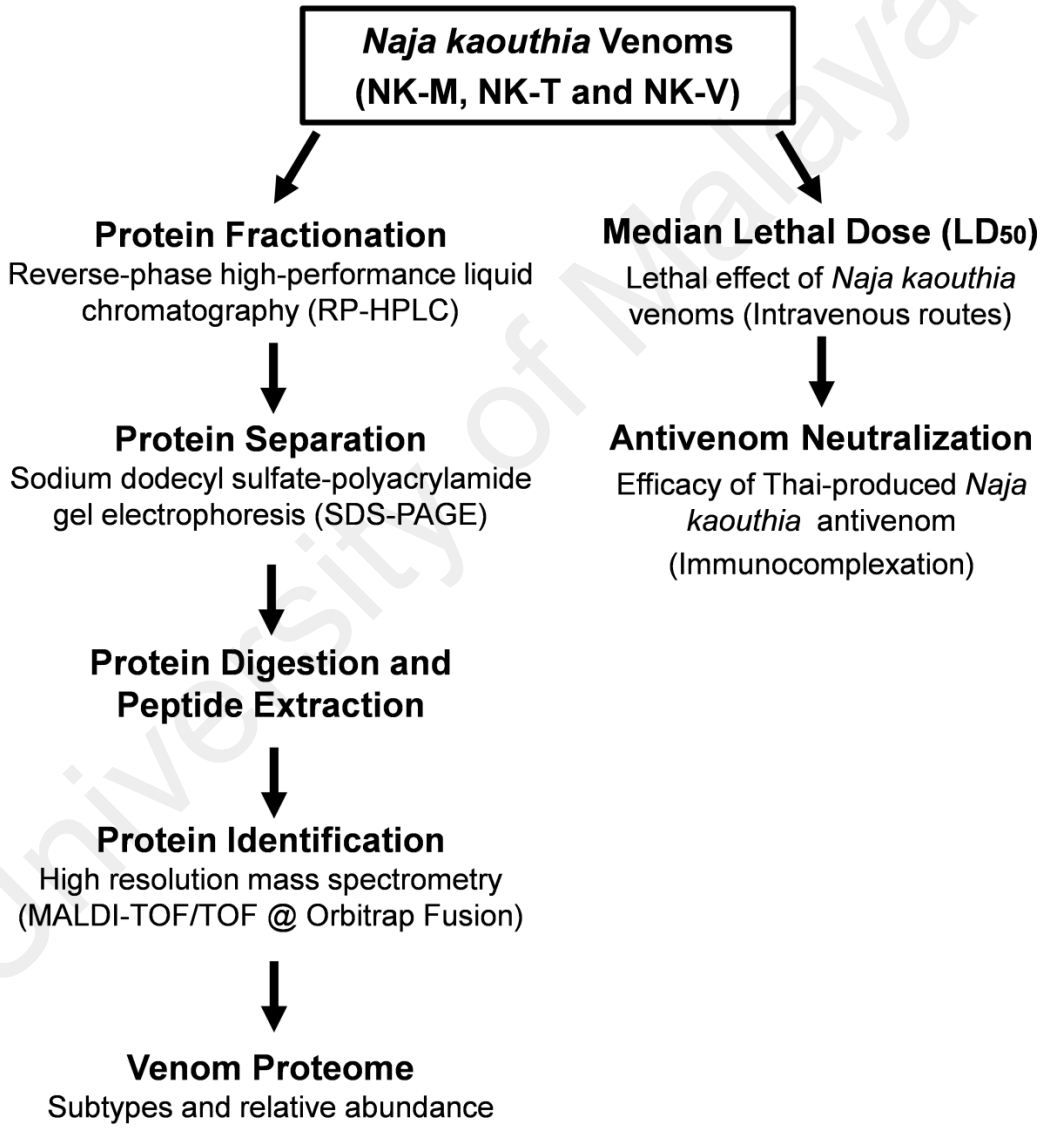
CHAPTER 4: PROTEOME OF *Naja kaouthia* (MONOCLED COBRA) VENOMS: INTRASPECIFIC GEOGRAPHICAL VARIATIONS AND IMPLICATIONS ON LETHALITY NEUTRALIZATION

4.1 Introduction

Many countries in Asia (including Malaysia) are unable to sustain domestic antivenom production due to the high manufacturing cost and small profit return. Instead, these countries rely on antivenom imported from foreign manufacturers. The antivenom is produced from the immunogen (venom or mixture of venoms) from snake species that are non-native to the importing countries. In Southeast Asia, Thailand is the only nation with the capacity to produce antivenom against *Naja kaouthia.* There are two products available: 1) *N. kaouthia* Monovalent Antivenom (NKMAV), and 2) Neuro Polyvalent Antivenom (NPAV). These two antivenoms are also imported by some other Southeast Asian countries for use in the treatment of *N. kaouthia* (and other *Naja* species) envenomation. However, the effectiveness of the Thai antivenoms to neutralize *N. kaouthia* from different geographical areas has not been rigorously examined.

Previous studies showed that *N. kaouthia* venoms from Malaysia and Thailand differ in their lethal activities and neutralizing responses to antivenom (Leong et al., 2012; Leong et al., 2014). It has also been demonstrated that envenomation by *N. kaouthia* in Malaysia and Thailand shows some discrepancies in the clinical manifestations of the envenomation (Bernheim et al., 2001; Khandelwal et al., 2007). This phenomenon has not been examined by in-depth study on the biogeographical variations of *N. kaouthia* venom, especially in term of the composition of toxin subtypes. Thus, there is a need to investigate the venom proteome of *N. kaouthia* sourced from different geographical regions. In this study, the venom proteomes of *N. kaouthia* sourced from Malaysia (NK-

M), Thailand (NK-T) and Vietnam (NK-V) were studied. The lethality of venoms and the neutralization by monovalent (NKMAV) and polyvalent antivenom (NPAV) were also evaluated, where the results were correlated to the compositional variations of the venoms. The experimental design of this study was summarized and shown in the following flow chart:



4.2 Methods

4.2.1 Protein Determination

The protein concentration of antivenoms (NKMAV and NPAV) was determined as described in **Section 3.2.1**.

4.2.2 Characterization of *Naja kaouthia* Venoms

4.2.2.1 C_{18} Reverse-phase High-performance Liquid Chromatography

Crude venoms (3 mg) of three *N. kaouthia* (NK-M, NK-T and NK-V) were subjected to fractionation using reverse-phase high-performance liquid chromatography (RP-HPLC) as described in **Section 3.2.2.1** and monitored at absorbance 215 nm. Protein fractions were collected manually and lyophilized.

4.2.2.2 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The lyophilized protein fractions obtained from reverse-phase HPLC were subjected to 15% SDS-PAGE (Laemmli, 1970) as according to **Section 3.2.3**.

4.2.2.3 In-gel Trypsin Digestion of Protein Bands and Peptides Extraction

The protein bands were excised from polyacrylamide gels and subjected to reduction, alkylation and "in-gel" digestion according to the protocol described in **Section 3.2.4.2**. The tryptic digested peptides were extracted and desalted as described in **Section 3.2.5**.

4.2.2.4 Protein Identification using Mass Spectrometry

The tryptic digested peptide samples were mixed with alpha-cyano-4-hydroxycinnamic acid matrix and spotted on OPTI-TOF™ LC/MALDI insert plate (123

mm x 81 mm). Protein identification was carried out using AB SCIEX 5800 TOF/TOF™ Plus Analyzer (MALDI-TOF/TOF) according to **Section 3.2.6.1**. Samples not identified by MALDI-TOF/TOF were subjected to Thermo Scientific™ Pierce™ Orbitrap Fusion™ Tribrid™ (nanoESI) as described in **Section 3.2.6.2**.

4.2.2.5 Estimation of Relative Abundance of Protein

The relative abundance of proteins from reverse-phase HPLC was estimated as described in **Section 3.2.7**. The relative abundances (in term of % total venom proteins) were then summed up according to the protein identity and family.

4.2.3 Determination of the Medium Lethal Dose (LD_{50})

The median lethal dose (LD_{50}) of *Naja kaouthia* venoms (NK-M, NK-T and NK-V) were determined by intravenous (*i.v.*) and subcutaneous (*s.c.*) routes as described in **Section 3.2.8**. The median lethal dose (LD_{50}) was calculated using Probit analysis (Finney, 1952) as described in **Section 3.2.10.1**.

4.2.4 Neutralization of *N. kaouthia* Venoms by Antivenoms using *In vitro* Immunocomplexation

In vitro immunocomplexation of the *N. kaouthia* venoms (NK-M, NK-T and NK-V) by *N. kaouthia* Monovalent Antivenom (NKMAV) and Neuro Polyvalent Antivenom (NPAV) was conducted according to **Section 3.2.9.1**. The median effective dose (ED_{50}), median effective ratio (ER_{50}), neutralizing potency (P) and normalized Potency (n-P) were determined as described in **Section 3.2.10.1**.

4.3 Results

4.3.1 Reverse-phase HPLC of the *N. kaouthia* Venoms

Reverse-phase HPLC of *N. kaouthia* venoms from Malaysia (NK-M), Thailand (NK-T) and Vietnam (NK-V) yielded 28 (NK-M), 28 (NK-T) and 29 (NK-V) fractions, respectively. From the chromatograms (Figure 4.1(a), (b) and (c)), the HPLC profiles revealed some distinct differences in the composition of the three *N. kaouthia* venoms, notably fraction 6 of NK-T (75-80 min) and fractions 17-19 of NK-V (105-125 min). On the other hand, the SDS-PAGE of the HPLC fractions indicated that all three *N. kaouthia* venoms contain mostly low molecular mass proteins (5-15 kDa) that were eluted over the initial 125 min of the chromatography, followed by the moderate to high molecular mass proteins (20-150 kDa). All visible gel bands in the SDS-PAGE were subsequently processed for tryptic digestion and peptide identification using high-resolution mass spectrometry. In total, 61 (NK-M), 51 (NK-T) and 68 (NK-V) proteins were identified from NK-M, NK-T and NK-V venom, respectively.

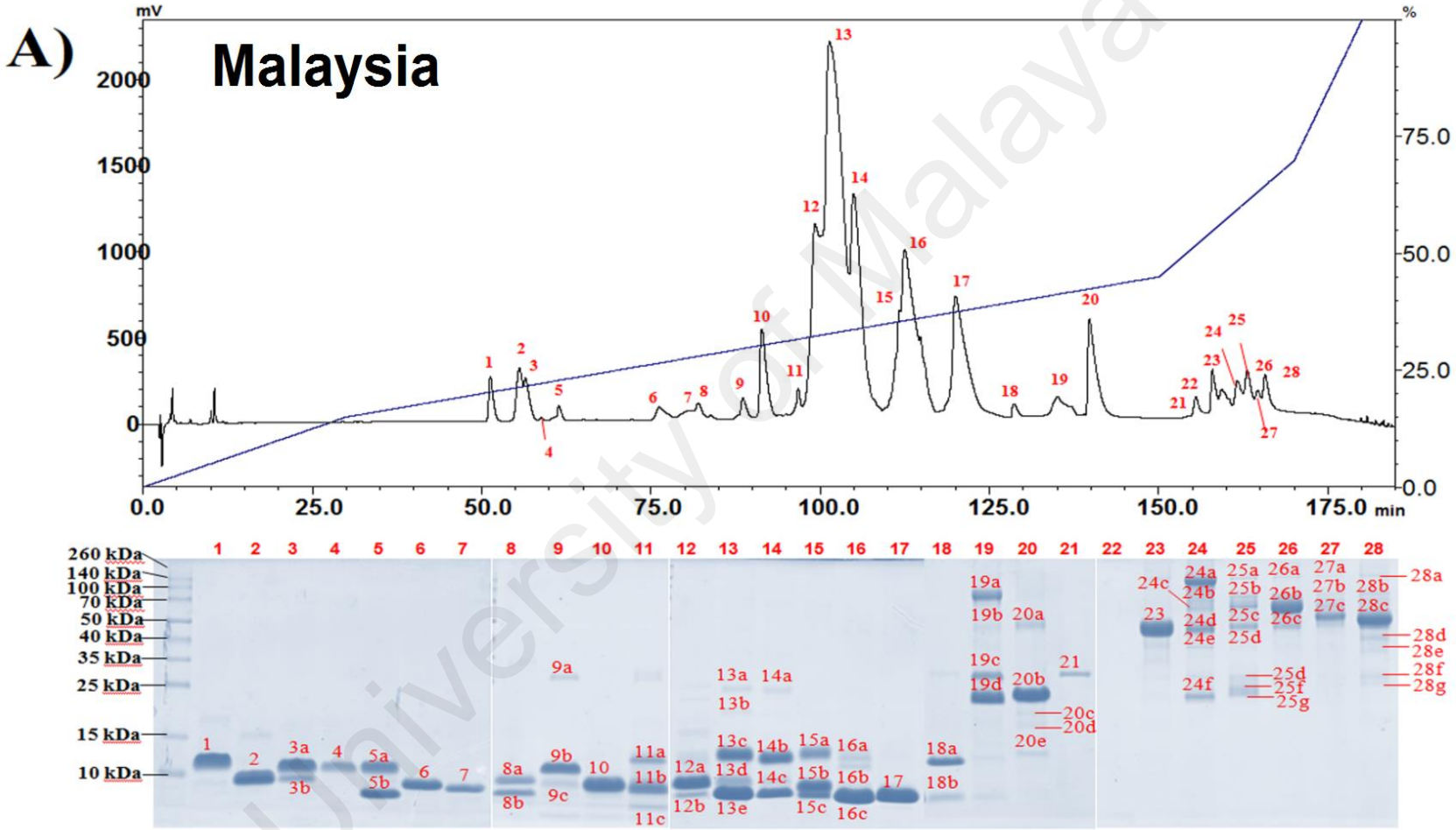


Figure 4.1(a) RP-HPLC chromatogram (TOP) from C_{18} reverse-phase fractionation of *N. kaouthia* venoms (3 mg) sourced from Malaysia and SDS-PAGE profiles (BOTTOM) of the individual fractions under reducing conditions. A broad range protein ladder (10 to 260 kDa) was used for calibration.

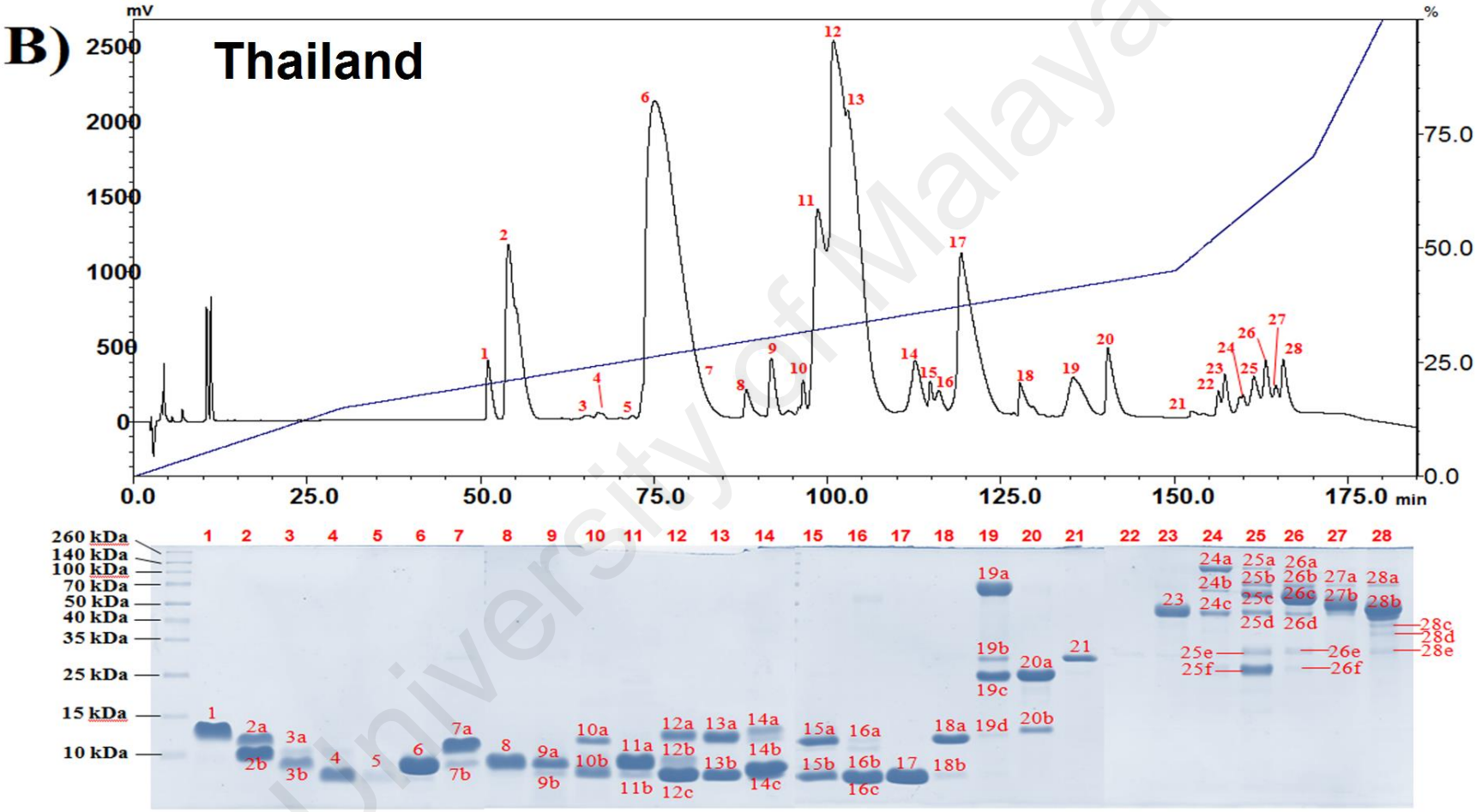


Figure 4.1(b) RP-HPLC chromatogram (TOP) of the C_{18} reverse-phase fractionation of *N. kaouthia* venoms (3 mg) sourced from Thailand and SDS-PAGE profiles (BOTTOM) of the individual fractions under reducing conditions. A broad range protein ladder (10 to 260 kDa) was used for calibration.

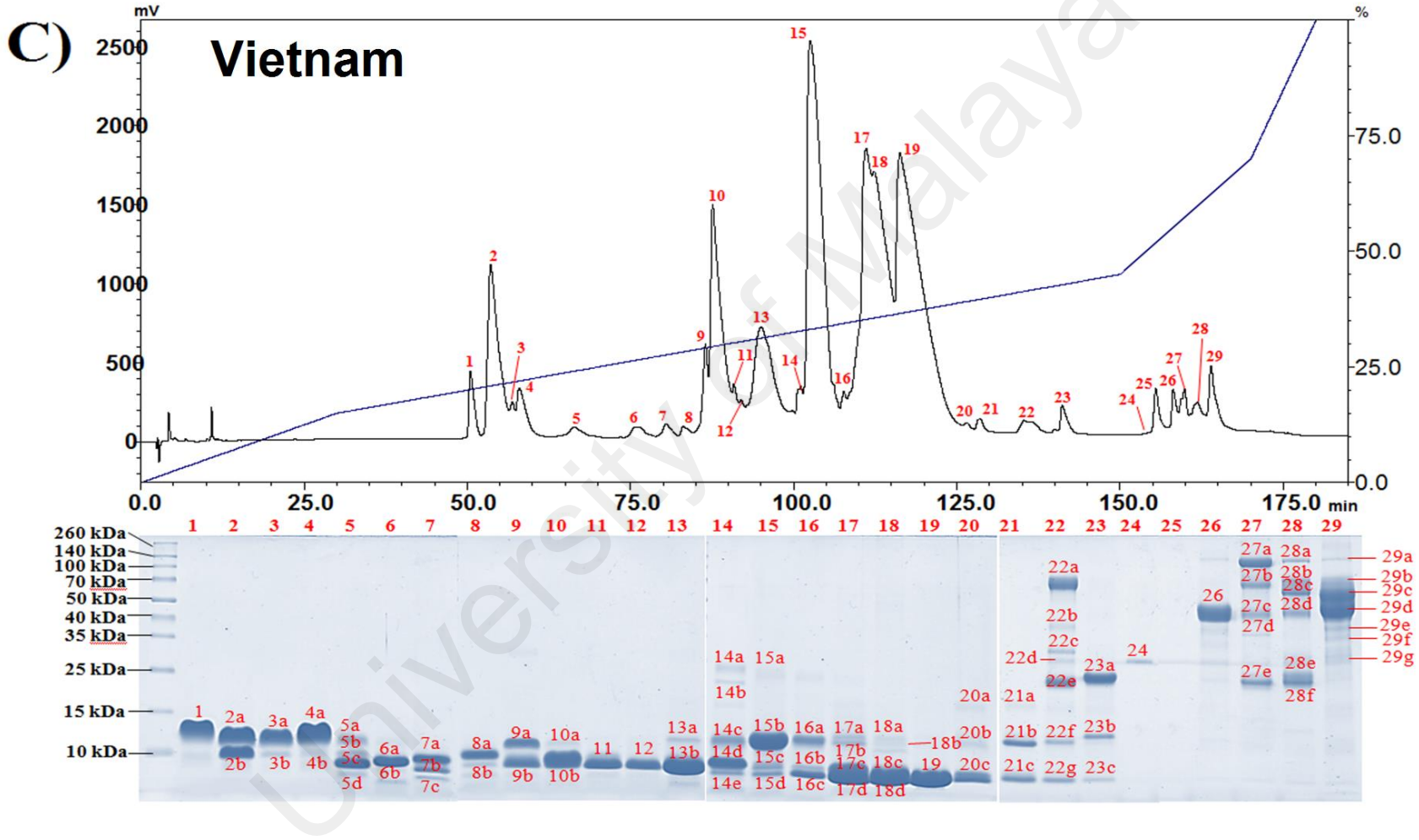


Figure 4.1(c) RP-HPLC chromatogram (TOP) from C_{18} reverse-phase fractionation of *N. kaouthia* venoms (3 mg) sourced from Vietnam and SDS-PAGE profiles (BOTTOM) of the individual fractions under reducing conditions. A broad range protein ladder (10 to 260 kDa) was used for calibration.

4.3.2 The Venom Proteomes of *N. kaouthia* Venoms

Mass spectrometry analysis of the RP-HPLC fractions revealed the presence of a total of 13 different toxin families in the *N. kaouthia* venoms (NK-M, NK-T and NK-V). These toxin families include three-finger toxin (3FTx), phospholipase A_2 (PLA_2), cysteine-rich secretory protein (CRISP), snake venom metalloproteinase (SVMP), L-amino acid oxidase (LAAO), cobra venom factor (CVF), Kunitz-type protease inhibitor (KUN), natriuretic peptide (NP), phosphodiesterase (PDE), 5'nucleotidase (5'NUC), vespryn, c-type lectin (CTL) and nerve growth factor (NGF) (Table 4.1 (a), (b) and (c); Table 4.2; Figure 4.2). Most of the proteins identified were annotated to proteins previously reported to be present in *N. kaouthia* or other closely related species from the genus *Naja*. It was noted that both 3FTx and PLA_2 constitute > 85% of the venom proteins for *N. kaouthia* venoms from all three geographical sources (NK-M, NK-T and NK-V). The content of three-finger toxin (3FTx) was higher in NK-T (78.3%) and NK-V (76.4%), but slightly lower in NK-M (63.7%). On the other hand, the content of PLA_2 was highest in NK-M (23.5%), followed by NK-V (17.4%) and NK-T (12.2%). The other toxin families generally exist in much smaller amount (< 3%) in all three geographical species.

Table 4.1(a): The proteins identified from the SDS-PAGE gel of reverse-phase isolated fractions of *N. kaouthia* (Malaysia) venom by using MALDI-TOF/TOF and nanoESI Orbitrap Fusion analysis.

Protein fraction	%	Protein family/subtype	MS/MS derived sequence	Matched MH+	Mass Δ (ppm)	Accession (species)	Matched peptide	Protein score
1	**1.0**	3FTx-SNTX (cobrotoxin)*	LECHNQQSSQTPTTTGCSGGETNCYK	2945.2082	-0.45	P60771 (*N. kaouthia*)	1	51
2	**1.6**	3FTx-SNTX (cobrotoxin-c)	LECHNQQSSQAPTTK	1727.7897	41	P59276 (*N. kaouthia*)	2	64
			KWWSDHR	1013.4831	51			
3a	**1.0**	3FTx-SNTX (cobrotoxin)*	LECHNQQSSQTPTTTGCSGGETNCYK	2945.2002	-3.18	P60771 (*N. kaouthia*)	4	452
			LECHNQQSSQTPTTTGCSGGETNCYKK	3073.2905	-4.56			
			NGIEINCCTTDR	1452.6174	-2.27			
			NGIEINCCTTDRCNN	1840.7310	-3.35			
3b	**0.3**	3FTx-SNTX (cobrotoxin-b)*	LECHNQQSSQTPTTK	1758.8016	-3.40	P59275 (*N. kaouthia*)	5	62
			VKPGVNLNCCR	1316.6531	-2.41			
			TCSGETNCYKK	1347.5627	-3.06			
			KWWSDHR	1014.4858	-4.64			
			TCSGETNCYK	1219.4677	-3.39			
4	**< 0.1**	3FTx-SNTX (cobrotoxin)*	LECHNQQSSQTPTTTGCSGGETNCYK	2945.1895	-6.79	P60771 (*N. kaouthia*)	2	11
			NGIEINCCTTDR	1452.6127	-5.46			
5a	**0.2**	3FTx-SNTX (cobrotoxin)*	LECHNQQSSQTPTTTGCSGGETNCYK	2945.1943	-5.17	P60771 (*N. kaouthia*)	4	91
			LECHNQQSSQTPTTTGCSGGETNCYKK	3073.2916	-4.20			
			LECHNQQSSQTPTTTGCSGGETNCYKKR	3229.3891	-5.13			
			NGIEINCCTTDR	1452.6143	-4.37			
5b	**0.2**	Kunitz-type inhibitor	FIYGGCGGNANR	1284.5669	26	P20229 (*Naja naja*)	1	99
6	**0.7**	3FTx-WTX (weak neurotoxin 6)*	LTCLICPEKYCNK	1698.7934	-4.63	P29180 (*N. naja*)	9	114
			YCNKVHTCLNGEK	1622.7345	-4.24			
			LTCLICPEK	1133.5643	-4.52			
			YIRGCADTCPVR	1467.6771	-4.15			
			GCADTCPVR	1035.4301	-4.42			
			YIRGCADTCPVRKPR	1848.9234	-4.69			
			GCADTCPVRKPR	1416.6775	-4.24			
			EIVQCCSTDK	1239.5302	-3.43			
			KLLGKR	714.4953	-4.51			
7	**0.6**	3FTx-LNTX (alpha-elapitoxin-Nk2a)*	TGVDIQCCSTDNCNPFPTR	2241.9258	-2.90	P01391 (*N. kaouthia*)	10	215
			VDLGCAATCPTVK	1391.6609	-3.51			
			RVDLGCAATCPTVK	1547.7612	-3.74			
			GKRVDLGCAATCPTVK	1732.8781	-3.05			
			TWCDAFCSIR	1315.5519	-2.99			

The proteins identified were sorted according to protein families. Cysteine residues determined in MS/MS analysis are carbamidomethylated.

* Results obtained from nanoESI Orbitrap Fusion analysis. Abbreviations: 3FTx: three-finger toxin; LNTX: long neurotoxin; SNTX: short neurotoxin; WTX: weak neurotoxin/toxin.

Table 4.1(a) *N. kaouthia* (Malaysia), continued.

Protein fraction	%	Protein family/subtype	MS/MS derived sequence	Matched MH+	Mass Δ (ppm)	Accession (species)	Matched peptide	Protein score
			IRCFITPDITSK	1450.7675	-3.32			
			TGVDIQCCSTDNCNPFPTRK	2370.0203	-2.92			
			CFITPDITSK	1181.5833	-3.25			
			CFITPDITSKDCPNGHVCYTK	2513.1152	-4.24			
			DCPNGHVCYTK	1350.5539	-1.99			
8a	**0.4**	3FTx-LNTX (alpha-elapitoxin-Nk2a)	TWCDAFCSIR	1314.5485	25	P01391 (*N. kaouthia*)	3	206
			RVDLGCAATCPTVK	1546.7596	20			
			TGVDIQCCSTDNCNPFPTR	2240.9249	24			
8b	**0.3**	Kunitz-type inhibitor*	TIDECNR	907.3939	0.07	P20229 (*N. naja*)	2	40
			TIDECNRTCVG	1324.5622	0.08			
9a	**0.1**	Not determined	-	-	-	-	-	-
9b	**0.5**	3FTx-MTLP (muscarinic toxin-like protein 2)	SIFGVTTEDCPDGQNLCFK	2186.9612	-74	P82463 (*N. kaouthia*)	1	76
9c	**0.1**	3FTx-LNTX (alpha-elapitoxin-Nk2a - deduced 8a)	-	-	-	-	-	-
10	**2.9**	3FTx-LNTX (alpha-elapitoxin-Nk2a)*	TGVDIQCCSTDNCNPFPTR	2241.9268	-2.43	P01391 (*N. kaouthia*)	4	42
			VDLGCAATCPTVK	1391.6630	-2.02			
			TWCDAFCSIR	1315.5535	-1.78			
			RVDLGCAATCPTVK	1547.7643	-1.69			
11a	**0.2**	vNGF	GIDSSHWNSYCTETDTFIK	2259.9743	-4	P61899 (*N. kaouthia*)	3	142
			ALTMEGNQASWR	1362.6350	3			
			ALTMEGNQASWR	1378.6299	3			
11b	**0.3**	3FTx-MTLP (muscarinic toxin-like protein 3)	TSETTEICPDSWYFCYK	2185.8972	-69	P82464 (*N. kaouthia*)	2	119
			ISLADGNDVR	1058.5356	-69			
11c	**0.1**	3FTx-CTX (cytotoxin 2)*	GCIDVCPK	948.4239	-4.14	P01445 (*N. kaouthia*)	6	26
			YVCCNTDR	1087.4262	-3.10			
			MFMVSNK	856.4023	-3.83			
			NSLLVK	673.4217	-3.94			
			NLCYK	697.3309	-4.11			
			TVPVKR	699.4482	-4.37			
12a	**8.4**	3FTx-WTX (weak toxin CM-9a)*	GCADTCPVGYPKEMIECCSTDK	2578.0270	-4.57	P25679 (*N. kaouthia*)	8	620
			LTCLNCPEMFCGK	1629.6835	-3.54			
			GCADTCPVGYPKEMIECCSTDKCNR	3008.2066	-2.30			

The proteins identified were sorted according to protein families. Cysteine residues determined in MS/MS analysis are carbamidomethylated.
* Results obtained from nanoESI Orbitrap Fusion analysis. Abbreviations: 3FTx: three-finger toxin; LNTX: long neurotoxin; CTX: cytotoxin; MTLP: muscarinic toxin-like protein; WTX: weak neurotoxin/toxin; vNGF: venom nerve growth factor.

Table 4.1(a) *N. kaouthia* (Malaysia), continued.

Protein fraction	%	Protein family/subtype	MS/MS derived sequence	Matched MH+	Mass Δ (ppm)	Accession (species)	Matched peptide	Protein score
			LTCLNCPEMFCGKFQICR	2334.0221	-4.28			
			YIRGCADTCPVGYPKEMIECCSTDK	3010.2753	-3.99			
			GCADTCPVGYPK	1324.5618	-3.23			
			KLHQR	681.4125	-4.45			
			NGEKICFK	995.4935	-4.49			
12b	**1.5**	3FTx-CTX (cytotoxin 2)*	YVCCNTDRCN	1361.4989	-3.09	P01445 (*N. kaouthia*)	6	106
			GCIDVCPK	948.4245	-3.49			
			YVCCNTDR	1087.4261	-3.22			
			MFMVSNK	856.4043	-1.55			
			NSLLVK	673.4221	-3.30			
			RGCIDVCPK	1104.5276	-1.16			
13a	**0.7**	PLA_2 (acidic 2)	SWWDFADYGCYCGR	1784.6711	-48	Q91133 (*Naja atra*)	3	128
			SWWDFADYGCYCGR	1841.6926	-44			
			LAAICFAGAPYNNNNYNIDLK	2355.1317	-42			
13b	**0.6**	PLA_2 (acidic 2)	SWWDFADYGCYCGR	1841.6926	-52	Q91133 (*N. atra*)	1	74
13c	**12.1**	PLA_2 (acidic 1)	NMIQCTVPNR	1247.5751	-2	P00596 (*N. kaouthia*)	2	171
			SWWDFADYGCYCGR	1841.6926	15			
13d	**4.8**	3FTx-CTX (cytotoxin 2)*	YVCCNTDRCN	1361.4988	-3.18	P01445 (*N. kaouthia*)	5	41
			GCIDVCPK	948.4237	-4.33			
			YVCCNTDR	1087.4257	-3.55			
			NSLLVK	673.4218	-3.76			
			MFMVSNK	856.4019	-4.33			
13e	**13.8**	3FTx-CTX (cytotoxin 2)	MFMVSNK	887.3881	15	P01445 (*N. kaouthia*)	3	124
			GCIDVCPK	947.4205	31			
			YVCCNTDR	1086.4223	20			
14a	**0.7**	PLA_2 (acidic 2 - deduced 13a)	-	-	-	-	-	-
14b	**6.7**	PLA_2 (acidic 2)	SWWDFADYGCYCGR	1841.6926	26	P15445 (*N. naja*)	3	274
			ISGCWPYFK	1156.5375	15			
			LAAICFAGAPYNDNNYNIDLK	2356.1157	24			
14c	**5.4**	3FTx-CTX (cytotoxin NK-CT1)	LVPLFYKTCPAGK	1492.8112	-44	P0CH80 (*N. kaouthia*)	3	110
			NSLVLKYVCCNTDR	1740.8287	11			
			YVCCNTDR	1086.4223	14			
15a	**0.7**	PLA_2 (acidic 1)	SWWDFADYGCYCGR	1784.6711	-75	P00598 (*N. atra*)	2	126
			SWWDFADYGCYCGR	1841.6926	-79			

The proteins identified were sorted according to protein families. Cysteine residues determined in MS/MS analysis are carbamidomethylated.
* Results obtained from nanoESI Orbitrap Fusion analysis. Abbreviations: 3FTx: three-finger toxin; CTX: cytotoxin; PLA_2: phospholipase A_2.

Table 4.1(a) *N. kaouthia* (Malaysia), continued.

Protein fraction	%	Protein family/subtype	MS/MS derived sequence	Matched MH+	Mass Δ (ppm)	Accession (species)	Matched peptide	Protein score
15b	**1.1**	3FTx-CTX (cytotoxin homolog)*	LKCHNTQLPFIYK	1661.8777	-3.37	P14541 (*N. kaouthia*)	12	894
			CHNTQLPFIYK	1420.7006	-2.54			
			KFPLKIPIK	1083.7261	-2.62			
			NSALLKYVCCSTDKCN	1932.8578	-1.79			
			NSALLKYVCCSTDK	1658.7800	-4.67			
			GCADNCPKNSALLK	1547.7236	-4.52			
			YVCCSTDKCN	1306.4825	-2.78			
			FPLKIPIK	955.6303	-3.87			
			FPLKIPIKR	1111.7322	-2.56			
			NLCFKATLK	1094.5998	-2.69			
			YVCCSTDK	1032.4097	-2.72			
			NSALLK	645.3910	-3.18			
15c	**0.5**	3FTx-CTX (cytotoxin 1)*	NSLLVKYVCCNTDRCN	2015.9048	-2.42	P60305 (*N. kaouthia*)	11	304
			YVCCNTDRCN	1361.5004	-2.01			
			MFMMSDLTIPVK	1412.6967	-1.43			
			GCIDVCPKNSLLVK	1602.8310	-2.06			
			GCIDVCPK	948.4257	-2.21			
			MFMMSDLTIPVKR	1568.7958	-2.57			
			CNKLIPIASK	1143.6529	-2.29			
			NSLLVKYVCCNTDR	1741.8333	-1.58			
			YVCCNTDR	1087.4269	-2.43			
			NSLLVK	673.4229	-2.13			
			RGCIDVCPK	1104.5252	-3.37			
16a	**1.1**	PLA_2 (acidic 2)	SWWDFADYGCYCGR	1841.6926	-75	Q91133 (*N. atra*)	1	118
16b	**0.9**	PLA_2 (acidic 1)*	TYSYECSQGTLTCK	1697.7239	5.50	P00596 (*N. kaouthia*)	5	52
			GDNDACAAAVCDCDR	1669.6087	5.19			
			CCQVHDNCYNEAEK	1826.7008	6.38			
			NMIQCTVPNR	1232.5931	4.52			
			GGSGTPVDDLDR	1188.5552	5.08			
16c	**10.1**	3FTx-CTX (cytotoxin 1)*	NSLLVKYVCCNTDR	1741.8315	-2.64	P60305 (*N. kaouthia*)	15	920
			MFMMSDLTIPVKR	1568.7937	-3.88			
			NLCYKMFMMSDLTIPVKR	2247.1058	-4.44			
			NSLLVKYVCCNTDRCN	2015.9055	-2.06			
			MFMMSDLTIPVK	1412.6954	-2.38			
			LKCNKLIPIASK	1384.8306	-2.86			

The proteins identified were sorted according to protein families. Cysteine residues determined in MS/MS analysis are carbamidomethylated.
* Results obtained from nanoESI Orbitrap Fusion analysis. Abbreviations: 3FTx: three-finger toxin; CTX: cytotoxin; PLA_2: phospholipase A_2.

Table 4.1(a) *N. kaouthia* (Malaysia), continued.

Protein fraction	%	Protein family/subtype	MS/MS derived sequence	Matched MH+	Mass Δ (ppm)	Accession (species)	Matched peptide	Protein score
			YVCCNTDRCN	1361.4999	-2.37			
			LIPIASKTCPAGK	1355.7675	-3.03			
			RGCIDVCPKNSLLVK	1758.9305	-2.78			
			GCIDVCPKNSLLVK	1602.8315	-1.76			
			GCIDVCPK	948.4253	-2.59			
			YVCCNTDR	1087.4269	-2.43			
			NSLLVK	673.4223	-3.03			
			RGCIDVCPK	1104.5259	-2.71			
			CNKLIPIASK	1143.6524	-2.71			
17	**8.4**	3FTx-CTX (cardiotoxin 2A precursor)*	YVCCNTDRCN	1361.5037	0.41	Q9PST4 (*N. kaouthia*)	14	792
			CNKLVPLFYK	1281.7033	0.67			
			LVPLFYKTCPAGK	1493.8218	2.20			
			MYMVATPK	940.4652	2.21			
			SSLLVKYVCCNTDRCN	1988.8976	-0.57			
			NLCYKMYMVATPK	1618.7793	0.17			
			GCIDVCPK	948.4280	0.17			
			SSLLVKYVCCNTDR	1714.8259	0.43			
			GCIDVCPKSSLLVKYVCCNTDR	2644.2363	0.45			
			RGCIDVCPK	1104.5294	0.46			
			SSLLVK	646.4131	-0.56			
			YVCCNTDR	1087.4296	0.04			
			VPVKRGCIDVCPK	1527.8155	1.29			
			GCIDVCPKSSLLVK	1575.8250	1.02			
18a	**0.3**	Vespryn (Thaicobrin)	FDGSPCVLGSPGFR	1437.6710	-31	P82885 (*N. kaouthia*)	2	111
			FDGSPCVLGSPGFR	1494.6925	-41			
18b	**0.1**	3FTx-CTX (cytotoxin 2)*	YVCCNTDR	1087.4312	1.50	Q98965 (*N. kaouthia*)	1	18
			GCIDVCPK	948.4288	1.08			
			SSLLVK	646.4139	0.77			
			FPVKR	646.4040	0.77			
19a	**0.4**	SVMP (atrase-A)	EHQEYLLR	1086.5458	19	D5LMJ3 (*N. atra*)	2	118
			ERPQCILNKPSR	1496.7881	19			
19b	**0.1**	Not determined	-	-	-	-	-	-

The proteins identified were sorted according to protein families. Cysteine residues determined in MS/MS analysis are carbamidomethylated.

* Results obtained from nanoESI Orbitrap Fusion analysis. Abbreviations: 3FTx: three-finger toxin; CTX: cytotoxin. SVMP: snake venom metalloproteinase; CRISP: cysteine-rich secretory protein.

Table 4.1(a) *N. kaouthia* (Malaysia), continued.

Protein fraction	%	Protein family/subtype	MS/MS derived sequence	Matched MH+	Mass Δ (ppm)	Accession (species)	Matched peptide	Protein score
19c	**0.3**	CRISP (natrin-1)	MEWYPEAASNAER	1552.6616	-35	Q7T1K6 (*N. atra*)	3	99
			VLEGIQCGESIYMSSNAR	2012.9295	-33			
			TWTEIIHLWHDEYK	1869.9050	-36			
19d	**0.6**	CRISP (natrin-2)	IGCGENLFMSSQPYAWSR	2117.9298	5	Q7ZZN8 (*N. atra*)	1	70
20a	**0.5**	CRISP (natrin-1)	NVDFNSESTR	1167.5156	32	Q7T1K6 (*N. atra*)	9	349
			QKEIVDLHNSLR	1450.7892	30			
			EIVDLHNSLR	1194.6357	31			
			MEWYPEAASNAER	1552.6616	29			
			MEWYPEAASNAER	1568.6565	29			
			WANTCSLNHSPDNLR	1783.8060	30			
			VLEGIQCGESIYMSSNAR	2012.9295	30			
			VLEGIQCGESIYMSSNAR	2028.9245	33			
			SNCPASCFCR	1257.4689	30			
20b	**2.9**	CRISP (natrin-1)	QKEIVDLHNSLR	1450.7892	28	Q7T1K6 (*N. atra*)	8	609
			EIVDLHNSLR	1194.6357	28			
			MEWYPEAASNAER	1552.6616	43			
			MEWYPEAASNAER	1568.6565	37			
			WANTCSLNHSPDNLR	1783.8060	36			
			VLEGIQCGESIYMSSNAR	2012.9295	39			
			VLEGIQCGESIYMSSNAR	2028.9244	34			
			SNCPASCFCR	1257.4689	39			
20c	**0.2**	Not determined	-	-	-	-	-	-
20d	**0.1**	Not determined	-	-	-	-	-	-
20e	**0.2**	CTL (BFL-1)	KYIWEWTDR	1295.6299	44	Q90WI8	2	138
			YIWEWTDR	1167.5349	35	(*Bungarus fasciatus*)		
21	**< 0.1**	EDCP*	GHLNPNGHQPDYSAK	1634.7662	-0.54	U3FCT9	2	27
			NDQNVVQK	944.4786	-1.12	(*Micrurus fulvius*)		
23	**1.0**	SVMP (kaouthiagin)	YIEFYVIVDNR	1429.7241	-38	P82942 (*N. kaouthia*)	1	130
24a	**0.4**	PDE*	NLHNCVNLILLADHGMEAISCNR	2664.2809	0.16	U3FAB3 (*M. fulvius*)	22	392
			MANVLCSCSEDCLTK	1787.7406	-1.41			
			AEYLETWDTLMPNINK	1937.9311	-0.18			
			RPDFSTLYIEEPDTTGHK	2106.0128	-0.53			
			YCSGGTHGYDNEFK	1634.6545	0.26			
			LWNYFHSTLLPK	1518.8096	-0.52			

The proteins identified were sorted according to protein families. Cysteine residues determined in MS/MS analysis are carbamidomethylated.

* Results obtained from nanoESI Orbitrap Fusion analysis. Abbreviations: SVMP: snake venom metalloproteinase; CRISP: cysteine-rich secretory protein; CTL: C-type lectin; PDE: phosphodiesterase; EDCP: endonuclease domain-containing protein.

Table 4.1(a) *N. kaouthia* (Malaysia), continued.

Protein fraction	%	Protein family/subtype	MS/MS derived sequence	Matched MH+	Mass Δ (ppm)	Accession (species)	Matched peptide	Protein score
			DFYTFDSEAIVK	1434.6783	-0.36			
			CSSITDLEAVNQR	1492.7065	0.25			
			AKRPDFSTLYIEEPDTTGHK	2305.1422	-1.65			
			VLSFILPHRPDNSESCADK	2185.0704	-0.15			
			IDKVNLMVDR	1202.6554	-0.66			
			AATYFWPGSEVK	1355.6624	-0.50			
			NPFYNPSPAK	1134.5579	0.03			
			YCLLHQTK	1062.5396	-0.47			
			TLGMLMEGLK	1092.5785	-0.64			
			YISAYSQDILMPLWNSYTISK	2493.2374	0.10			
			AYLAKDLPK	1018.5914	-1.75			
			NVPKDFYTFDSEAIVK	1872.9366	-0.71			
			VNLMVDR	846.4493	-1.09			
			DCCTDYK	961.3382	-0.81			
			YGPVSGQVIK	1047.5832	-0.18			
			SLQMADR	820.3973	-1.15			
24b	**0.1**	Not determined	-	-	-	-	-	-
24c	**0.1**	Not determined	-	-	-	-	-	-
24d	**0.2**	SVMP (kaouthiagin)	TAPAFQFSSCSIR	1470.6926	38	P82942 (*N. kaouthia*)	3	140
			DYQEYLLR	1098.5345	40			
			GCFDLNMR	1011.4266	39			
24e	**< 0.1**	5'-NUC	ETPVLSNPGPYLEFR	1717.8675	24	B6EWW8	2	64
			ETPVLSNPGPYLEFRDEVEELQK	2688.3282	23	(*Gloydius brevicaudus*)		
24f	**0.2**	GPX*	AKVDCYDSVK	1184.5562	-4.58	V8P395 (*O. hannah*)	4	40
			VDCYDSVK	985.4254	-4.22			
			LVILGFPCNQFGK	1492.7928	-3.61			
			FLVNPQGKPVMR	1385.7668	-3.98			
25a	**0.1**	Not determined	-	-	-	-	-	-
25b	**0.1**	CVF	FFYIDGNENFHVSITAR	2028.9693	-24	Q91132 (*N. kaouthia*)	2	77
			FVAYYQVGNNEIVADSVWVDVK	2514.2430	-26			
25c	**0.3**	5'-NUC	ETPVLSNPGPYLEFR	1717.8675	17	B6EWW8	4	124
			ETPVLSNPGPYLEFRDEVEELQK	2688.3282	15	(*G. brevicaudus*)		
			FHECNLGNLICDAVIYNNVR	2420.1365	14			
			VVSLNVLCTECR	1448.7116	18			

The proteins identified were sorted according to protein families. Cysteine residues determined in MS/MS analysis are carbamidomethylated.

* Results obtained from nanoESI Orbitrap Fusion analysis. Abbreviations: SVMP: snake venom metalloproteinase; 5'NUC: 5'nucleotidase; CVF: cobra venom factor; GPX: glutathione peroxidase.

Table 4.1(a) *N. kaouthia* (Malaysia), continued.

Protein fraction	%	Protein family/subtype	MS/MS derived sequence	Matched MH+	Mass Δ (ppm)	Accession (species)	Matched peptide	Protein score
25d	**0.3**	CVF	INYENALLAR	1175.6298	14	Q91132 (*N. kaouthia*)	6	341
			HFEVGFIQPGSVK	1443.7511	10			
			CAGETCSSLNHQER	1647.6729	6			
			IDVPLQIEK	1053.6070	16			
			THQYISQR	1031.5148	18			
			VNDDYLIWGSR	1336.6412	14			
25e	**0.1**	CVF	DSITTWVVLAVSFTPTK	1863.9982	-23	Q91132 (*N. kaouthia*)	4	161
			GICVAEPYEIR	1305.6387	-53			
			AVPFVIVPLEQGLHDVEIK	2102.1775	-17			
			ASVQEALWSDGVR	1416.6997	-38			
25f	**0.1**	CVF	GICVAEPYEIR	1305.6387	13	Q91132 (*N. kaouthia*)	3	206
			AILHNYVNEDIYVR	1717.8787	12			
			ASVQEALWSDGVR	1416.6997	13			
25g	**0.2**	Not determined	-	-	-	-	-	-
26a	**0.1**	CVF	QLDIFVHDFPR	1385.7092	9	Q91132 (*N. kaouthia*)	8	343
			QNQYVVVQVTGPQVR	1713.9162	8			
			GIYTPGSPVLYR	1321.7030	8			
			KYVLPSFEVR	1236.6866	8			
			YVLPSFEVR	1108.5917	7			
			FFYIDGNENFHVSITAR	2028.9694	6			
			RDGQNLVTMNLHITPDLIPSFR	2536.3220	10			
			DGQNLVTMNLHITPDLIPSFR	2380.2209	5			
26b	**1.1**	LAAO	EGWYVNMGPMR	1338.5849	-1	A8QL58 (*N. atra*)	3	158
			TFVTADYVIVCSTSR	1717.8345	-3			
			RIYFEPPLPPK	1355.7601	-2			
26c	**0.3**	Not determined	-	-	-	-	-	-
27a	**< 0.1**	CVF	QLDIFVHDFPR	1385.7092	15	Q91132 (*N. kaouthia*)	7	344
			QNQYVVVQVTGPQVR	1713.9162	16			
			GIYTPGSPVLYR	1321.7030	17			
			KYVLPSFEVR	1236.6866	17			
			YVLPSFEVR	1108.5917	15			
			TNHGDLPR	908.4464	16			
			IKLEGDPGAR	1054.5771	18			
27b	**0.1**	Not determined	-	-	-	-	-	-

The proteins identified were sorted according to protein families. Cysteine residues determined in MS/MS analysis are carbamidomethylated.
Abbreviations: CVF: cobra venom factor; LAAO: L-amino acid oxidase.

Table 4.1(a) *N. kaouthia* (Malaysia), continued.

Protein fraction	%	Protein family/subtype	MS/MS derived sequence	Matched MH+	Mass Δ (ppm)	Accession (species)	Matched peptide	Protein score
27c	**0.5**	SVMP (mocarhagin-1)	VYEMVNALNTMYR	1602.7534	8	Q10749	2	74
			VYEMVNALNTMYR	1618.7483	5	(*Naja mossambica*)		
28a	**< 0.1**	CVF	QLDIFVHDFPR	1385.7092	17	Q91132(*N. kaouthia*)	10	328
			QNQYVVVQVTGPQVR	1713.9162	17			
			GIYTPGSPVLYR	1321.7030	19			
			KYVLPSFEVR	1236.6866	18			
			YVLPSFEVR	1108.5917	18			
			FFYIDGNENFHVSITAR	2028.9694	13			
			RDGQNLVTMNLHITPDLIPSFR	2536.3220	18			
			DGQNLVTMNLHITPDLIPSFR	2380.2209	14			
			DGQNLVTMNLHITPDLIPSFR	2396.2158	12			
			IKLEGDPGAR	1054.5771	20			
28b	**0.1**	Not determined	-	-	-	-	-	-
28c	**0.9**	SVMP (cobrin)	ATLDLFGEWR	1206.6033	-7	Q9PVK7(*N. naja*)	1	68
28d	**0.1**	SVMP (cobrin)	VYEMINTMNMIYR	1676.7724	17	Q9PVK7(*N. naja*)	6	161
			VYEMINTMNMIYR	1692.7673	20			
			ATLDLFGEWR	1206.6033	24			
			TKPAYQFSSCSVR	1529.7297	20			
			DDCDLPELCTGQSAECPTDVFQR	2712.1102	15			
			DSCFTLNQR	1139.5030	35			
28e	**0.1**	SVMP (cobrin)	ATLDLFGEWR	1206.6033	41	Q9PVK7(*N. naja*)	3	65
			TKPAYQFSSCSVR	1529.7297	35			
			DSCFTLNQR	1139.5030	51			
28f	**0.1**	CVF	DDNEDGFIADSDIISR	1780.7751	6	Q91132(*N. kaouthia*)	5	250
			GICVAEPYEIR	1305.6387	11			
			AILHNYVNEDIYVR	1717.8787	8			
			AVPFVIVPLEQGLHDVEIK	2102.1776	5			
			ASVQEALWSDGVR	1416.6997	9			
28g	**< 0.1**	CVF	TMSFYLR	916.4477	23	Q91132(*N. kaouthia*)	6	295
			TMSFYLR	932.4426	21			
			GICVAEPYEIR	1305.6387	19			
			AILHNYVNEDIYVR	1717.8787	18			
			AVPFVIVPLEQGLHDVEIK	2102.1776	14			
			ASVQEALWSDGVR	1416.6997	19			

The proteins identified were sorted according to protein families. Cysteine residues determined in MS/MS analysis are carbamidomethylated.
Abbreviations: SVMP: snake venom metalloproteinase; CVF: cobra venom factor.

Table 4.1(b): The proteins identified from the SDS-PAGE gel of reverse-phase isolated fractions of *N. kaouthia* (Thailand) venom by using MALDI-TOF/TOF and nanoESI Orbitrap Fusion analysis.

Protein fraction	%	Protein family/subtype	MS/MS derived sequence	Matched MH+	Mass Δ (ppm)	Accession (species)	Matched peptide	Protein score
1	**0.9**	3FTx-SNTX (cobrotoxin)*	LECHNQQSSQTPTTTGCSGGETNCYK LECHNQQSSQTPTTTGCSGGETNCYKK	2945.2123 3073.3065	0.92 0.66	P60771 (*N. kaouthia*)	2	70
2a	**2.7**	3FTx-SNTX (cobrotoxin)*	LECHNQQSSQTPTTTGCSGGETNCYK NGIEINCCTTDR	2945.1965 1452.6156	-4.43 -3.44	P60771 (*N. kaouthia*)	2	64
2b	**4.0**	3FTx-SNTX (cobrotoxin-c)	WWSDHR VKPGVNLNCCR	885.3882 1315.6489	-37 -33	P59276 (*N. kaouthia*)	2	68
3a	**< 0.1**	Not determined	-	-	-	-	-	-
3b	**< 0.1**	NP (natriuretic peptide Na-NP)*	GSSCFGQK IGSMSGMGCR	870.3773 1055.4429	-0.18 -0.22	D9IX97 (*N. atra*)	2	27
4	**0.1**	NP (natriuretic peptide Na-NP)*	GSSCFGQK	870.3768	-0.74	D9IX97 (*N. atra*)	1	12
5	**< 0.1**	Not determined	-	-	-	-	-	-
6	**32.0**	3FTx-LNTX (alpha-elapitoxin-Nk2a)	TWCDAFCSIR TWCDAFCSIR RVDLGCAATCPTVK TGVDIQCCSTDNCNPFPTR TGVDIQCCSTDNCNPFPTR	1257.5271 1314.5485 1546.7596 2183.9034 2240.9249	-36 -32 -43 -42 -44	P01391 (*N. kaouthia*)	5	224
7a	**0.5**	3FTx-MTLP (muscarinic toxin-like protein 2)	SIFGVTTEDCPDGQNLCFK GCAATCPIAENR GCAATCPIAENR	2186.9612 1261.5543 1318.5758	-17 5 -2	P82463 (*N. kaouthia*)	3	69
7b	**0.1**	3FTx-LNTX (alpha-elapitoxin-Nk2a)	TWCDAFCSIR TWCDAFCSIR	1257.5271 1314.5485	-28 -27	P01391 (*N. kaouthia*)	2	57
8	**1.1**	3FTx-LNTX (alpha-elapitoxin-Nk2a)	TWCDAFCSIR TGVDIQCCSTDNCNPFPTR	1314.5485 2240.9249	-15 -16	P01391 (*N. kaouthia*)	2	69
9a	**0.1**	3FTx-LNTX (alpha-elapitoxin-Nk2a)	TWCDAFCSIR TWCDAFCSIR TGVDIQCCSTDNCNPFPTR TGVDIQCCSTDNCNPFPTRK	1257.5271 1314.5485 2240.9249 2254.9769	-20 -16 -15 -33	P01391 (*N. kaouthia*)	4	224
9b	**< 0.1**	3FTx-MTLP (muscarinic toxin-like protein 3 - deduced 10b)	-	-	-	-	-	-
10a	**0.2**	vNGF (venom nerve growth factor 2)	QYFFETK GIDSSHWNSYCTETDTFIK ALTMEGNQASWR	961.4545 2259.9742 1378.6299	100 94 100	Q5YF89 (*Naja sputatrix*)	3	103

The proteins identified were sorted according to protein families. Cysteine residues determined in MS/MS analysis are carbamidomethylated.

* Results obtained from nanoESI Orbitrap Fusion analysis. Abbreviations: 3FTx: three-finger toxin; LNTX: long neurotoxin; SNTX: short neurotoxin; MTLP: muscarinic toxin-like protein; NP: Natriuretic peptide.

Table 4.1(b) *N. kaouthia* (Thailand), continued.

Protein fraction	%	Protein family/subtype	MS/MS derived sequence	Matched MH+	Mass Δ (ppm)	Accession (species)	Matched peptide	Protein score
10b	**0.3**	3FTx-MTLP (muscarinic toxin-like protein 3)	TSETTEICPDSWYFCYK	2185.8972	97	P82464 (*N. kaouthia*)	2	95
			GCTFTCPELRPTGIYVYCCR	2509.1010	96			
11a	**6.4**	3FTx-WTX (weak toxin CM-9a)*	GCADTCPVGYPKEMIECCSTDK	2578.0351	-1.45	P25679 (*N. kaouthia*)	5	85
			LTCLNCPEMFCGK	1629.6877	-0.99			
			LTCLNCPEMFCGKFQICR	2334.0381	2.54			
			NGEKICFK	995.4972	-0.69			
			KLHQR	681.4148	-0.95			
11b	**0.7**	3FTx-CTX	(cytotoxin 3 - deduced 12c)	-	-	-	-	-
12a	**5.0**	PLA_2 (acidic 2)	SWWDFADYGCYCGR	1841.6926	100	Q91133 (*N. atra*)	2	146
			LAAICFAGAPYNNNNYNIDLK	2355.1317	99			
12b	**2.5**	3FTx-WTX	(weak toxin CM-9a - deduced 11a)	-	-	-	-	-
12c	**9.1**	3FTx-CTX (cytotoxin 3)	NSLLVKYVCCNTDR	1740.8287	-13	P01446 (*N. atra*)	2	111
			YVCCNTDR	1086.4223	-14			
13a	**6.8**	PLA_2 (acidic 2)	SWWDFADYGCYCGR	1841.6926	-69	Q91133 (*N. atra*)	1	97
13b	**7.3**	3FTx-CTX (cytotoxin 3)*	LKCNKLIPLAYK	1460.8674	1.05	P01446 (*N. atra*)	14	162
			NSLLVKYVCCNTDR	1741.8370	0.52			
			YVCCNTDRCN	1361.5037	0.41			
			NSLLVKYVCCNTDRCN	2015.9079	-0.87			
			GCIDACPKNSLLVK	1574.8029	-0.05			
			MFMVSNKTVPVKR	1536.8411	1.38			
			MFMVSNKTVPVK	1380.7389	0.77			
			LIPLAYKTCPAGK	1431.8047	1.28			
			NLCYKMFMVSNK	1534.7238	1.46			
			GCIDACPK	920.3970	0.50			
			YVCCNTDR	1087.4318	2.06			
			MFMVSNK	856.4055	-0.13			
			NSLLVK	673.4235	-1.22			
			CNKLIPLAYK	1219.6889	1.73			
14a	**0.3**	PLA_2 (acidic 1)*	GDNDACAAAVCDCDR	1669.5940	-3.58	P00596 (*N. kaouthia*)	6	91
			TYSYECSQGTLTCK	1697.7074	-4.20			
			CCQVHDNCYNEAEK	1826.6810	-4.45			
			GGSGTPVDDLDR	1188.5441	-4.27			
			LAAICFAGAPYNNNNYNIDLK	2356.1268	-5.23			
			SWWDFADYGCYCGR	1842.6928	-3.89			

The proteins identified were sorted according to protein families. Cysteine residues determined in MS/MS analysis are carbamidomethylated.

* Results obtained from nanoESI Orbitrap Fusion analysis. Abbreviations: 3FTx: three-finger toxin; CTX: cytotoxin; WTX: weak neurotoxin/toxin; MTLP: muscarinic toxin-like protein; PLA_2: phospholipase A_2.

Table 4.1(b) *N. kaouthia* (Thailand), continued.

Protein fraction	%	Protein family/subtype	MS/MS derived sequence	Matched MH+	Mass Δ (ppm)	Accession (species)	Matched peptide	Protein score
14b	**0.1**	vNGF	EDHPVHNLGEHSVCDSVSAWVTK	2602.1871	-21	P01140 (*N. naja*)	4	140
			GIDSSHWNSYCTETDTFIK	2259.9743	-25			
			ALTMEGNQASWR	1362.6350	-22			
			ALTMEGNQASWR	1378.6299	-24			
14c	**1.2**	3FTx-CTX (cytotoxin homolog)*	LKCHNTQLPFIYK	1661.8725	-6.45	P14541 (*N. kaouthia*)	11	185
			KFPLKIPIK	1083.7236	-4.90			
			CHNTQLPFIYK	1420.6972	-4.94			
			CHNTQLPFIYKTCPEGK	2092.9911	-1.58			
			NSALLKYVCCSTDK	1658.7804	-4.38			
			YVCCSTDKCN	1306.4808	-4.09			
			FPLKIPIK	955.6314	-2.72			
			FPLKIPIKR	1111.7317	-3.04			
			YVCCSTDK	1032.4080	-4.37			
			GCADNCPKNSALLK	1547.7258	-3.10			
			GCADNCPK	921.3525	-3.14			
15a	**0.2**	vNGF 2	QYFFETK	961.4545	-10	Q5YF89 (*N. sputatrix*)	4	159
			GIDSSHWNSYCTETDTFIK	2259.9743	-5			
			ALTMEGNQASWR	1362.6350	-5			
			ALTMEGNQASWR	1378.6299	-4			
15b	**0.2**	3FTx-CTX (cardiotoxin 2A)*	YVCCNTDRCN	1361.5093	4.53	Q9PST4 (*N. sputatrix*)	5	92
			GCIDVCPK	948.4309	3.26			
			MYMVATPK	940.4684	5.65			
			YVCCNTDR	1087.4328	2.96			
			SSLLVK	646.4154	3.03			
16a	**< 0.1**	Not determined	-	-	-	-	-	-
16b	**< 0.1**	Not determined	-	-	-	-	-	-
16c	**0.4**	3FTx-CTX (cytotoxin I-like T-15)	MFMMSDLTIPVKR	1567.7924	-28	Q91136 (*N. atra*)	4	116
			GCIDVCPK	947.4205	-32			
			YVCCNTDR	1029.4008	-19			
			YVCCNTDR	1086.4223	-22			
17	**8.6**	3FTx-CTX (cytotoxin 5a)	LVPLFYK	878.5265	-34	O73857 (*N. sputatrix*)	3	68
			YVCCNTDR	1029.4008	-24			
			YVCCNTDR	1086.4223	-28			

The proteins identified were sorted according to protein families. Cysteine residues determined in MS/MS analysis are carbamidomethylated.

* Results obtained from nanoESI Orbitrap Fusion analysis. Abbreviations: 3FTx: three-finger toxin; CTX: cytotoxin; vNGF: venom nerve growth factor.

Table 4.1(b) *N. kaouthia* (Thailand), continued.

Protein fraction	%	Protein family/subtype	MS/MS derived sequence	Matched MH+	Mass Δ (ppm)	Accession (species)	Matched peptide	Protein score
18a	**0.7**	Vespryn (Thaicobrin)	TVENVGVSQVAPDNPER	1809.8856	-11	P82885	5	192
			FDGSPCVLGSPGFR	1437.6710	-8	(*N. kaouthia*)		
			FDGSPCVLGSPGFR	1494.6925	-3			
			HFFEVK	805.4122	-21			
			EWAVGLAGK	929.4970	-17			
18b	**0.1**	3FTx-CTX (cytotoxin 3)	LVPLFYK	878.5265	-25	P60301 (*N. atra*)	3	80
			YVCCNTDR	1029.4008	-27			
			YVCCNTDR	1086.4223	-23			
19a	**1.0**	SVMP (atrase-B)	DDCDLPELCTGQSAECPTDSLQR	2666.0894	95	D6PXE8 (*N. atra*)	1	113
19b	**0.2**	CRISP (natrin-1)*	SNCPASCFCR	1258.4703	-4.74	Q7T1K6 (*N. atra*)	6	47
			EIVDLHNSLR	1195.6368	-5.22			
			LTNCDSLLK	1063.5389	-6.02			
			QSSCQDDWIK	1266.5361	-4.67			
			QKEIVDLHNSLR	1451.7905	-4.21			
			NVDFNSESTR	1168.5179	-4.35			
19c	**0.7**	CRISP (natrin-2)	CSFAHSPPHLR	1307.6193	-25	Q7ZZN8 (*N. atra*)	2	147
			IGCGENLFMSSQPYAWSR	2101.9349	-31			
19d	**0.1**	Not determined	-	-	-	-	-	-
20a	**1.4**	CRISP (natrin-1)	QKEIVDLHNSLR	1450.7892	-22	Q7T1K6 (*N. atra*)	9	564
			EIVDLHNSLR	1194.6357	-21			
			MEWYPEAASNAER	1552.6616	-22			
			WANTCSLNHSPDNLR	1783.8060	-26			
			VLEGIQCGESIYMSSNAR	2012.9295	-21			
			TWTEIIHLWHDEYK	1869.9050	-23			
			NFVYGVGANPPGSVTGHYTQIVWYQTYR	3173.5358	-34			
			AGCAVSYCPSSAWSYFYVCQYCPSGNFQGK	3500.4358	-25			
			SNCPASCFCR	1257.4689	-21			
20b	**0.4**	CTL (BFL-1)	KYIWEWTDR	1295.6299	-19	Q90WI8 (*B. fasciatus*)	2	127
			YIWEWTDR	1167.5349	-9			
21	**0.2**	EDCP*	VNRPSHLWSAACCLIDNNHLR	2533.2174	-4.97	V8N4Y2	8	346
			GRVNRPSHLWSAACCLIDNNHLR	2746.3456	-2.54	(*Ophiophagus hannah*)		
			LAQLYNVNHVSLFHSDCPR	2270.1009	-5.57			
			SSTFTLTNIVPQFIK	1695.9272	-2.65			
			LNGGAWNNYEQTTMQQMTR	2243.0066	4.34			

The proteins identified were sorted according to protein families. Cysteine residues determined in MS/MS analysis are carbamidomethylated.

* Results obtained from nanoESI Orbitrap Fusion analysis. Abbreviations: 3FTx: three-finger toxin; CTX: cytotoxin; SVMP: snake venom metalloproteinase; CRISP: cysteine-rich secretory protein; CTL: C-type lectin; EDCP: endonuclease domain-containing protein.

Table 4.1(b) *N. kaouthia* (Thailand), continued.

Protein fraction	%	Protein family/subtype	MS/MS derived sequence	Matched MH+	Mass Δ (ppm)	Accession (species)	Matched peptide	Protein score
			LAQLYNVNHVSLFHSDCPRQ	2398.1629	-3.86			
			GCQQTFAVVGAVPGDTYIAR	2110.0294	-4.41			
			SWAILAR	816.4786	7.27			
23	**0.5**	SVMP (kaouthiagin)	QTVLLPR	825.5072	-14	P82942 (*N. kaouthia*)	5	329
			TAPAFQFSSCSIR	1470.6925	-8			
			DYQEYLLR	1098.5345	-9			
			HDCDLPELCTGQSAECPTDSLQR	2688.1214	-9			
			GCFDLNMR	1011.4266	-12			
24a	**0.2**	PDE (phosphodiesterase 1)*	AEYLETWDTLMPNINK	1937.9270	-2.26	U3FAB3 (*M. fulvius*)	14	149
			DFYTFDSEAIVK	1434.6758	-2.06			
			YCSGGTHGYDNEFK	1634.6498	-2.57			
			LWNYFHSTLLPK	1518.8068	-2.36			
			YCLLHQTK	1062.5434	3.09			
			NLHNCVNLILLADHGMEAISCNR	2664.2833	1.09			
			NPFYNPSPAK	1134.5546	-2.87			
			AYLAKDLPK	1018.5878	-5.35			
			RPDFSTLYIEEPDTTGHK	2106.0044	-4.53			
			YGPVSGQVIK	1047.5822	-1.11			
			VNLMVDR	846.4474	-3.33			
			AATYFWPGSEVK	1355.6591	-2.93			
			TLGMLMEGLK	1092.5754	-3.55			
			DCCTDYK	961.3359	-3.28			
24b	**< 0.1**	CVF	QLDIFVHDFPR	1385.7092	-6	Q91132 (*N. kaouthia*)	3	200
			QNQYVVVQVTGPQVR	1713.9162	-6			
			GIYTPGSPVLYR	1321.7030	-1			
24c	**0.1**	CVF	INYENALLAR	1175.6298	-9	Q91132 (*N. kaouthia*)	2	119
			VNDDYLIWGSR	1336.6411	-10			
25a	**< 0.1**	PDE (phosphodiesterase 1 - deduced 24a)	-	-	-	-	-	-
25b	**0.1**	CVF	QLDIFVHDFPR	1385.7092	-11	Q91132 (*N. kaouthia*)	6	453
			QNQYVVVQVTGPQVR	1713.9162	-10			
			GIYTPGSPVLYR	1321.7030	-9			
			YVLPSFEVR	1108.5916	-11			
			FFYIDGNENFHVSITAR	2028.9693	-8			
			LILNIPLNAQSLPITVR	1874.1353	-12			

The proteins identified were sorted according to protein families. Cysteine residues determined in MS/MS analysis are carbamidomethylated.

* Results obtained from nanoESI Orbitrap Fusion analysis. Abbreviations: SVMP: snake venom metalloproteinase; PDE: phosphodiesterase; CVF: cobra venom factor.

Table 4.1(b) *N. kaouthia* (Thailand), continued.

Protein fraction	%	Protein family/subtype	MS/MS derived sequence	Matched MH+	Mass Δ (ppm)	Accession (species)	Matched peptide	Protein score
25c	**0.2**	5'NUC	ETPVLSNPGPYLEFR	1717.8675	-15	B6EWW8	4	225
			ETPVLSNPGPYLEFRDEVEELQK	2688.3282	-9	(*G. brevicaudus*)		
			FHECNLGNLICDAVIYNNVR	2420.1365	-7			
			VVSLNVLCTECR	1448.7116	-14			
25d	**0.1**	CVF	INYENALLAR	1175.6298	-8	Q91132 (*N. kaouthia*)	7	456
			ATMTILTFYNAQLQEK	1870.9498	-12			
			HFEVGFIQPGSVK	1443.7510	-10			
			VYSYYNLDEK	1292.5924	4			
			VNDDYLIWGSR	1336.6411	-4			
			NTWIER	817.4082	-9			
			WPHEDECQEEEFQK	1889.7526	-8			
25e	**0.1**	CVF	SWLWLTK	932.5120	-28	Q91132 (*N. kaouthia*)	5	360
			GICVAEPYEIR	1305.6387	-17			
			AILHNYVNEDIYVR	1717.8787	-20			
			AVPFVIVPLEQGLHDVEIK	2102.1775	-18			
			ASVQEALWSDGVR	1416.6997	-19			
25f	**0.3**	CVF	GICVAEPYEIR	1305.6387	-16	Q91132 (*N. kaouthia*)	3	231
			AILHNYVNEDIYVR	1717.8787	-20			
			ASVQEALWSDGVR	1416.6997	-18			
26a	**< 0.1**	PDE (phosphodiesterase 1 - deduced 24a)	-	-	-	-	-	-
26b	**0.1**	CVF	QLDIFVHDFPR	1385.7092	-12	Q91132 (*N. kaouthia*)	6	358
			QNQYVVVQVTGPQVR	1713.9162	-13			
			GIYTPGSPVLYR	1321.7030	-12			
			YVLPSFEVR	1108.5917	-13			
			FFYIDGNENFHVSITAR	2028.9694	-11			
			YVLPSFEVR	1874.1353	-12			
26c	**0.6**	LAAO	VTLLEASER	1016.5502	78	A8QL58 (*N. atra*)	6	227
			EGWYVNMGPMR	1338.5849	83			
			RIYFEPPLPPK	1355.7601	80			
			IYFEPPLPPK	1199.6590	75			
			REIQALCYPSIK	1476.7758	84			
			EIQALCYPSIK	1320.6747	55			

The proteins identified were sorted according to protein families. Cysteine residues determined in MS/MS analysis are carbamidomethylated.

* Results obtained from nanoESI Orbitrap Fusion analysis. Abbreviations: PDE: phosphodiesterase; 5'NUC: 5'nucleotidase; CVF: cobra venom factor; LAAO: L-amino acid oxidase.

Table 4.1(b) *N. kaouthia* (Thailand), continued.

Protein fraction	%	Protein family/subtype	MS/MS derived sequence	Matched MH+	Mass Δ (ppm)	Accession (species)	Matched peptide	Protein score
26d	**0.1**	CVF	QNQYVVVQVTGPQVR	1713.9162	0	Q91132 (*N. kaouthia*)	6	193
			GIYTPGSPVLYR	1321.7030	2			
			DLNLDITIELPDR	1525.7988	1			
			INYENALLAR	1175.6298	3			
			HFEVGFIQPGSVK	1443.7511	-5			
			VNDDYLIWGSR	1336.6412	4			
26e	**0.1**	CVF (deduced 25e)	-	-	-	-	-	-
26f	**< 0.1**	CVF (deduced 25f)	-	-	-	-	-	-
27a	**< 0.1**	CVF	QLDIFVHDFPR	1385.7092	-17	Q91132 (*N. kaouthia*)	7	314
			QNQYVVVQVTGPQVR	1713.9162	-16			
			GIYTPGSPVLYR	1321.7030	-14			
			KYVLPSFEVR	1236.6866	-17			
			YVLPSFEVR	1108.5917	-15			
			LILNIPLNAQSLPITVR	1874.1353	-16			
			TNHGDLPR	908.4464	-15			
27b	**0.4**	LAAO*	TCADIVINDLSLIHDLPK	2037.0641	-2.23	A8QL58 (*N. atra*)	7	76
			TFVTADYVIVCSTSR	1718.8355	-3.70			
			EIQALCYPSIK	1321.6883	4.68			
			REIQALCYPSIK	1477.7784	-3.27			
			KFWEADGIHGGK	1344.6632	-4.76			
			IYFEPPLPPK	1200.6617	-3.92			
			EGWYVNMGPMR	1339.5892	-2.32			
28a	**0.1**	CVF	TDTEEQILVEAHGDSTPK	1968.9276	-37	Q91132 (*N. kaouthia*)	10	535
			QLDIFVHDFPR	1385.7092	-35			
			QNQYVVVQVTGPQVR	1713.9162	-31			
			GIYTPGSPVLYR	1321.7030	-32			
			KYVLPSFEVR	1236.6866	-36			
			YVLPSFEVR	1108.5917	-37			
			FFYIDGNENFHVSITAR	2028.9694	-28			
			LILNIPLNAQSLPITVR	1874.1353	-29			
			YFTYLILNK	1173.6434	-41			
			DGQNLVTMNLHITPDLIPSFR	2396.2158	-34			

The proteins identified were sorted according to protein families. Cysteine residues determined in MS/MS analysis are carbamidomethylated.

* Results obtained from nanoESI Orbitrap Fusion analysis. Abbreviations: SVMP: snake venom metalloproteinase; CVF: cobra venom factor; LAAO: L-amino acid oxidase.

Table 4.1(b) *N. kaouthia* (Thailand), continued.

Protein fraction	%	Protein family/subtype	MS/MS derived sequence	Matched MH+	Mass Δ (ppm)	Accession (species)	Matched peptide	Protein score
28b	**0.8**	SVMP (cobrin)	ATLDLFGEWR	1206.6033	70	Q9PVK7 (*N. naja*)	6	514
			TKPAYQFSSCSVR	1529.7296	69			
			AAKDDCDLPELCTGQSAECPTDVFQR	2982.2793	65			
			DDCDLPELCTGQSAECPTDVFQR	2712.1102	69			
			CPIMTNQCIALR	1475.7047	66			
			DSCFTLNQR	1139.5029	71			
28c	**0.1**	SVMP (cobrin)	VYEMINTMNMIYR	1708.7622	79	Q9PVK7 (*N. naja*)	7	445
			ATLDLFGEWR	1206.6033	77			
			TSAAVVQDYSK	1167.5771	77			
			GFCTCGFNK	1089.4371	75			
			TKPAYQFSSCSVR	1529.7296	77			
			LQHEAQCDSEECCEK	1921.7240	75			
			DSCFTLNQR	1139.5029	79			
28d	**< 0.1**	SVMP (cobrin)	ATLDLFGEWR	1206.6033	82	Q9PVK7 (*N. naja*)	6	372
			GFCTCGFNK	1089.4371	77			
			RTKPAYQFSSCSVR	1685.8307	73			
			TKPAYQFSSCSVR	1529.7296	79			
			LQHEAQCDSEECCEK	1921.7240	77			
			DSCFTLNQR	1139.5029	76			
28e	**0.1**	SVMP (cobrin)	TSAAVVQDYSK	1167.5771	73	Q9PVK7 (*N. naja*)	5	375
			GFCTCGFNK	1089.4371	74			
			TKPAYQFSSCSVR	1529.7296	76			
			LQHEAQCDSEECCEK	1921.7240	74			
			DSCFTLNQR	1139.5029	76			

The proteins identified were sorted according to protein families. Cysteine residues determined in MS/MS analysis are carbamidomethylated.
Abbreviations: SVMP: snake venom metalloproteinase.

Table 4.1(c): The proteins identified from the SDS-PAGE gel of reverse-phase isolated fractions of *N. kaouthia* (Vietnam) venom by using MALDI-TOF/TOF and nanoESI Orbitrap Fusion analysis.

Protein fraction	%	Protein family/subtype	MS/MS derived sequence	Matched MH+	Mass Δ (ppm)	Accession (species)	Matched peptide	Protein score
1	**1.1**	3FTx-SNTX (cobrotoxin)*	LECHNQQSSQTPTTTGCSGGETNCYK	2945.2179	2.85	P60771 (*N. kaouthia*)	3	387
			LECHNQQSSQTPTTTGCSGGETNCYKK	3073.3191	4.73			
			NGIEINCCTTDR	1452.6249	2.94			
2a	**3.5**	3FTx-SNTX (cobrotoxin)	LECHNQQSSQTPTTTGCSGGETNCYK	2944.2022	78	P60771 (*N. kaouthia*)	1	58
2b	**2.5**	3FTx-SNTX (cobrotoxin-c)*	LECHNQQSSQAPTTK	1728.8064	5.45	P59276 (*N. kaouthia*)	5	542
			VKPGVNLNCCR	1316.6587	1.83			
			TCSGETNCYKK	1347.5691	1.74			
			KWWSDHR	1014.4933	2.76			
			TCSGETNCYK	1219.4750	2.62			
3a	**0.5**	3FTx-SNTX (cobrotoxin)*	LECHNQQSSQTPTTTGCSGGETNCYK	2945.2097	0.05	P60771 (*N. kaouthia*)	8	131
			LECHNQQSSQTPTTTGCSGGETNCYKK	3073.3055	0.33			
			GCGCPSVKNGIEINCCTTDRCNN	2686.0942	1.72			
			NGIEINCCTTDRCNN	1840.7374	0.10			
			NGIEINCCTTDR	1452.6221	1.01			
			LECHNQQSSQTPTTTGCSGGETNCYKKR	3229.4111	1.71			
			TERGCGCPSVK	1250.5617	0.02			
			GCGCPSVK	864.3707	0.43			
3b	**0.0**	3FTx-SNTX (cobrotoxin-b)*	LECHNQQSSQTPTTK	1758.8071	-0.30	P59275 (*N. kaouthia*)	7	178
			VKPGVNLNCCTTDRCNN	2021.9035	4.16			
			TCSGETNCYKK	1347.5673	0.38			
			VKPGVNLNCCTTDR	1633.7792	0.41			
			KWWSDHR	1014.4897	-0.76			
			WWSDHRGTIIER	1555.7756	-0.60			
			TCSGETNCYK	1219.4714	-0.38			
4a	**1.4**	3FTx-SNTX (cobrotoxin)	LECHNQQSSQTPTTTGCSGGETNCYK	2944.2022	71	P60771 (*N. kaouthia*)	1	85
4b	**0.1**	3FTx-SNTX (cobrotoxin-b)	(deduced 3b)	-	-	-	-	-
5a	**0.1**	3FTx-SNTX (cobrotoxin)*	LECHNQQSSQTPTTTGCSGGETNCYK	2945.2018	-2.62	P60771 (*N. kaouthia*)	4	28
			NGIEINCCTTDR	1452.6163	-3.02			
			NGIEINCCTTDRCNN	1840.7328	-2.35			
			GCGCPSVK	864.3668	-4.02			
5b	**0.1**	Not determined	-	-	-	-	-	-
5c	**0.2**	3FTx-WTX (weak toxin S4C11)*	LTCLICPEKYCNK	1698.7963	-2.91	P01400	6	153
			EIVECCSTDKCNH	1651.6437	-4.38	(*Naja melanoleuca*)		

The proteins identified were sorted according to protein families. Cysteine residues determined in MS/MS analysis are carbamidomethylated.

* Results obtained from nanoESI Orbitrap Fusion analysis. Abbreviations: 3FTx: three-finger toxin; SNTX: short neurotoxin; WTX: weak neurotoxin/toxin.

Table 4.1(c) *N. kaouthia* (Vietnam), continued.

Protein fraction	%	Protein family/subtype	MS/MS derived sequence	Matched MH+	Mass Δ (ppm)	Accession (species)	Matched peptide	Protein score
			LTCLICPEK	1133.5654	-3.55			
			LTCLICPEKYCNKVHTCR	2352.0957	-5.23			
			YCNKVHTCR	1237.5513	-4.19			
			EIVECCSTDK	1240.5130	-4.42			
5d	**0.0**	Not determined	-	-	-	-	-	-
6a	**0.3**	3FTx-WTX (weak neurotoxin 6)*	LTCLICPEKYCNKVHTCLNGEK	2737.3044	4.19	P29180 (*N. naja*)	10	191
			LTCLICPEKYCNK	1698.7981	-1.83			
			YCNKVHTCLNGEK	1622.7333	-4.99			
			YIRGCADTCPVR	1467.6762	-4.77			
			LTCLICPEK	1133.5654	-3.55			
			GCADTCPVR	1035.4308	-3.71			
			EIVQCCSTDK	1239.5301	-3.53			
			GCADTCPVRKPR	1416.6781	-3.81			
			EIVQCCSTDKCNH	1650.6595	-4.53			
			VHTCLNGEKICFK	1605.7879	0.14			
6b	**0.0**	Kunitz-type inhibitor*	FRTIDECNR	1210.5597	-3.07	P20229 (*N. naja*)	3	31
			TIDECNR	907.3915	-2.55			
			TIDECNRTCVG	1324.5574	-3.51			
7a	**0.2**	3FTx-WTX (weak neurotoxin 7)	RGCAATCPEAKPR	1472.6976	79	P29181 (*N. naja*)	3	139
			GCAATCPEAKPR	1316.5965	82			
			EIVQCCSTDK	1238.5271	81			
7b	**0.1**	3FTx-WTX (probable weak neurotoxin NNAM2)	GCAATCPETKPR	1346.6071	81	Q9YGI4 (*N. atra*)	3	67
			DMVECCSTDR	1271.4581	83			
			DMVECCSTDR	1287.4530	82			
7c	**0.0**	Not determined	-	-	-	-	-	-
8a	**0.2**	3FTx-WTX (weak neurotoxin 7)	RGCAATCPEAKPR	1472.6976	79	P29181 (*N. naja*)	3	147
			GCAATCPEAKPR	1316.5965	80			
			EIVQCCSTDK	1238.5271	80			
8b	**0.0**	3FTx-WTX (weak toxin S4C11)*	EIVECCSTDKCNH	1282.6065	-2.44	P01400 (*N. melanoleuca*)	4	31
			EIVECCSTDK	1026.4467	-2.82			
			LTCLICPEK	2287.8871	-1.72			
			GCAATCPEAKPR	1083.4753	-2.13			
9a	**0.7**	3FTx-MTLP (muscarinic toxin-like protein 2)	SIFGVTTEDCPDGQNLCFK	2186.9613	93	P82463 (*N. kaouthia*)	6	229
			RWHMIVPGR	1150.6182	95			
			RWHMIVPGR	1166.6131	96			

The proteins identified were sorted according to protein families. Cysteine residues determined in MS/MS analysis are carbamidomethylated.

* Results obtained from nanoESI Orbitrap Fusion analysis. Abbreviations: 3FTx: three-finger toxin; WTX: weak neurotoxin/toxin; MTLP: muscarinic toxin-like protein.

Table 4.1(c) *N. kaouthia* (Vietnam), continued.

Protein fraction	%	Protein family/subtype	MS/MS derived sequence	Matched MH+	Mass Δ (ppm)	Accession (species)	Matched peptide	Protein score
			WHMIVPGR	994.5171	103			
			WHMIVPGR	1010.5120	102			
			GCAATCPIAENR	1318.5758	98			
9b	**0.8**	3FTx-WTX (weak neurotoxin 6)*	GCAATCPGTKPR	1275.5905	-2.21	O42256 (*N. sputatrix*)	3	23
			DMVECCSTDR	1272.4620	-2.70			
			RPFSLR	775.4552	-2.74			
10a	**0.4**	3FTx-MTLP (muscarinic toxin-like protein 2)	(deduced 9a)	-	-	-	-	-
10b	**7.4**	3FTx-WTX (weak neurotoxin 6 precursor)*	GCAATCPGTKPR	1275.5907	-2.02	O42256 (*N. sputatrix*)	2	19
			DMVECCSTDR	1272.4627	-2.13			
11	**0.8**	3FTx-MTLP (muscarinic toxin-like protein)	TPETTEICPDSWYFCYK	2195.9180	88	Q9W727 (*Bungarus multicinctus*)	5	214
			ISLADGNDVR	1058.5356	98			
			RGCTFTCPELRPTGK	1778.8556	85			
			GCTFTCPELRPTGK	1622.7545	90			
			YVYCCR	919.3680	98			
12	**0.6**	3FTx-MTLP (muscarinic toxin-like protein)	ISLADGNDVR	1058.5356	119	Q9W727 (*B. multicinctus*)	1	67
13a	**0.6**	vNGF (nerve growth factor beta chain precursor)	QYFFETK	961.4545	86	A59218 (*N. kaouthia*)	2	78
			NPNPEPSGCR	1126.4825	89			
13b	**7.0**	3FTx-WTX (weak toxin CM-9a)*	LTCLNCPEMFCGKFQICR	2334.0254	-2.87	P25679 (*N. kaouthia*)	3	281
			LTCLNCPEMFCGK	1629.6845	-2.94			
			KLHQR	681.4133	-3.19			
14a	**0.0**	PLA_2 (acidic 1)*	GGNNACAAAVCDCDR	1610.6054	-3.18	P00598 (*N. atra*)	5	35
			CCQVHDNCYNEAEK	1826.6835	-3.07			
			ISGCWPYFK	1157.5428	-1.81			
			NMIQCTVPSR	1205.5738	-2.34			
			TYSYECSQGTLTCK	1697.7105	-2.40			
14b	**0.0**	PLA_2 (acidic 1)*	TYSYECSQGTLTCK	1697.7148	0.11	P00598 (*N. atra*)	5	48
			GGNNACAAAVCDCDR	1610.6091	-0.91			
			CCQVHDNCYNEAEK	1826.6859	-1.74			
			NMIQCTVPSR	1205.5763	-0.21			
			GGSGTPVDDLDR	1188.5477	-1.29			
14c	**0.2**	PLA_2 (acidic 1)	NMIQCTVPSR	1204.5692	16	P00598 (*N. atra*)	7	249
			SWWDFADYGCYCGR	1841.6926	13			
			GGSGTPVDDLDR.C	1187.5418	20			
			ISGCWPYFK.T	1156.5375	30			

The proteins identified were sorted according to protein families. Cysteine residues determined in MS/MS analysis are carbamidomethylated.

* Results obtained from nanoESI Orbitrap Fusion analysis. Abbreviations: 3FTx: three-finger toxin; WTX: weak neurotoxin/toxin; MTLP: muscarinic toxin-like protein; PLA_2: phospholipase A_2.

Table 4.1(c) *N. kaouthia* (Vietnam), continued.

Protein fraction	%	Protein family/subtype	MS/MS derived sequence	Matched MH+	Mass Δ (ppm)	Accession (species)	Matched peptide	Protein score
			TYSYECSQGTLTCK	1696.7073	14			
			GGNNACAAAVCDCDR	1609.6031	17			
			LAAICFAGAPYNNNNYNIDLK	2355.1317	12			
14d	**0.5**	3FTx-MTLP (muscarinic toxin-like protein 1)*	FLFSETTETCPDGQNVCFNQAHLIYPGK	3273.4922	-1.81	P82462 (*N. kaouthia*)	11	522
			EKFLFSETTETCPDGQNVCFNQAHLIYPGK	3530.6495	3.93			
			LQNRDVIFCCSTDKCNL	2142.9717	-0.59			
			LQNRDVIFCCSTDK	1755.8172	1.05			
			DVIFCCSTDKCNL	1631.7018	9.52			
			FLFSETTETCPDGQNVCFNQAHLIYPGKYKR	3720.7860	7.67			
			DVIFCCSTDK	1244.5278	-0.71			
			TRGCAATCPKLQNR	1632.8071	0.81			
			TRGCAATCPK	1121.5197	0.54			
			GCAATCPKLQNR	1375.6626	4.12			
			GCAATCPK	864.3711	0.93			
14e	**0.1**	3FTx-CTX (cardiotoxin-1f)	LVPLFYK	878.5265	-27	P85429 (*N. atra*)	1	63
15a	**0.5**	PLA_2 (acidic 1 - deduced 14a)	-	-	-	-	-	-
15b	**13.5**	PLA_2 (acidic 1)	NMIQCTVPSR	1204.5693	-88	P00598 (*N. atra*)	6	227
			NMIQCTVPSR	1220.5642	-85			
			SWWDFADYGCYCGR	1841.6926	-86			
			GGSGTPVDDLDR	1187.5419	-89			
			ISGCWPYFK	1156.5376	-67			
			GGNNACAAAVCDCDR	1609.6032	-81			
15c	**3.0**	3FTx-WTX (weak neurotoxin 6)*	GCAATCPGTKPR	1275.5968	2.77	O42256 (*N. sputatrix*)	3	98
			DMVECCSTDR	1272.4684	2.38			
			RPFSLR	775.4590	2.06			
15d	**1.9**	3FTx-CTX (cytotoxin 4N)*	MFMVSNLTVPVK	1365.7280	0.72	Q9W6W9 (*N. atra*)	9	77
			YVCCNTDRCN	1361.5051	1.39			
			RGCIDVCPK	1104.5306	1.49			
			MFMVSNLTVPVKR	1521.8317	2.38			
			SSLLVKYVCCNTDR	1714.8292	2.35			
			GCIDVCPK	948.4292	1.53			
			YVCCNTDR	1087.4313	1.61			
			SSLLVK	646.4136	0.29			
			LVPLFYKTCPAGK	1493.8207	1.46			

The proteins identified were sorted according to protein families. Cysteine residues determined in MS/MS analysis are carbamidomethylated.

* Results obtained from nanoESI Orbitrap Fusion analysis. Abbreviations: 3FTx: three-finger toxin; CTX: cytotoxin; WTX: weak neurotoxin/toxin; PLA_2: phospholipase A_2.

Table 4.1(c) *N. kaouthia* (Vietnam), continued.

Protein fraction	%	Protein family/subtype	MS/MS derived sequence	Matched MH+	Mass Δ (ppm)	Accession (species)	Matched peptide	Protein score
16a	**0.4**	PLA_2 (acidic 1)	NMIQCTVPSR	1204.5692	19	P00598 (*N. atra*)	8	353
			NMIQCTVPSR	1220.5642	20			
			SWWDFADYGCYCGR	1841.6926	15			
			GGSGTPVDDLDR	1187.5418	21			
			ISGCWPYFK	1156.5375	32			
			TYSYECSQGTLTCK	1696.7073	16			
			GGNNACAAAVCDCDR	1609.6031	19			
			LAAICFAGAPYNNNNYNIDLK	2355.1317	15			
16b	**0.1**	3FTx-CTX (cytotoxin - deduced 17c)	-	-	-	-	-	-
16c	**0.3**	3FTx-CTX (cytotoxin 5a)	LVPLFYK	878.5265	-34	O73857 (*N. sputatrix*)	1	78
17a	**0.7**	PLA_2 (acidic 1)*	TYSYECSQGTLTCK	1697.7167	1.26	P00598 (*N. atra*)	6	84
			GGNNACAAAVCDCDR	1610.6135	1.82			
			CCQVHDNCYNEAEK	1826.6903	0.66			
			NMIQCTVPSR	1205.5778	1.00			
			GGSGTPVDDLDR	1188.5503	0.97			
			ISGCWPYFK	1157.5446	-0.23			
17b	**0.8**	PLA_2 (acidic 2)	SWWDFADYGCYCGR	1784.6711	-27	P15445 (*N. naja*)	4	151
			SWWDFADYGCYCGR	1841.6926	-14			
			ISGCWPYFK	1156.5375	-20			
			LAAICFAGAPYNDNNYNIDLK	2356.1157	-31			
17c	**3.8**	3FTx-CTX (cytotoxin homolog)*	LKCHNTQLPFIYK	1661.8829	-0.24	P14541 (*N. kaouthia*)	8	109
			CHNTQLPFIYK	1420.7034	-0.56			
			KFPLKIPIK	1083.7289	0.00			
			FPLKIPIKR	1111.7342	-0.80			
			FPLKIPIK	955.6314	-2.72			
			YVCCSTDKCN	1306.4842	-1.47			
			YVCCSTDK	1032.4108	-1.65			
			NSALLK	645.3921	-1.38			
17d	**5.0**	3FTx-CTX (cytotoxin 5a)	LVPLFYK	878.5265	-2	O73857 (*N. sputatrix*)	4	184
			MFMVSNLTVPVKR	1520.8207	10			
			GCIDVCPK	947.4205	2			
			YVCCNTDR	1086.4223	7			
18a	**0.6**	PLA_2 (acidic 1 - deduced 17a)	-	-	-	-	-	-
18b	**0.6**	PLA_2 (acidic 2 - deduced 17b)	-	-	-	-	-	-

The proteins identified were sorted according to protein families. Cysteine residues determined in MS/MS analysis are carbamidomethylated.

* Results obtained from nanoESI Orbitrap Fusion analysis. Abbreviations: 3FTx: three-finger toxin; CTX: cytotoxin; PLA_2: phospholipase A_2.

Table 4.1(c) *N. kaouthia* (Vietnam), continued.

Protein fraction	%	Protein family/subtype	MS/MS derived sequence	Matched MH+	Mass Δ (ppm)	Accession (species)	Matched peptide	Protein score
18c	**0.7**	vNGF (venom nerve growth factor)*	GNTVTVMENVNLDNK	1647.8051	2.65	P61899 (*N. kaouthia*)	8	90
			ALTMEGNQASWR	1363.6436	0.90			
			IETACVCVITK	1293.6550	0.60			
			TTATDIKGNTVTVMENVNLDNK	2378.1894	1.04			
			IETACVCVITKK	1421.7497	0.37			
			CKNPNPEPSGCR	1415.6172	1.22			
			NPNPEPSGCR	1127.4904	0.46			
			TTATDIK	749.4039	-0.18			
18d	**10.5**	3FTx-CTX (cytotoxin 5a)	LVPLFYK	878.5265	-3	O73857 (*N. sputatrix*)	5	158
			MFMVSNLTVPVKR	1520.8207	6			
			GCIDVCPK	947.4205	1			
			YVCCNTDR	1029.4008	1			
			YVCCNTDR	1086.4223	2			
19	**22.9**	3FTx-CTX (cytotoxin 3)	LVPLFYK	878.5265	-41	P60301 (*N. atra*)	5	155
			MFMVATPK	923.4608	-38			
			MFMVATPK	939.4558	-39			
			GCIDVCPK	947.4205	-28			
			YVCCNTDR	1086.4223	-35			
20a	**0.0**	PLA_2 (acidic 1)	SWWDFADYGCYCGR	1784.6711	-32	P00598 (*N. atra*)	3	173
			SWWDFADYGCYCGR	1841.6926	-30			
			LAAICFAGAPYNNNNYNIDLK	2355.1317	-32			
20b	**0.0**	PLA_2 (acidic 1)	SWWDFADYGCYCGR	1784.6711	9	P00598 (*N. atra*)	3	147
			SWWDFADYGCYCGR	1841.6926	13			
			LAAICFAGAPYNNNNYNIDLK	2355.1317	-4			
20c	**0.0**	3FTx-CTX (cytotoxin 3)	K.LVPLFYK.T	878.5265	-40	P60301 (*N. atra*)	1	34
21a	**0.0**	PLA_2 (acidic 1 - deduced 20a)	-	-	-	-	-	-
21b	**0.1**	Vespryn (Thaicobrin)	SPPGNWQK	912.4454	-46	P82885 (*N. kaouthia*)	4	320
			ADVTFDSNTAFESLVVSPDKK	2269.1114	-44			
			TVENVGVSQVAPDNPER	1809.8857	-46			
			FDGSPCVLGSPGFR	1494.6926	-46			
21c	**0.1**	3FTx-CTX (cytotoxin 4N)*	YVCCNTDRCN	1361.5037	0.41	Q9W6W9 (*N. atra*)	11	102
			MFMVSNLTVPVK	1365.7280	0.72			
			MFMVSNLTVPVKR	1521.8278	-0.19			
			GCIDVCPK	948.4277	-0.15			

The proteins identified were sorted according to protein families. Cysteine residues determined in MS/MS analysis are carbamidomethylated.

* Results obtained from nanoESI Orbitrap Fusion analysis. Abbreviations: 3FTx: three-finger toxin; CTX: cytotoxin; vNGF: venom nerve growth factor; PLA_2: phospholipase A_2.

Table 4.1(c) *N. kaouthia* (Vietnam), continued.

Protein fraction	%	Protein family/subtype	MS/MS derived sequence	Matched MH+	Mass Δ (ppm)	Accession (species)	Matched peptide	Protein score
			GCIDVCPKSSLLVK	1575.8255	1.33			
			SSLLVKYVCCNTDR	1714.8277	1.50			
			SSLLVKYVCCNTDRCN	1988.8989	0.08			
			RGCIDVCPK	1104.5280	-0.87			
			YVCCNTDR	1087.4294	-0.18			
			CNKLVPLFYK	1281.7022	-0.19			
			SSLLVK	646.4130	-0.65			
22a	**0.2**	SVMP (atrase-A)*	LQPHAQCDSEECCEK	1890.7373	-2.24	D5LMJ3 (*N. atra*)	23	503
			RTILMASTMAHELGHNMGIHHDK	2600.2511	-5.11			
			TRVYEMVNYLNTK	1630.8285	1.63			
			TILMASTMAHELGHNMGIHHDKANCR	2945.3591	-5.43			
			TILMASTMAHELGHNMGIHHDK	2444.1516	-4.76			
			ERPQCILNKPSR	1497.7876	-5.29			
			VYEMVNYLNTK	1373.6775	0.39			
			NGHPCQNNQGYCYNGK	1910.7581	-3.99			
			TRVYEMVNYLNTKYR	1949.9886	-0.83			
			CGTLYCTEIKK	1372.6562	-2.77			
			CGTLYCTEIK	1244.5601	-3.94			
			TGCIVPVSPR	1085.5738	-3.19			
			KTGCIVPVSPR	1213.6674	-3.96			
			KGDDVSHCR	1073.4755	-3.54			
			FKGAETECR	1097.5003	-3.81			
			IPCAAKDEK	1031.5154	-3.53			
			EHQEYLLR	1087.5478	-4.90			
			RTILMASTMAHELGHNMGIHHDKANCR	3101.4840	2.50			
			GAETECR	822.3382	-3.46			
			GDDVSHCR	945.3827	-1.76			
			SQCVKV	720.3692	-2.40			
			SFAEWR	795.3782	-0.29			
			VYEMVNYLNTKYR	1692.8417	0.17			
22b	**0.0**	Not determined	-	-	-	-	-	-
22c	**0.0**	SVMP (atrase-A)*	VYEMVNYLNTK	1373.6764	-0.41	D5LMJ3 (*N. atra*)	10	27
			ERPQCILNKPSR	1497.7907	-3.21			
			NGHPCQNNQGYCYNGK	1910.7599	-3.04			
			KGDDVSHCR	1073.4745	-4.52			

The proteins identified were sorted according to protein families. Cysteine residues determined in MS/MS analysis are carbamidomethylated.
* Results obtained from nanoESI Orbitrap Fusion analysis. Abbreviations: SVMP: snake venom metalloproteinase.

Table 4.1(c) *N. kaouthia* (Vietnam), continued.

Protein fraction	%	Protein family/subtype	MS/MS derived sequence	Matched MH+	Mass Δ (ppm)	Accession (species)	Matched peptide	Protein score
			TGCIVPVSPR	1085.5724	-4.43			
			TRVYEMVNYLNTK	1630.8182	-4.65			
			LQPHAQCDSEECCEK	1890.7335	-4.28			
			CGTLYCTEIK	1244.5672	1.75			
			KTGCIVPVSPR	1213.6689	-2.76			
			EHQEYLLR	1087.5497	-3.11			
22d	**0.0**	TTPA*	LIGIISWGVGCGK	1359.7413	-3.04	V8NYC7 (*O. hannah*)	7	87
			LIGIISWGVGCGKK	1487.8387	-1.14			
			NMLCAGDTR	1037.4454	-4.80			
			TVTKNMLCAGDTR	1466.7033	-3.95			
			LKVVLGR	784.5367	-4.65			
			KNTPGVYTR	1035.5525	-5.47			
			LKEGHVR	838.4869	-2.95			
22e	**0.2**	CRISP (natrin-2)	QIVDKHNALR	1192.6676	-51	Q7ZZN8 (*N. atra*)	3	101
			RSVRPTAR	941.5519	-47			
			VIQSWYDENKK	1408.6987	-54			
22f	**0.0**	Vespryn (Thaicobrin)	TVENVGVSQVAPDNPER	1809.8857	-51	P82885 (*N. kaouthia*)	2	66
			FDGSPCVLGSPGFR	1494.6926	-51			
22g	**0.1**	3FTx-CTX (cardiotoxin 2A)*	YVCCNTDRCN	1361.4974	-4.25	Q9PST4 (*N. sputatrix*)	6	35
			GCIDVCPK	948.4260	-1.95			
			MYMVATPK	940.4581	-5.39			
			SSLLVKYVCCNTDR	1714.8261	0.55			
			SSLLVK	646.4119	-2.44			
			YVCCNTDR	1087.4260	-3.33			
23a	**0.4**	CRISP (natrin-1)	QKEIVDLHNSLR	1450.7892	-57	Q7T1K6 (*N. atra*)	8	515
			EIVDLHNSLR	1194.6357	-55			
			VSPTASNMLK	1046.5430	-53			
			MEWYPEAASNAER	1552.6616	-58			
			WANTCSLNHSPDNLR	1726.7845	-51			
			WANTCSLNHSPDNLR	1783.8060	-52			
			VLEGIQCGESIYMSSNAR	2012.9295	-59			
			TWTEIIHLWHDEYK	1869.9050	-56			

The proteins identified were sorted according to protein families. Cysteine residues determined in MS/MS analysis are carbamidomethylated.

* Results obtained from nanoESI Orbitrap Fusion analysis. Abbreviations: 3FTx: three-finger toxin; CTX: cytotoxin; CRISP: cysteine-rich secretory protein; TTPA: tissue-type plasminogen activator.

Table 4.1(c) *N. kaouthia* (Vietnam), continued.

Protein fraction	%	Protein family/subtype	MS/MS derived sequence	Matched MH+	Mass Δ (ppm)	Accession (species)	Matched peptide	Protein score
23b	**0.1**	CRISP (natrin-1)*	VLEGIQCGESIYMSSNAR	2013.9321	-2.42	Q7T1K6 (*N. atra*)	10	64
			LGPPCGDCPSACDNGLCTNPCTIYNK	2941.1861	-6.18			
			MEWYPEAASNAER	1553.6716	1.65			
			WANTCSLNHSPDNLR	1784.8173	2.21			
			NVDFNSESTR	1168.5197	-2.78			
			QKEIVDLHNSLR	1451.8013	3.28			
			RVSPTASNMLK	1203.6507	-0.66			
			SNCPASCFCR	1258.4736	-2.12			
			VSPTASNMLK	1047.5458	-4.33			
			LTNCDSLLK	1063.5456	0.30			
23c	**0.0**	3FTx-CTX (cardiotoxin 2A)*	YVCCNTDRCN	1361.5016	-1.12	Q9PST4 (*N. sputatrix*)	5	31
			GCIDVCPK	948.4273	-0.53			
			MYMVATPK	940.4612	-2.01			
			YVCCNTDR	1087.4262	-3.10			
			SSLLVK	646.4112	-3.48			
24	**0.0**	Not determined	-	-	-	-	-	-
25	**0.5**	SVMP (kaouthiagin)	YIEFYVIVDNR	1429.7241	-25	P82942 (*N. kaouthia*)	7	296
			YYNYDKPAIK	1273.6342	-29			
			IHIALIGLEIWSNEDKFEVKPAASVTLK	3120.7222	-24			
			QTVLLPR	825.5072	-40			
			TAPAFQFSSCSIR	1470.6925	-25			
			DYQEYLLR	1098.5345	-29			
			GCFDLNMR	1011.4266	-34			
27a	**0.3**	PDE (phosphodiesterase 1)*	SKNVPKDFYTFDSEAIVK	2088.0807	7.59	U3FAB3 (*M. fulvius*)	41	1217
			YISAYSQDILMPLWNSYTISK	2493.2294	-3.08			
			YKYCSGGTHGYDNEFK	1925.8078	-2.36			
			RPDFSTLYIEEPDTTGHK	2106.0088	-2.44			
			MANVLCSCSEDCLTK	1787.7381	-2.85			
			NLHNCVNLILLADHGMEAISCNR	2664.2719	-3.21			
			AEYLETWDTLMPNINK	1937.9454	7.22			
			EACCWDYQDICVLPTQSWSCNK	2820.1526	0.17			
			AKRPDFSTLYIEEPDTTGHK	2305.1449	-0.47			
			MANVLCSCSEDCLTKK	1915.8322	-3.10			
			CSSITDLEAVNQR	1492.7009	-3.51			

The proteins identified were sorted according to protein families. Cysteine residues determined in MS/MS analysis are carbamidomethylated.

* Results obtained from nanoESI Orbitrap Fusion analysis. Abbreviations: 3FTx: three-finger toxin; CTX: cytotoxin; SVMP: snake venom metalloproteinase; CRISP: cysteine-rich secretory protein; PDE: phosphodiesterase.

Table 4.1(c) *N. kaouthia* (Vietnam), continued.

Protein fraction	%	Protein family/subtype	MS/MS derived sequence	Matched MH+	Mass Δ (ppm)	Accession (species)	Matched peptide	Protein score
			LWNYFHSTLLPK	1518.8035	-4.53			
			EACCWDYQDICVLPTQSWSCNKLR	3089.3280	-3.04			
			RMANVLCSCSEDCLTKK	2071.9336	-2.75			
			RMANVLCSCSEDCLTK	1943.8366	-3.96			
			YCSGGTHGYDNEFK	1634.6495	-2.80			
			NVPKDFYTFDSEAIVK	1872.9334	-2.39			
			VLSFILPHRPDNSESCADK	2185.0821	5.20			
			DFYTFDSEAIVK	1434.6770	-1.21			
			IDKVNLMVDR	1202.6534	-2.39			
			SLQMADRTLGMLMEGLKQR	2178.1140	-2.42			
			YCLLHQTKYISAYSQDILMPLWNSYTISK	3536.7521	-2.05			
			MANVLCSCSEDCLTKKDCCTDYK	2858.1513	-2.81			
			TLGMLMEGLK	1092.5793	0.03			
			VNLMVDRQWLAVR	1599.8783	-0.34			
			AEYLETWDTLMPNINKLK	2179.1050	-2.49			
			LWNYFHSTLLPKYATER	2139.1090	3.17			
			YCLLHQTK	1062.5375	-2.42			
			IDKVNLMVDRQWLAVR	1956.0866	0.87			
			DQCASSSAAQCPEGFDQSPLILFSMDGFR	3221.3759	-6.66			
			NPFYNPSPAK	1134.5533	-4.06			
			TLGMLMEGLKQR	1376.7358	-2.31			
			AYLAKDLPK	1018.5890	-4.09			
			EKNEVTSFENIEVYNLMCDLLK	2688.2955	2.18			
			LKTCGTHAK	1015.5320	-3.30			
			DCCTDYK	961.3358	-3.35			
			VNLMVDR	846.4486	-1.96			
			KDCCTDYK	1089.4299	-3.79			
			AATYFWPGSEVK	1355.6686	4.09			
			SKNVPK	672.4017	-3.38			
			MQTHTAR	844.4064	-3.57			
27b	**0.1**	CSA*	LLEECCQSEHHVQCLHGGEQVFK	2824.2588	-0.45	Q91134 (*N. naja*)	57	1506
			FREIMEEQEYTCYNLKK	2281.0581	-2.06			
			KILETCCAEADKDACIHEK	2291.0419	-2.04			
			SKPNISEEELAATILTFR	2019.0724	-1.66			
			FREIMEEQEYTCYNLK	2152.9665	-0.63			

The proteins identified were sorted according to protein families. Cysteine residues determined in MS/MS analysis are carbamidomethylated.
* Results obtained from nanoESI Orbitrap Fusion analysis. Abbreviations: CSA: cobra albumin serum.

Table 4.1(c) *N. kaouthia* (Vietnam), continued.

Protein fraction	%	Protein family/subtype	MS/MS derived sequence	Matched MH+	Mass Δ (ppm)	Accession (species)	Matched peptide	Protein score
			DAISSNVGHCCEKPLVERPNCLATLANDAR	3367.5839	-3.05			
			WECISNLGPDLSFVPPTFNPK	2418.1705	-3.93			
			SNCDSYKELGDYFFTNEFLVK	2576.1714	2.47			
			AALSQYVCEHKDAISSNVGHCCEKPLVERPNCLATLANDAR	4654.1951	-1.48			
			EIMEEQEYTCYNLKK	1977.8886	-2.38			
			TMDNPEKLCSTSEDTVQK	2082.9276	-2.07			
			RYPTALSVVILESTK	1676.9555	-1.62			
			TSSTGTISPFVHPNAEEACQAFQNDRDSVLAQYIFELSR	4386.0807	1.68			
			YPTALSVVILESTK	1520.8516	-3.60			
			TSSTGTISPFVHPNAEEACQAFQNDR	2864.2837	-2.40			
			CVASEFSDPPCTKPLGIVFLDVLCHNEEFSNK	3709.7164	-4.66			
			ILETCCAEADKDACIHEK	2162.9451	-3.00			
			ELGDYFFTNEFLVK	1721.8407	-0.83			
			ENYKESFLFTLTR	1647.8332	-2.78			
			ETEACTTYTEQR	1488.6232	-2.65			
			KLSAEIIELHKK	1408.8497	-1.84			
			KLSAEIIELHK	1280.7538	-2.76			
			MMPQAPTSFLIELTEK	1835.9423	7.65			
			KILETCCAEADK	1437.6669	-3.04			
			LSAEIIELHKK	1280.7549	-1.87			
			LCSTSEDTVQK	1267.5799	-2.86			
			KCVASEFSDPPCTKPLGIVFLDVLCHNEEFSNK	3837.8181	-2.74			
			LSAEIIELHK	1152.6603	-1.76			
			ADPDRNECVLSHK	1540.7111	-4.05			
			AALSQYVCEHK	1305.6220	-2.79			
			HVDDQHSTIR	1207.5791	-1.91			
			ILETCCAEADK	1309.5727	-2.79			
			EIQKLCCEAENK	1521.6996	-2.65			
			YGKDKLYALK	1198.6811	-1.66			
			ESFLFTLTR	1113.5916	-2.09			
			ETEACTTYTEQRENYK	2022.8625	-4.20			
			KGLLSELVK	986.6218	-2.80			
			DSVLAQYIFELSR	1540.7981	-1.62			
			SKKGLLSELVK	1201.7487	-2.32			
			YGINDCCAK	1100.4473	-2.42			
			LCCEAENKK	1151.5148	-3.12			

The proteins identified were sorted according to protein families. Cysteine residues determined in MS/MS analysis are carbamidomethylated.

Table 4.1(c) *N. kaouthia* (Vietnam), continued.

Protein fraction	%	Protein family/subtype	MS/MS derived sequence	Matched MH+	Mass Δ (ppm)	Accession (species)	Matched peptide	Protein score
			NECVLSHK	986.4687	-3.74			
			KFREIMEEQEYTCYNLK	2281.0598	-1.34			
			LCCEAENKKECFDK	1830.7792	-1.52			
			SPDLPPPSEEILKETEACTTYTEQR	2891.3499	-4.05			
			RYPTALSVVILESTKTYK	2069.1624	-0.84			
			SPDLPPPSEEILK	1421.7494	-2.02			
			LCCEAENK	1023.4195	-3.87			
			DKLYALK	850.5007	-3.12			
			GLLSELVK	858.5280	-1.79			
			EIMEEQEYTCYNLK	1849.7970	-0.70			
			VMGSICK	794.3873	-3.32			
			LYITKINEVVK	1319.7917	-1.28			
			INEVVK	701.4170	-3.24			
			TMDNPEK	834.3617	-5.47			
			NHPELSK	824.4238	-2.87			
			LYITK	637.3901	-2.96			
27c	**0.1**	SVMP (kaouthiagin)	YIEFYVIVDNR	1429.7241	-21	P82942 (*N. kaouthia*)	4	169
			QTVLLPR	825.5072	-36			
			DYQEYLLR	1098.5345	-20			
			NTMSCLIPPNPDGIMAEPGTK	2217.0115	-40			
27d	**0.0**	Not determined	-	-	-	-	-	-
27e	**0.2**	GPX*	LVILGFPCNQFGKQEPGQNSEILQGIK	3014.6014	8.10	V8P395 (*O. hannah*)	12	194
			TDRLVILGFPCNQFGK	1864.9734	-0.25			
			LVILGFPCNQFGK	1492.7997	1.06			
			TNVSTVKNDIIR	1359.7597	0.43			
			FLVNPQGKPVMR	1385.7760	2.72			
			QEPGQNSEILQGIK	1540.7950	-1.08			
			AKVDCYDSVK	1184.5643	2.20			
			IHDIKWNFEK	1329.6965	1.06			
			GDVNGENEQK	1089.4802	-0.56			
			VDCYDSVK	985.4291	-0.50			
			TNVSTVK	748.4197	-0.33			
			NDIIR	630.3566	-0.58			

The proteins identified were sorted according to protein families. Cysteine residues determined in MS/MS analysis are carbamidomethylated.

* Results obtained from nanoESI Orbitrap Fusion analysis. Abbreviations: SVMP: snake venom metalloproteinase; GPX: glutathione peroxidase.

Table 4.1(c) *N. kaouthia* (Vietnam), continued.

Protein fraction	%	Protein family/subtype	MS/MS derived sequence	Matched MH+	Mass Δ (ppm)	Accession (species)	Matched peptide	Protein score
28a	**0.1**	PDE (phosphodiesterase 1)*	RPDFSTLYIEEPDTTGHK	2106.0161	1.04	U3FAB3 (*M. fulvius*)	24	425
			MANVLCSCSEDCLTK	1787.7431	-0.05			
			AKRPDFSTLYIEEPDTTGHK	2305.1454	-0.25			
			AEYLETWDTLMPNINK	1937.9307	-0.37			
			CSSITDLEAVNQR	1492.7074	0.90			
			EACCWDYQDICVLPTQSWSCNK	2820.1436	-3.04			
			SKNVPKDFYTFDSEAIVK	2088.0657	0.40			
			LWNYFHSTLLPK	1518.8097	-0.44			
			DFYTFDSEAIVK	1434.6790	0.15			
			NVPKDFYTFDSEAIVK	1872.9371	-0.44			
			YCSGGTHGYDNEFK	1634.6536	-0.26			
			YKYCSGGTHGYDNEFK	1925.8141	0.93			
			VLSFILPHRPDNSESCADK	2185.0711	0.19			
			RMANVLCSCSEDCLTK	1943.8429	-0.73			
			TLGMLMEGLK	1092.5790	-0.20			
			MANVLCSCSEDCLTKK	1915.8379	-0.11			
			IDKVNLMVDR	1202.6563	0.05			
			YCLLHQTK	1062.5401	-0.01			
			YISAYSQDILMPLWNSYTISK	2493.2356	-0.58			
			NPFYNPSPAK	1134.5585	0.57			
			DCCTDYK	961.3391	0.08			
			MQTHTAR	844.4096	0.19			
			VNLMVDR	846.4495	-0.88			
			AATYFWPGSEVK	1355.6629	-0.14			
28b	**0.2**	CVF	QLDIFVHDFPR	1385.7092	-50	Q91132 (*N. kaouthia*)	6	347
			VVLLSYQSSFLFIQTDK	1987.0666	-60			
			FFYIDGNENFHVSITAR	2028.9693	-48			
			YLYGEEVEGVAFVLFGVK	2018.0399	-60			
			LILNIPLNAQSLPITVR	1874.1353	-55			
			FVAYYQVGNNEIVADSVWVDVK	2514.2430	-50			
28c	**0.2**	5'NUC	ETPVLSNPGPYLEFR	1717.8675	-41	B6EWW8 (*G. brevicaudus*)	4	213
			YLGYLNVIFDDK	1458.7394	-36			
			FHECNLGNLICDAVIYNNVR	2420.1365	-36			
			VVSLNVLCTECR	1448.7115	-32			

The proteins identified were sorted according to protein families. Cysteine residues determined in MS/MS analysis are carbamidomethylated.

* Results obtained from nanoESI Orbitrap Fusion analysis. Abbreviations: PDE: phosphodiesterase; 5'NUC: 5'nucleotidase; CVF: cobra venom factor.

Table 4.1(c) *N. kaouthia* (Vietnam), continued.

Protein fraction	%	Protein family/subtype	MS/MS derived sequence	Matched MH+	Mass Δ (ppm)	Accession (species)	Matched peptide	Protein score
28d	**0.2**	CVF	DLNLDITIELPDR	1525.7988	-56	Q91132 (*N. kaouthia*)	7	413
			DLNLDITIELPDREVPIR	2120.1477	-52			
			ATMTILTFYNAQLQEK	1870.9498	-66			
			YLGEVDSTMTIIDISMLTGFLPDAEDLTR	3215.5617	-57			
			VAVIIYLNK	1031.6379	-69			
			IEEQDGNDIYVMDVLEVIK	2221.0823	-59			
			VNDDYLIWGSR	1336.6411	-47			
28e	**0.1**	CVF	DSITTWVVLAVSFTPTK	1863.9982	-65	Q91132 (*N. kaouthia*)	2	47
			ASVQEALWSDGVR	1416.6997	-62			
28f	**0.2**	Not determined	-	-	-	-	-	-
29a	**0.0**	Not determined	-	-	-	-	-	-
29b	**0.2**	CVF	QLDIFVHDFPR	1385.7092	-24	Q91132 (*N. kaouthia*)	4	132
			VVLLSYQSSFLFIQTDK	1987.0666	-48			
			FFYIDGNENFHVSITAR	2028.9693	-31			
			YLYGEEVEGVAFVLFGVK	2018.0399	-47			
29c	**0.5**	LAAO	VTLLEASER	1016.5502	-62	A8QL58 (*N. atra*)	6	256
			LNEFFQENENAWYYINNIR	2476.1447	-57			
			VIEELKR	885.5283	-62			
			FDEIVGGFDQLPISMYQAIAEMVHLNAR	3163.5470	-48			
			FDEIVGGFDQLPISMYQAIAEMVHLNAR	3179.5419	-53			
			STTDLPSR	875.4349	-67			
29d	**0.6**	SVMP (atragin)	KYIEFYVVVDNIMYR	1950.9913	-35	D3TTC2 (*N. atra*)	9	465
			YIEFYVVVDNIMYR	1822.8963	-35			
			YIEFYVVVDNIMYR	1838.8912	-39			
			KVYEMINTMNMIYR	1804.8674	-39			
			VYEMINTMNMIYR	1676.7724	-39			
			LNFHIALIGLEIWSNINEINVQSDVR	3006.5926	-33			
			ATLNLFGEWR	1205.6193	-33			
			TSAAVVQDYSSR	1282.6153	-33			
			CPIMTNQCIALR	1475.7047	-36			
29e	**0.1**	SVMP (atragin)	YIEFYVVVDNIMYR	1822.8963	-48	D3TTC2 (*N. atra*)	2	47
			ATLNLFGEWR	1205.6193	-56			
29f	**0.0**	SVMP (atragin)	YIEFYVVVDNIMYR	1822.8963	-17	D3TTC2 (*N. atra*)	1	65
29g	**0.1**	CVF	DSITTWVVLAVSFTPTK	1863.9982	-64	Q91132 (*N. kaouthia*)	2	77
			VFFIDLQMPYSVVK	1684.8898	-55			

The proteins identified were sorted according to protein families. Cysteine residues determined in MS/MS analysis are carbamidomethylated.
* Results obtained from nanoESI Orbitrap Fusion analysis. Abbreviations: SVMP: snake venom metalloproteinase; 5'NUC: 5'nucleotidase; CVF: cobra venom factor; LAAO: L-amino acid oxidase.

Table 4.2: Toxin protein family subtypes and relative abundance (%) in venoms of *N. kaouthia* Malaysia (NK-M), Thailand (NK-T) and Vietnam (NK-V).

Protein family	Subtype	Accession (species)	NK-M (%)	NK-T (%)	NK-V (%)
3FTx			**63.7 (14)**	**78.3 (11)**	**76.4 (18)**
	LNTX		**3.9 (1)**	**33.3 (1)**	**-**
	alpha-elapitoxin-Nk2a	P01391 (*N. kaouthia*)	3.9	33.3	-
	SNTX		**4.2 (3)**	**7.7 (2)**	**9.2 (3)**
	cobrotoxin	P60771 (*N. kaouthia*)	2.3	3.6	6.6
	cobrotoxin-b	P59275 (*N. kaouthia*)	0.3	-	0.1
	cobrotoxin-c	P59276 (*N. kaouthia*)	1.6	4.1	2.5
	CTX		**45.7 (6)**	**27.6 (6)**	**44.9 (6)**
	cardiotoxin 2A	Q9PST4 (*N. sputatrix*)	8.4	0.2	0.1
	cardiotoxin-1f	P85429 (*N. atra*)	-	-	0.1
	cytotoxin 1	P60305 (*N. kaouthia*)	10.6	-	-
	cytotoxin 2	Q98965 (*N. kaouthia*)	0.1	-	-
	cytotoxin 2	P01445 (*N. kaouthia*)	20.1	-	-
	cytotoxin 3	P01446 (*N. atra*)	-	17.1	-
	cytotoxin 3	P60301 (*N. atra*)	-	0.1	23.0
	cytotoxin 4N	Q9W6W9 (*N. atra*)	-	-	2.0
	cytotoxin 5a	O73857 (*N. sputatrix*)	-	8.6	15.8
	cytotoxin homolog	P14541 (*N. kaouthia*)	1.1	1.2	3.9
	cytotoxin I-like T-15	Q91136 (*N. atra*)	-	0.4	-
	cytotoxin NK-CT1	P0CH80 (*N. kaouthia*)	5.4	-	-
	MTLP		**0.8 (2)**	**0.8 (1)**	**3.0 (3)**
	muscarinic toxin-like protein	Q9W727 (*B. multicinctus*)	-	-	1.4
	muscarinic toxin-like protein 1	P82462 (*N. kaouthia*)	-	-	0.5
	muscarinic toxin-like protein 2	P82463 (*N. kaouthia*)	0.5	0.5	1.1
	muscarinic toxin-like protein 3	P82464 (*N. kaouthia*)	0.3	0.3	-
	WTX		**9.1 (2)**	**8.9 (1)**	**19.3 (6)**
	weak neurotoxin NNAM2	Q9YGI4 (*N. atra*)	-	-	0.1
	weak neurotoxin 6	P29180 (*N. naja*)	0.7	-	0.3
	weak neurotoxin 6	O42256 (*N. sputatrix*)	-	-	11.2
	weak neurotoxin 7	P29181 (*N. naja*)	-	-	0.4
	weak toxin CM-9a	P25679 (*N. kaouthia*)	8.4	8.9	7.0
	weak toxin S4C11	P01400 (*N. melanoleuca*)	-	-	0.3
PLA_2			**23.5 (4)**	**12.2 (2)**	**17.4 (2)**
	acidic phospholipase A_2 1	P00596 (*N. kaouthia*)	13.0	0.3	-
	acidic phospholipase A_2 1	P00598 (*N. atra*)	0.7	-	16.0
	acidic phospholipase A_2 2	P15445 (*N. naja*)	6.7	-	1.4
	acidic phospholipase A_2 2	Q91133 (*N. atra*)	3.1	11.9	-
CRISP			**4.3 (2)**	**2.3 (2)**	**0.8 (2)**
	natrin-1	Q7T1K6 (*N. atra*)	3.7	1.6	0.6
	natrin-2	Q7ZZN8 (*N. atra*)	0.6	0.7	0.2
SVMP			**3.3 (4)**	**2.5 (3)**	**1.6 (3)**
	atragin	D3TTC2 (*N. atra*)	-	-	0.7
	atrase-A	D5LMJ3 (*N. atra*)	0.4	-	0.3
	atrase-B	D6PXE8 (*N. atra*)	-	1.0	-
	cobrin	Q9PVK7 (*N. naja*)	1.1	1.0	-
	kaouthiagin	P82942 (*N. kaouthia*)	1.3	0.5	0.6
	mocarhagin-1	Q10749 (*N. mossambica*)	0.5	-	-
LAAO	L-amino acid oxidase	A8QL58 (*N. atra*)	**1.1 (1)**	**1.0 (1)**	**0.5 (1)**
CVF	cobra venom factor	Q91132 (*N. kaouthia*)	**0.8 (1)**	**1.1 (1)**	**0.7 (1)**
KUN	Kunitz-type inhibitor	P20229 (*N. naja*)	**0.5 (1)**	**-**	**< 0.1 (1)**
NP	natriuretic peptide Na-NP	D9IX97 (*N. atra*)	**-**	**0.2 (1)**	**-**
PDE	phosphodiesterase 1	U3FAB3 (*M. fulvius*)	**0.4 (1)**	**0.3 (1)**	**0.4 (1)**
5'-NUC	snake venom 5'-nucleotidase	B6EWW8 (*G. brevicaudus*)	**0.3 (1)**	**0.2 (1)**	**0.2 (1)**
Vespryn	Thaicobrin	P82885 (*N. kaouthia*)	**0.3 (1)**	**0.7 (1)**	**0.2 (1)**
CTL	BFL-1	Q90WI8 (*B. fasciatus*)	**0.2 (1)**	**0.4 (1)**	**-**

Table 4.2, continued.

Protein family	Subtype	Accession (species)	NK-M (%)	NK-T (%)	NK-V (%)
NGF			**0.2 (1)**	**0.5 (2)**	**1.3 (2)**
	venom nerve growth factor	P61899 (*N. kaouthia*)	0.2	-	0.7
	venom nerve growth factor 2	Q5YF89 (*N. sputatrix*)	-	0.4	-
	venom nerve growth factor	P01140 (*N. naja*)	-	0.1	-
	nerve growth factor precursor	A59218 (*N. kaouthia*)	-	-	0.6
N.T.			**0.2**	**0.2**	**0.4**
N.D.			**1.2**	**0.1**	**0.4**

Parentheses indicated the number of protein subtypes detected. Abbreviations: 3FTx; three-finger toxin, LNTX; long-chain neurotoxin, SNTX; short-chain neurotoxin, CTX; cytotoxin/cardiotoxin, MTLP; muscarinic toxin-like protein, WTX; weak neurotoxin/toxin, PLA_2; phospholipase A_2, CRISP; cysteine-rich secretory protein, SVMP; snake venom metalloproteinase, LAAO; L-amino acid oxidase, CVF; cobra venom factor, KUN; Kunitz-type protease inhibitor, NP; natriuretic peptide, PDE; phosphodiesterase, 5'NUC; 5'nucleotidase, CTL; c-type lectin, NGF; nerve growth factor, N.T.; Non-toxin, N.D.; Not determined.

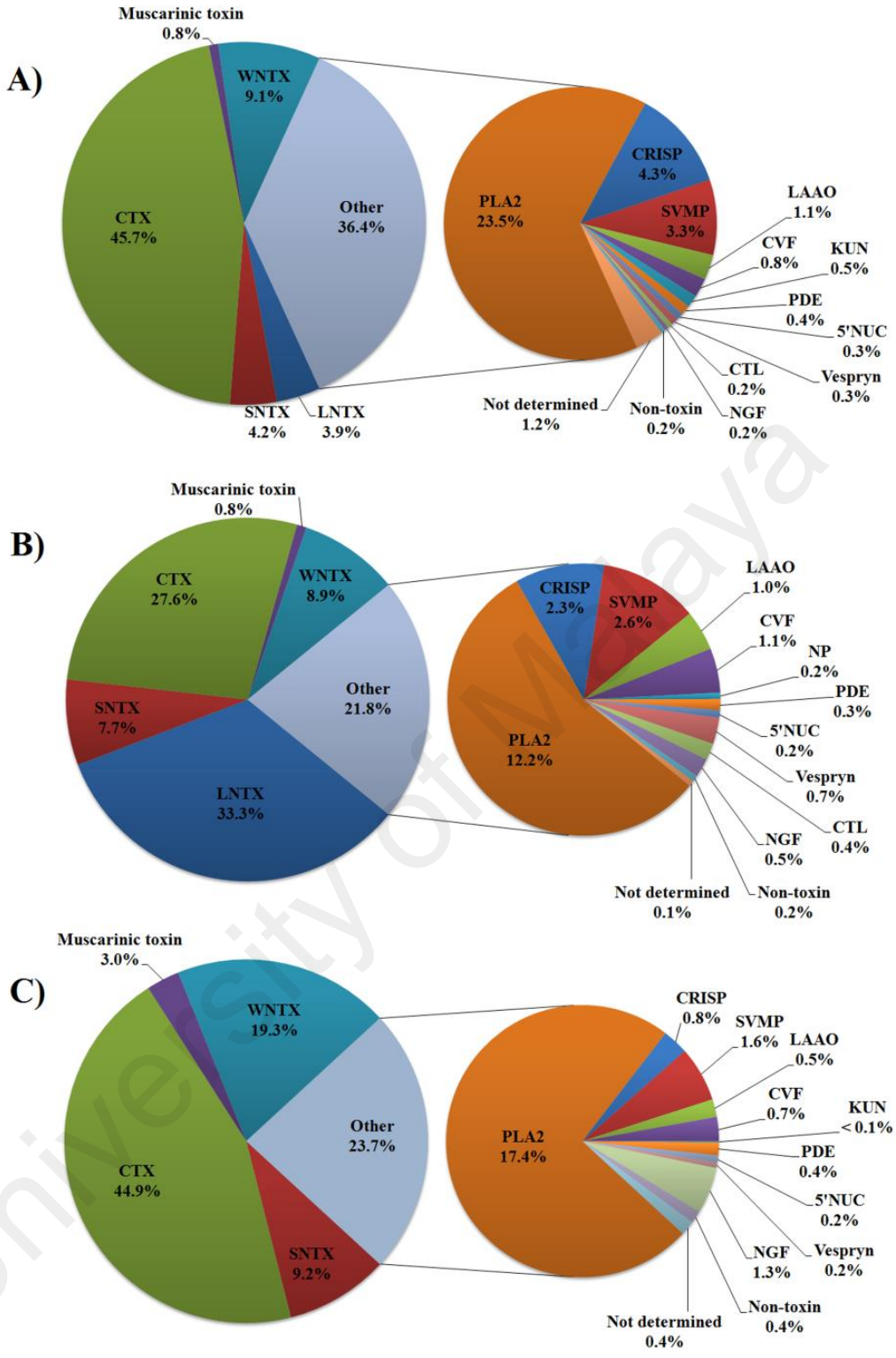


Figure 4.2 Relative abundances of venom protein families identified by mass spectrometry following reverse-phase HPLC and SDS-PAGE of *N. kaouthia* venoms. A) Malaysia. B) Thailand. C) Vietnam.

Left: 3FTx subtypes; **Right:** Others protein families in the venoms. Abbreviations: 3FTx; three-finger toxin, LNTX; long-chain neurotoxin, SNTX; short-chain neurotoxin, CTX; cytotoxin/cardiotoxin, MTLP; muscarinic toxin-like protein, WTX; weak neurotoxin/toxin, PLA_2; phospholipase A_2, CRISP; cysteine-rich secretory protein, SVMP; snake venom metalloproteinase, LAAO; L-amino acid oxidase, CVF; cobra venom factor, KUN; Kunitz-type protease inhibitor, NP; natriuretic peptide, PDE; phosphodiesterase, 5'NUC; 5'nucleotidase, CTL; c-type lectin, and NGF; nerve growth factor.

4.3.3 Median Lethal Dose (LD_{50}) of *N. kaouthia* Venoms

The median lethal doses (LD_{50}) of *N. kaouthia* venoms were shown in Table 4.3. The results showed that the Thai *N. kaouthia* (NK-T) venom has the lowest LD_{50} (~0.2 µg/g), which is approximately 5x more lethal than that for Malaysian (NK-M) and Vietnamese (NK-V) samples (LD_{50} ~0.9 µg/g each). The LD_{50} of the venoms determined by intravenous injection and subcutaneous injection were comparable.

4.3.4 Neutralization by Antivenoms – *In vitro* Immunocomplexation

The neutralization potency of the antivenoms produced from Thai *N. kaouthia* species (NKMAV and NPAV) was examined using *in vitro* immunocomplexation (pre-incubation) approach, against the three *N. kaouthia* venoms (NK-M, NK-T and NK-V). The results indicated that both antivenoms were able to neutralize the lethality of all three *N. kaouthia* venoms examined effectively. The neutralization efficacy of the antivenoms (neutralization potency, P) was in the range of 0.70 to 1.14 mg/ml antivenom (amount of venom neutralized completely by one ml of antivenom).

Since the protein content of different antivenom products differs, the protein concentration of the reconstituted NKMAV and NPAV were determined and shown in Table 4.4. The protein concentration data were used to normalize the neutralization potency (P) values and expressed as the amount of venom (mg) completely neutralized per unit amount of antivenom protein (g). The normalized neutralization potency (n-P) values of the two antivenoms were in the range of 11.7 to 24.5 mg/g (Table 4.5). The findings showed that the two antivenoms have comparable immunoreactivity against the *N. kaouthia* venoms from three different geographical regions.

Table 4.3: The median lethal dose (LD_{50}) of *N. kaouthia* venoms from Malaysia (NK-M), Thailand (NK-T) and Vietnam (NK-V) administrated by intravenous (*i.v.*) or subcutaneous (*s.c.*) routes.

Venom	*i.v.* LD_{50} (µg/g) [a]	*s.c.* LD_{50} (µg/g) [a]
***N. kaouthia* (Malaysia)**	0.90 (0.59-1.36)	1.00 (0.88-1.14)
***N. kaouthia* (Thailand)**	0.18 (0.12-0.27)	0.20 (0.16-0.25)
***N. kaouthia* (Vietnam)**	0.90 (0.59-1.36)	1.11 (0.73-1.69)

Values of 95% C.I. are in parentheses

[a] LD_{50} : median lethal dose (µg/g)

Table 4.4: Protein concentrations of *N. kaouthia* Monovalent Antivenom (NKMAV) and Neuro Polyvalent Antivenom (NPAV).

Antivenom	Protein concentration (mg/ml)
NKMAV	45.0 ± 0.6
NPAV	75.3 ± 0.6

Results are presented as mean ± S.E.M of triplicate experiments.

Table 4.5: Neutralization of lethality of *N. kaouthia* venoms from different geographical regions by *N. kaouthia* Monovalent Antivenom (NKMAV) and Neuro Polyvalent Antivenom (NPAV).

Venom	*i.v.* LD_{50}[a]	Challenge dose[b]	NKMAV				NPAV			
			ED_{50}[c] (µl)	ER_{50}[d] (mg/ml)	Potency (mg/ml)[e]	Normalized P (mg/g)[f]	ED_{50}[c] (µl)	ER_{50}[d] (mg/ml)	Potency (mg/ml)[e]	Normalized P (mg/g)[f]
***N. kaouthia* (Malaysia)**	0.90 (0.59-1.36)	5x LD_{50}	78.29	1.38 (0.90-2.08)	1.10	24.44	70.68	1.43 (0.94-2.16)	1.14	15.14
***N. kaouthia* (Thailand)**	0.18 (0.12-0.27)	5x LD_{50}	18.75	1.15 (0.77-1.73)	0.92	20.44	17.67	1.17 (0.78-1.76)	0.94	12.48
***N. kaouthia* (Vietnam)**	0.90 (0.59-1.36)	5x LD_{50}	120.86	0.87 (0.57-1.32)	0.70	15.55	89.89	1.10 (0.72-1.66)	0.88	11.69

Values of 95% C.I. were in parentheses

[a] LD_{50} : median lethal dose (µg/g)

[b] Challenge dose : challenge dose (µg/g), all challenge doses were proven to be above 100% lethal dose (LD_{100}) when given intravenously

[c] ED_{50} : median effective dose, the antivenom dose (µl) at which 50% of mice survived

[d] ER_{50} : median effective ratio, the ratio of the amount of venom (mg) to the volume dose of antivenom (ml) at which 50% of mice survived

[e] Potency, P : neutralization potency of the antivenom (mg/ml), the amount of venom (mg) completely neutralized by one ml antivenom (ml)

[f] Normalized P, n-P : normalized neutralization potency of the antivenom (mg/g), the amount of venom (mg) completely neutralized per unit amount of antivenom protein (g)

4.4 Discussion

4.4.1 Proteomics Characterization of *N. kaouthia* Venoms from Malaysia, Thailand and Vietnam

The results obtained showed that the use of MALDI-TOF/TOF in combination with the high-resolution Orbitrap Fusion analysis was able to identify most of the toxins/proteins detected by RP-HPLC and SDS-PAGE, including those that exist only in trace amount. The present study has revealed the presence of several novel toxin families (KUN, NP, PDE, 5'NUC and CTL) in *N. kaouthia* venoms that have not been hitherto reported for the venom proteome of *N. kaouthia*. The presence of PDE and 5'NUC is consistent with findings from earlier studies on enzymatic activities of the venom (Tan & Tan, 1988b; Yap et al., 2011).

All three *N. kaouthia* venoms contain the protein families that are commonly found in cobra venom, including 3FTx, PLA_2, CRISP, SVMP and CVF. Among these protein families, 3FTx was the principal toxin family due to their high abundance and high lethality. It is interesting to note that there are considerable geographical variations in the composition of 3FTx of the venoms. Also, some low abundance proteins were not detected in all three venoms. For example, KUN was only present in NK-M and NK-V; NP was exclusively present in NK-T; while CTL was only detected in NK-M and NK-T (Figure 4.2; Table 4.2). For certain venom samples, some of these proteins existed in very small amounts and hence were not detected by the techniques used. The clinical significance of these observations is unclear as the roles of these minor toxins in the pathogenesis of *Naja* envenomation is yet to be established. It was also noted in an earlier enzymatic study, the activities of hyaluronidase, alkaline phosphomonoesterase and acetylcholinesterase were present in *N. kaouthia* venom (Yap et al., 2011), but the presence of the proteins was not detected in this present study. This may be due to

exceptionally low amounts of these enzymes in the venom, or due to the lack of specific sequence information in the database.

4.4.2 Comparison of the Composition of 3FTx of the three *N. kaouthia* Venoms

Three-finger toxin (3FTx) constituted 63.7-78.3% of the total venom proteins in the three *N. kaouthia* venoms. Many subtypes of 3FTx have been identified from snake venoms based on the variation in peptide sequences and biological activities, although in general, they are structurally similar by having a similar protein scaffold. 3FTx subtypes exhibit a wide range of pharmacological activities, including neurotoxicity, cytotoxicity/cardiotoxicity, anticoagulant and antiplatelet effects (Chanda et al., 2013; Kini & Doley, 2010). The examination of the 3FTx composition of the three *N. kaouthia* venoms revealed substantial differences in terms of their subtypes and relative abundances (Figure 4.2; Table 4.2). For *N. kaouthia* venom, the 3FTx can be further classified into five different subtypes, namely long-chain alpha-neurotoxin (LNTX, long neurotoxin), short-chain alpha-neurotoxin (SNTX, short neurotoxin), weak toxin/neurotoxin (WTX), muscarinic toxin-like protein (MTLP) and cytotoxin/cardiotoxin (CTX) (Figure 4.2; Table 4.2).

4.4.2.1 Neurotoxins

Table 4.2 shows that the three *N. kaouthia* venoms differ substantially in their relative abundance of long (LNTX) and short (SNTX) alpha-neurotoxins. Functionally, both LNTX and SNTX are antagonists of muscular nicotinic acetylcholine receptor (nAChR) although LNTX is more specific towards neuronal-type nAChR (alpha7, alpha8 and alpha9) (Endo & Tamiya, 1991; Servent & Menez, 2001; Servent et al., 1997). These alpha-neurotoxins are rapid-acting toxins that can cause flaccid paralysis, respiratory failure and consequently death in envenomed victims, as seen in some cases

of *N. kaouthia* envenomation in Thailand (Wongtongkam et al., 2005). The short and long alpha-neurotoxins of elapid venoms differ in their amino acid sequence length, conserved cysteine residues, the number of disulfide bridges, and also in the reversibility of receptor binding. Generally, the neuromuscular blockage caused by the long alpha-neurotoxin is less reversible (Barber et al., 2013). Alpha-elapitoxin-Nk2a (also known as the alpha-cobratoxin) is the sole LNTX subtype found in the *N. kaouthia* venom. It is also the major neurotoxin in NK-T venom (33.3% of venom protein). In contrast, the content of LNTX in NK-M sample is much lower (3.9%) and not detectable in NK-V sample. The occurrence of alpha-elapitoxin-Nk2a in *N. kaouthia* venom was first reported by Karlsson et al. (Karlsson & Eaker, 1972) as the principal neurotoxin that comprises one fourth of crude venom weight from the venom of Thai cobra (which was known as *Naja naja siamensis* in earlier years).

On the other hand, SNTXs have also been isolated from Thai cobra venom. Three isoforms were identified but all in very low content (Chiou et al., 1989; Karlsson & Eaker, 1972), which is consistent with the results of the present study. This study showed that the SNTX content of the three *N. kaouthia* venoms is all less than 10%: 4.2% in NK-M, 7.7% in NK-T and 9.2% in NK-V samples, respectively. The relative abundances of the three SNTX subtypes (cobrotoxin, cobrotoxin-b, cobrotoxic-c) in the three *N. kaouthia* venoms also differ. The three SNTX subtypes however are similar to the three SNTX isolated from the venom of Chinese *N. kaouthia* previously (Yunnan, China) (Cheng et al., 2002; Meng et al., 2002).

It is interesting to note that the NK-V venom contains weak neurotoxin (WTX) as its major type of neurotoxin. The content of WTX (19.3%) in NK-V is markedly higher than that in NK-T and NK-M venoms (≤ 9%). Besides, NK-V venom also contains relatively more subtypes of WTX. The most highly expressed isoform, weak neurotoxin 6, is similar to that cloned from *N. sputatrix* venom (UniProtKB: O42256) (Poh et al.,

2002) (Table 4.2). It has been reported that the WTX isolated from *N. kaouthia* (geographical origin unspecified) venom was antagonist of human and rat nicotinic receptors (Utkin et al., 2001a; Utkin et al., 2001b), but less lethal (LD_{50} ~5-80 µg/g) as compared to the typical alpha-neurotoxin (LD_{50} ~0.1 µg/g).

On the other hand, a small amount of muscarinic toxin-like protein (MTLP) was detected in all three venoms. Like WNTX, MTLP also acts on the cholinergic receptors system. However, the MTLP selectively antagonizes distinct subtypes of muscarinic acetylcholine receptors (mAChRs) (Kukhtina et al., 2000), while the WTX (as non-conventional neurotoxin) possesses low affinity toward both muscular-type and neuronal-type of nicotinic acetylcholine receptors (nAChRs) (Poh et al., 2002; Utkin et al., 2001a). It has also been demonstrated that intravenous (*i.v.*) injection of WTX induced a dose-dependent decrease in blood pressure and heart rate in rodents (Ogay et al., 2005). This may be related to autonomic disturbances induced by the interaction of WTX with mAChRs (Mordvintsev et al., 2007; Poh et al., 2002; Utkin et al., 2001a), although the specific clinical effect has not been reported in *N. kaouthia* envenomation.

4.4.2.2 Cytotoxin

Another interesting finding from the current proteomic study is the substantial difference in cytotoxin (CTX) content of the three *N. kaouthia* venoms. Cytotoxin constitutes approximately 45% of the total venom proteins for NK-M and NK-V venoms; but only 27.6% for NK-T (Figure 4.2; Table 4.2). It is usually less lethal (LD_{50} ~1.0-2.5 µg/g) as compared to the alpha-neurotoxin of most elapids (LD_{50} ~0.1 µg/g) (Leong et al., 2015; Tan, 1991). Cytotoxin exhibits a wide range of pharmacological activities (Hegde et al., 2009; Yap et al., 2014a; Yap et al., 2011), but its main pathological action in envenomation is most likely related to its cytotoxic and cytolytic actions that lead to tissue destruction (Feofanov et al., 2005; Konshina et al., 2011;

Osipov et al., 2008). Extensive and severe tissue necrosis can result in crippling deformity even when the patient survives a cobra bite. Necrosis and the resultant local tissue destruction are irreversible and the patient may need surgical intervention including skin grafting and amputation. Therefore, this is the reason that pressure bandage immobilization is contraindicated in *N. kaouthia* envenomation (though commonly used in Australian elapid envenomation). In the previous *in vitro* studies, cobra's cytotoxin had been shown to exhibit cardiotoxic activity (that probably had earned it the name "cardiotoxin"), however, this effect is rarely reported clinically in cobra envenomation (Tan, 1982).

The varied composition of 3FTx in the three *N. kaouthia* venoms is reflected in the differences in their lethality. The Thai *N. kaouthia* venom, which contains the highest amount of highly lethal LNTX, has a much lower LD_{50} (0.2 µg/g) as compared to the venoms of Malaysian and Vietnamese *N. kaouthia* (LD_{50} = 0.9 µg/g for both) (Leong et al., 2012; Yap et al., 2011) (Table 4.3).

4.4.3 Other Toxin Components in *N. kaouthia* Venoms

Other than the three-finger toxin (3FTx), phospholipase A_2 (PLA_2) is the second most abundant toxin protein family in all three *N. kaouthia* venoms examined (17.4-23.5%), followed by the protein families of CRISP, SVMP, LAAO, CVF, KUN, NP, PDE, 5'NUC, vespryn, CTL and NGF. The other very minor toxin families each constitutes less than 1% of total venom proteins (Figure 4.2). Their low abundances and toxic properties that are less established suggest that these toxins are likely to have only minor roles in the pathogenesis of *N. kaouthia* envenomation.

4.4.3.1 Phospholipase A_2 (PLA_2)

PLA_2 presents in most snake venoms and usually found in multiple isoforms with varied pharmacological activities. The PLA_2 content in all three *N. kaouthia* venoms are rather high (12-23%), consistent with the reports that showed a high content of PLA_2 in the venoms of Thai cobra (Mukherjee & Maity, 2002) and several other cobras in Southeast Asia (Yap et al., 2011). All PLA_2 isoforms of *N. kaouthia* venoms identified in this study belong to Group IA (acidic subtypes). Generally, the acidic PLA_2 (Group IA) is less toxic compared to the basic PLA_2 (Joubert & Taljaard, 1980b; Karlsson, 1979), although some toxic effects (cytotoxicity and anticoagulant activity) had been reported in acidic PLA_2 isolated from Indian *N. kaouthia* venom (Mukherjee, 2007; Mukherjee et al., 2014). In addition, cobra venom PLA_2 is known to interact synergistically with cytotoxins/cardiotoxins, thereby potentiating the toxic effect of the venom (Gasanov et al., 2014; Rakhimov et al., 1981; Tan, 1991; Yap et al., 2014b).

4.4.3.2 Cysteine-rich Secretory Protein (CRISP)

The CRISP protein family accounts for 2-5% of total venom proteins in NK-M and NK-T venoms. CRISP is a single chain polypeptide widely distributed in numerous animal tissues and reptilian venoms (Heyborne & Mackessy, 2009). It exhibits a wide range of biological activities, including blockage of various ion channels, induction of hypothermia in prey animals and specific proteolysis. CRISP isoforms identified in the *N. kaouthia* venoms were homologous to natrin isolated from *N. atra* (Table 4.2), which is an antagonist of the high-conductance calcium-activated potassium (BKca) channel and ryanodine (RyR1) receptors (Chang et al., 2005; Wang et al., 2006; Wang et al., 2005; Zhou et al., 2008).

4.4.3.3 Snake Venom Metalloproteinase (SVMP)

SVMP is a high molecular mass toxin that is abundant in many viperid/crotalid venoms but it is typically a minor component in elapid venoms, including all three *N. kaouthia* venoms investigated. The SVMPs identified in all the three *N. kaouthia* venoms have a molecular mass above 40 kDa (Figure 4.1(a), (b) and (c)) and were annotated as SVMP P-III subtypes. These metalloproteinases are involved in the destruction of basal membrane, causing hemorrhage and presumably, the digestion of prey (Fox & Serrano, 2005, 2008b; Fox & Serrano, 2009; Markland & Swenson, 2013). The low expression level of SVMP and the lack of hemorrhagic syndrome in cobra-envenomed patients suggest that the biological role of SVMPs in *N. kaouthia* venom is mainly for prey digestion, though they may also contribute to the local tissue-damaging effect.

4.4.3.4 L-amino Acid Oxidase (LAAO)

In all three *N. kaouthia* venoms, LAAO constitutes only 0.5-1.1% of total venom protein. This is consistent with the low level of LAAO activity reported for Thai *N. kaouthia* venom (Yap et al., 2011). LAAO is usually a minor constituent in most snake venoms (including cobra (*Naja* sp.) venoms) (Mackessy, 2002a; Tan, 1998); its expression level however, can be exceptionally high in certain species. For instance, in *Ophiophagus hannah*, *Calloselasma rhodostoma* and *Hypnale hypnale*, the venom's LAAO content can exceed 10% of the total venom proteins (Fox, 2013; Tan et al., 2015c). The biological role of snake venom LAAO may be related to its cytotoxic and antimicrobial properties (Lee et al., 2011). LAAO isolated from *N. naja kaouthia* venom (unknown geographical origins) was shown to exhibit platelet aggregation activity (Sakurai et al., 2001; Tan & Swaminathan, 1992). Clinically, this is unlikely to be significant as *N. kaouthia* envenomation rarely results in blood coagulation defect.

4.4.3.5 Cobra Venom Factor (CVF)

CVF is a complement-activating protein found commonly in cobra venoms, with structural and functional homology to complement C3. It is typically an ancillary toxin and the content of CVF in all three *N. kaouthia* venoms is very low (0.7-1.1%). It has been shown that CVF can cause the release of anaphylatoxins such as C3a and C5a that promote local pro-inflammatory response through vasodilation, and the chemotaxis and activation of leucocyte (Vogel & Fritzinger, 2010). These reactions increase the vascular permeability and blood flow at the bite site, thus enhancing the absorption and spread of venom toxins.

4.4.3.6 Vespryn (Thaicobrin)

Thaicobrin is a protein toxin belonged to vespryn family and its content in all three *N. kaouthia* venoms is very low (0.2-0.7%). The protein has not been well characterized. Nonetheless, it was found to exhibit high sequence homology to ohanin, a vespryn isolated from the king cobra (*Ophiophagus hannah*) venom. As such, it is likely that Thaicobrin also possesses similar pharmacological properties as ohanin, i.e. the ability to induce hyperalgesia and hypolocomotion which may be beneficial in predation (Pung et al., 2006; Pung et al., 2005).

4.4.3.7 Novel Protein Families Found in *N. kaouthia* Venoms

The proteomic approach and data mining adopted in this study have successfully unveiled the presence of several novel protein families i.e. KUN, NP, PDE, 5'NUC and CTL in *N. kaouthia* venom(s). These toxin families have not been detected in the previous proteomic characterization of *N. kaouthia* venom (Kulkeaw et al., 2007; Vejayan et al., 2014), although the presence of PDE and 5'NUC in the venom had been

demonstrated enzymatically (Tan, 1991; Yap et al., 2011). From the results, KUN (Kunitz-type protease inhibitor) was detected in the venoms of NK-M and NK-V, and appeared to be similar to the serine protease inhibitor isolated from *Naja naja* venom (Shafqat et al., 1990). On the other hand, natriuretic peptide (NP) was detected only in NK-T venom. This protein is a minor toxin component and is reported to be able to increase cGMP formation in cultured rabbit endocardial endothelial cells and induce rapid relaxation of phenylephrine-precontracted rat aortic strips (Zhang et al., 2011). Both PDE and 5'NUC were detected in minute amounts (0.2-0.4%) in all three *N. kaouthia* venoms. These two enzymes were suggested to liberate nucleosides and may be involved in prey immobilization (Aird, 2002, 2009; Dhananjaya & D'Souza, 2010), presumably via hypotensive effect. Also, the injection of PDE had also been shown to result in a drop in arterial pressure and locomotor depression in animals (Russell et al., 1963).

The present study also revealed the presence of CTL in two *N. kaouthia* venoms (NK-M and NK-T). CTL is a toxin that targets a wide range of plasma components and cells particularly platelets, and could cause either platelet aggregation activation or inhibition (Du & Clemetson, 2009). CTL is usually found in considerable amount in viperid venoms and plays a significant role in venom-induced coagulopathy. However, in cobra envenomation, there is usually no coagulopathy, indicating that CTL has a negligible role in the pathogenesis of cobra envenomation in human, presumably because of its low content. In addition, NGF is also present in all three venoms examined, with a content of 0.2-1.3%. This is a venom protein that has been shown to be cytotoxic (Lavin et al., 2009). Again, this minor component is unlikely to play an important role in the lethal action of the cobra venom.

4.4.4 Comparison of the Proteomes of other Cobra Venoms (*Naja* genus)

The proteomic findings revealed the presence of more toxin families (a total of 13 families) in *N. kaouthia* venom as compared to the reported venom proteomes of several other cobras: Chinese *Naja atra* (3 protein families) (Li et al., 2004), African spitters *Naja nigricollis, Naja katiensis, Naja pallida, Naja nubiae, Naja mossambica* (6 protein families) (Petras et al., 2011), Pakistani *N. naja* (6 protein families) (Ali et al., 2013), Moroccan *Naja haje* (10 protein families) (Malih et al., 2014) and Malaysian *Naja sumatrana* (10 protein families) (Yap et al., 2014a). This is probably due to the differences in resolution of the proteomic approach or the improvement of the database used. Nevertheless, both 3FTx (particularly for neurotoxins and cytotoxins/cardiotoxins) and PLA_2 constitute the bulk of the venom proteins in all venom proteomes of African and Asian cobras studied thus far.

It is interesting to note that the Thai *N. kaouthia* venom contains highest amount of alpha-neurotoxin (> 40%) as compared to the venoms of African spitters (0.4-15%), the Morrocan *N. haje* (13%), the Malaysian *N. sumatrana* (15%), and the Pakistani *N. naja.* This finding is consistent with the clinical reports that showed a high percentage of patients envenomed by *N. kaouthia* in Thailand experienced severe neurotoxic symptom (Viravan et al., 1986). On the other hand, *N. kaouthia* from Vietnam and Malaysia, both have a higher content of cytotoxin/cardiotoxin (CTX) (~45%) than *N. kaouthia* from Thailand (28%). The very high content of CTX and low content of neurotoxin in the venoms of African spitting cobras suggest that these African spitting cobra venoms are mainly cytotoxic (Petras et al., 2011).

The proteomic studies of cobra (*Naja*) venoms mentioned above also revealed a relatively high PLA_2 content in all *Naja* venoms (15-30%), except for the Moroccan *N. haje* (4%). Besides, the Southeast Asian spitting cobras (*N. sumatrana*, *N. siamensis*, *N.*

sputatrix) were reported to contain a substantial amount of basic PLA_2 (Yap et al., 2011), similar to the venom of African spitting cobras, *N. nigricollis* (Fletcher et al., 1982). In contrast, basic PLA_2 was not detected in the non-spitting *N. kaouthia* venom (Yap et al., 2011), where only acidic PLA_2 isoforms were detected.

4.4.5 Comparison of Median Lethal Doses (LD_{50}) of the three *N. kaouthia* Venoms

The mouse lethality assay showed that the three *N. kaouthia* venoms differ in their median lethal doses (LD_{50}). This variation in LD_{50} can be correlated to the variation in 3FTx composition of the venoms. The very high lethality of Thai *N. kaouthia* venom (NK-T; LD_{50} ~0.2 µg/g) is likely due to the high abundance (33.3%) of LNTX in the venom. On the other hand, both the Malaysian and Vietnamese *N. kaouthia* venoms are less lethal (LD_{50} ~0.9 µg/g), presumably because they both have a much lower content of lethal alpha-neurotoxin. It is also interesting to note that the LD_{50} of the venom determined by subcutaneous (*s.c.*) injection is comparable to that administered via intravenous (*i.v.*) routes (Table 4.3). The findings indicate that the bioavailability of the principal lethal toxins of *N. kaouthia* venoms injected subcutaneously is in the vicinity of 100%.

Interestingly, the Vietnamese venom sample possesses a high content of non-conventional weak neurotoxin (WTX). A previous study showed that kaouthiotoxin (KTX), a WTX from *N. kaouthia* venom could interact non-covalently with PLA_2 to cause more intense membrane damage by forming a synergistic complex (Mukherjee, 2008). Therefore, it is hypothesized that with high WTX (19.3%) and PLA_2 content (17.4%), along with a high abundance of CTX (44.9%), NK-V venom may exert a more potent cytotoxic effect compared to NK-M and NK-T venoms.

4.4.6 Neutralization of *N. kaouthia* Venoms by two Antivenoms

The *in vitro* immunocomplexation results showed that both antivenoms produced from Thai cobra venom (monovalent, NKMAV; polyvalent, NPAV) were able to effectively neutralize the lethality of all three *N. kaouthia* venoms examined (Table 4.5). The efficacy of NKMAV and NPAV against the venoms appears to be comparable, indicating that the venom proteins of the three *N. kaouthia* possess similar antigenicity, even though there are variations in the venom proteomes (in particular the content of major lethal toxins). The findings revealed that NPAV is slightly more effective than NKMAV based on the volume-to-volume comparison, in term of neutralization potency (P, mg venom neutralized per ml reconstituted antivenom). However, the normalized values (expressed in term of mg venom neutralized per gram antivenom protein) that considering the protein content of antivenom suggest that NKMAV is more potent in neutralizing the *N. kaouthia* venom, whereas NPAV with higher protein content is less potent. This is mainly because the NPAV is pharmaceutically prepared as a polyvalent antivenom, and thus it is formulated with a higher immunoglobulin content to meet the need to neutralize multiple venoms.

It is interesting to note that both antivenoms (NKMAV and NPAV) were able to neutralize the venoms of Malaysian *N. kaouthia* (NK-M) with a higher potency than the venom of Thai species (NK-T), even though the antivenoms were produced using venom of the Thai species as immunogen. The findings suggest the possibility to further optimize the dosing of antivenom used in the treatment of *N. kaouthia* envenomation in Malaysia particularly (lower dose needed). Nevertheless, the results based on animal experiment are only indicative and must be validated clinically in future studies.

4.5 Conclusion

The proteomic findings in this study revealed as many as 13 different toxin families in the venoms of *Naja kaouthia* from three Southeast Asian regions (Malaysia, Thailand and Vietnam). Marked geographical variations were noted in the toxin composition of the venoms of same species sourced from three localities. These compositional variations particularly in the 3FTx correlate well with the lethality of the three venoms and explain the discrepancies in envenoming effect and lethality reported previously for *N. kaouthia* venom. Despite their variations in the venom composition, all three venoms can be effectively neutralized by the two commercial antivenoms (NKMAV and NPAV) raised against *N. kaouthia* venom of the Thai origin.

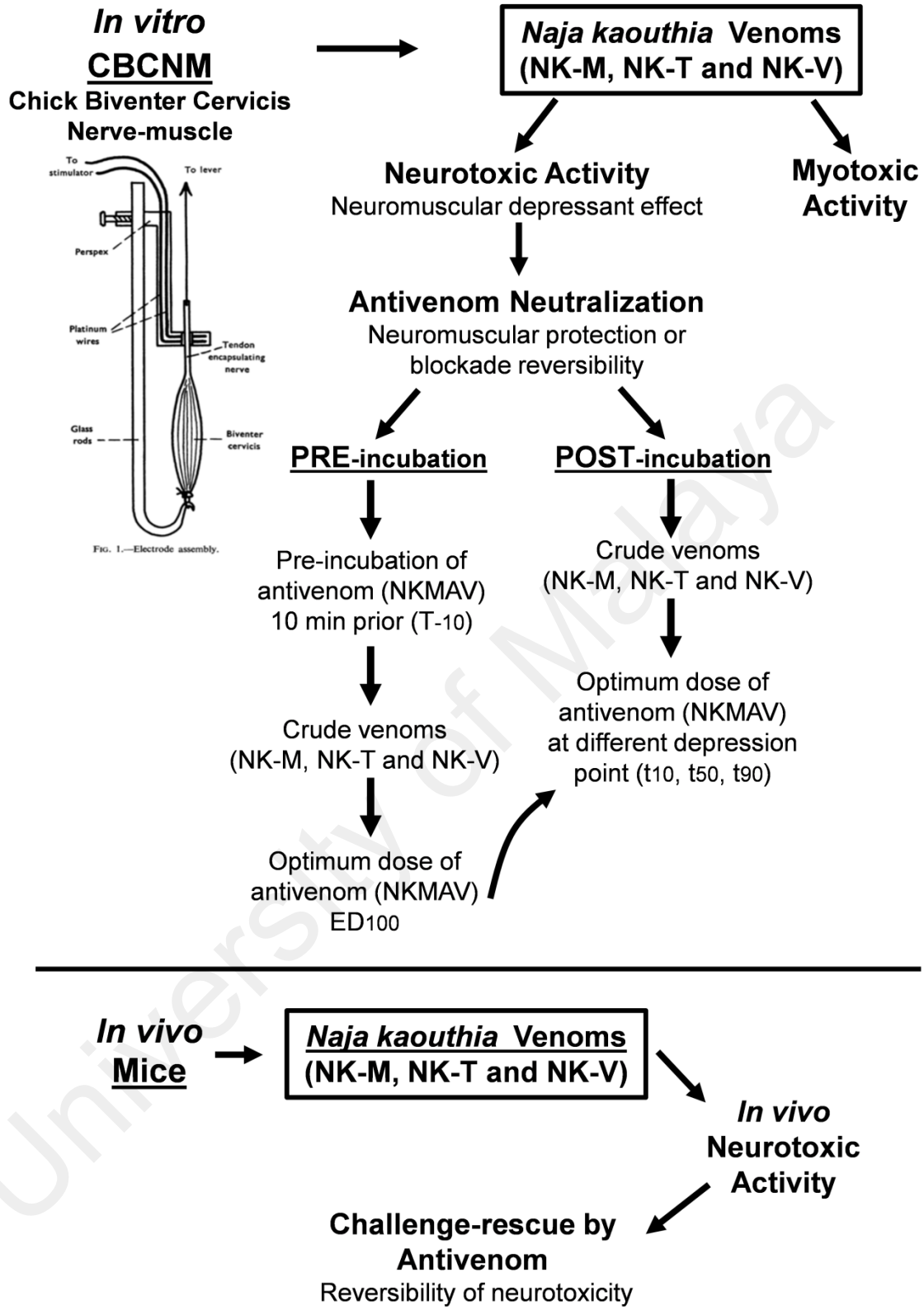
CHAPTER 5: NEUROMUSCULAR DEPRESSANT ACTIVITY OF *Naja kaouthia* VENOMS FROM THREE SOUTHEAST ASIA REGIONS

5.1 Introduction

Neurotoxicity and tissue necrosis constitute the major clinical syndromes of *Naja kaouthia* envenomation (Bernheim et al., 2001; Wongtongkam et al., 2005). The development of neurotoxicity in *N. kaouthia* envenomation is mainly attributed to the presence of alpha-neurotoxins in the venom. These curare-like polypeptides belong to the three-finger toxin (3FTx) family and are capable of binding to post-synaptic nicotinic receptors at the motor end plate, resulting in blockade of neuromuscular transmission and paralysis (Ranawaka et al., 2013). The neurotoxic and myotoxic properties of *Naja naja kaouthia* venoms had been studied using chick biventer cervicis nerve-muscle (CBCNM) preparation (Barfaraz & Harvey, 1994; Harvey et al., 1994). However, the lack of stringent authentication of the species and locale, as well as the uncertain species status of many Asiatic cobras in the earlier days has rendered the interpretation of the mechanistic findings difficult. For example, the Thai cobra was once termed either as *N. naja kaouthia* or *Naja naja siamensis*, but it is now well established that *N. kaouthia* and *N. siamensis* are two separate species (Wüster, 1996). As such, it is not known if the venom used in the earlier study was from the authentic *N. kaouthia* and the locality was unclear.

The comparative proteomic study in **Chapter 4** has revealed substantial variations in the venom proteomes of *N. kaouthia* from three Southeast Asia regions (Malaysia, NK-M; Thailand, NK-T; Vietnam, NK-V). In view of the diverse neurotoxin and cytotoxin subtypes and their varied expression levels, there is a need to investigate the neuro/myotoxic mechanisms of these *N. kaouthia* venoms, and how the toxic effects can be reversed or neutralized by the specific antivenom (*N. kaouthia* Monovalent

Antivenom, NKMAV). In this study, the chick biventer cervicis nerve-muscle preparation (CBCNM) was used for investigation of the neuro/myotoxic mechanisms, and an *in vivo* challenge-rescue rodent model was established to provide correlation with the findings from the *in vitro* isolated tissue studies. It is hoped that these findings will provide further insights into the pathophysiology and treatment optimization for *N. kaouthia* envenomation in different Southeast Asia regions. The experimental design of this study was summarized and shown in the following flow chart (next page):



5.2 Methods

5.2.1 Chick Biventer Cervicis Nerve-Muscle (CBCNM) Preparation

5.2.1.1 Experimental Procedure – Direct and Indirect Twitches

Male chicks (4-10 days old) were euthanized by isoflurane inhalation and both biventer cervicis nerve-muscles were removed (Figure 5.1). The tissues were mounted under 1 gram tension (gt) in 15 ml organ baths containing physiological solution of the following composition (per liter):

i. 118.4 mM sodium chloride (NaCl) : 6.92 g NaCl
ii. 4.7 mM potassium chloride (KCl) : 0.35 g KCl
iii. 1.2 mM magnesium sulfate ($MgSO_4$) : 0.29 g $MgSO_4$
iv. 1.2 mM potassium sulfate monobasic (KH_2PO_4) : 0.16 g KH_2PO_4
v. 2.5 mM calcium chloride ($CaCl_2$) : 0.28 g $CaCl_2$
vi. 25.0 mM sodium bicarbonate ($NaHCO_3$) : 2.10 g $NaHCO_3$
vii. 11.1 mM glucose : 2.10 g glucose

The physiological salt solution was aerated with carbogen (5% CO_2 and 95% O_2) and maintained at 34 ºC. The tissues for indirect nerve stimulation (indirect nerve-evoked twitches) were stimulated every 10 s with pulses of 0.2 ms duration at a supramaximal voltage using a Grass S5 stimulator (Grass Technologies, USA) attached to silver ring electrodes. The nerve-evoked indirect stimulation of the tissues was confirmed by the abolishment of twitches by d-tubocurarine (d-TC; 10 µM). For the direct muscle stimulation (direct muscle-evoked twitches), an electrode was placed on the muscle's belly and was stimulated every 10 s with pulses of 2 ms duration at a supramaximal voltage. The muscle twitch tensions were measured via force transducers (MLT050/D, ADInstrument, USA) that were attached to Quad Bridge Amp (ADInstruments, USA), and recorded on a PowerLab data acquisition system (ADInstruments, USA). The

change in muscle twitch tensions was expressed as a percentage of the initial nerve-evoked response prior to the addition of venom (mean ± SEM). The change in the baseline (muscle contracture) was measured from the initial baseline prior to the addition of venom. Data analysis was performed by using one-way analysis of variants (ANOVA), followed by Dunnett's multiple comparison tests (SPSS Version 16.0, IBM, USA) as described in **Section 3.2.10.2**.

5.2.1.2 Neuromuscular Depressant and Myotoxic Activity of *Naja kaouthia* Venoms

The responses of the tissues to exogenous agonists: acetylcholine (ACh; 1 mM for 30 s), carbachol (CCh; 20 µM for 60 s) and potassium chloride (KCl; 40 mM for 30 s) were obtained in the absence of electrical stimulation both prior to the addition of venom and at the end of the experiment (Harvey et al., 1994). In both indirect nerve-evoked and direct muscle-evoked stimulation experiments, the venom samples (NK-M, NK-T and NK-V) doses (1, 3 and 5 µg/ml) were left in contact with the preparation until a complete twitch blockade or a maximum of 180 min period. Differing from the indirect nerve-evoked stimulation, the effect of venoms (5 µg/ml) on the direct muscle twitches was examined in CBCNM preparation with the presence of d-tubocurarine (d-TC; 10 µM) to ensure selective direct stimulation of the muscle.

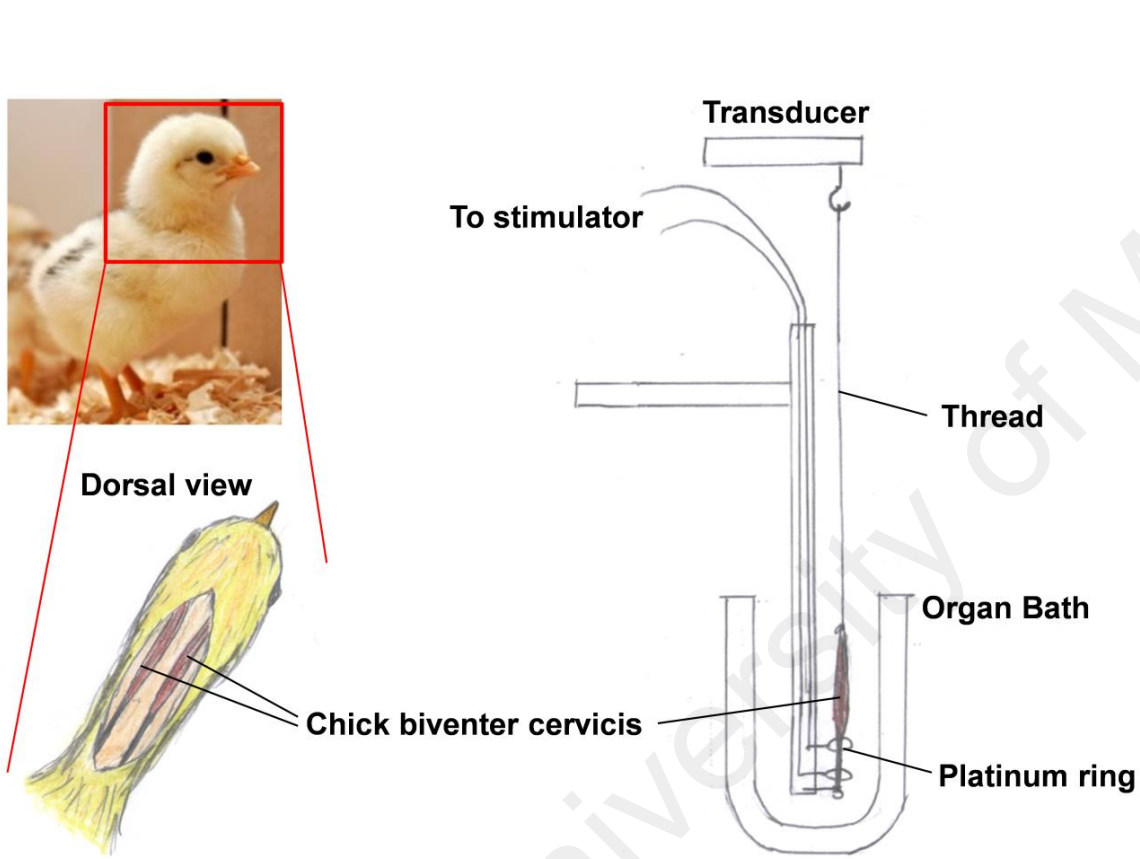


Figure 5.1 Experimental setup of chick biventer cervicis nerve-muscle (CBCNM) preparation.

5.2.1.3 Neutralization of Venom-Induced Neurotoxic Effect in CBCNM Preparation by Antivenom

(a) ***Pre-incubation Study***

N. kaouthia monovalent antivenom (NKMAV) was incubated with the CBCNM preparation 10 min prior (T_{-10}) to the addition of venoms (5 µg/ml). The tissues were pre-incubated with appropriate antivenom doses, in which the doses were estimated from the NKMAV potency values obtained from the *in vitro* immunocomplexation neutralization studies in mice (Chapter 4, Table 4.5) (Potency: NK-M, 1.10 mg/ml; NK-T, 0.92 mg/ml and NK-V, 0.70 mg/ml, respectively). The doses of NKMAV used in this CBCNM pre-incubation study were expressed in terms of the number of potencies (P), calculated as the following:

$$1\text{x Potency of NKMAV used in CBCNM pre} - \text{incubation neutralization (ml)} = \frac{\text{Amount of venom added into the preparation (mg)}}{\textit{In vitro}\ \text{immunocomplexation potency of NKMAV in mice (mg/ml)}}$$

The amount of venoms (NK-T, NK-M or NK-V) added to the tissue preparation was standardized to give a final bath concentration of 5 µg/ml. The venom in the preparation was individually subjected to neutralization by three different doses of pre-incubating antivenom, which included the highest effective titer. The highest effective titer is defined as the number of "potency" that was able to retain 100% of the initial indirect twitches throughout the experiment and indicates as the dose that produces maximal neutralization efficacy in this pre-incubation assay (abbreviated as ED_{100} in the subsequent description).

(b) ***Challenge-rescue Study***

The CBCNM preparation was first challenged with *N. kaouthia* venom at 5 µg/ml (NK-T, NK-M or NK-V). The depression of indirect twitches was monitored, and a rescue dose of NKMAV equivalent to ED_{100} of the antivenom was added at the time when the twitches were reduced by 10%, 50% or 90% (i.e. t_{10}, t_{50} or t_{90}) separately. The added antivenom was then kept in contact with the respective preparation throughout the remaining time of the experiment (up to 180 min). The effect of the addition of NKMAV at different time points after the onset of twitch depression was monitored and compared among the three venoms.

5.2.2 *In vivo* Neurotoxic Activity Study in Mice

ICR mice (20-25 g, n = 5) were subcutaneously injected with 5x LD_{50} of *N. kaouthia* venoms (NK-T, NK-M or NK-V) as described in Chapter 4, Table 4.3 (*s.c.* LD_{50}: NK-M, 1.00 µg/g; NK-T, 0.20 µg/g and NK-V, 1.11 µg/g). The neurological signs were closely observed and scored according to a modified matrix for the onset time of venom neurotoxicity (Rodriguez-Acosta et al., 2006). The development of syndromes was recorded according to the following indicators: grooming behavior, posterior limb paralysis, dyspnea, flaccid paralysis, urinary sphincter relaxation and death.

5.2.3 *In vivo* Challenge-rescue Experiment in Mice

In a separate series of experiments, ICR mice (20-25 g, n = 6) were subcutaneously envenomed with 5x LD_{50} of *N. kaouthia* venoms (*s.c.* LD_{50}: NK-M, 1.00 µg/g; NK-T, 0.20 µg/g and NK-V, 1.11 µg/g) and were rescued with NKMAV (injected intravenously with the amount of NKMAV corresponding to ED_{100}) at the early sign of posterior limb paralysis (as described in **Section 3.2.9.2**). The mice were observed for clinical recovery for a period of 48 hours, during which they were allowed access to food and water *ad libitum*.

5.3 Results

5.3.1 Neurotoxic Effects of *N. kaouthia* Venoms

5.3.1.1 Effect of the Venoms on Nerve-evoked Indirect Muscle Twitches

All three *N. kaouthia* venoms (NK-M, NK-T and NK-V) (1, 3 and 5 µg/ml) abolished the indirect twitches of the CBCNM preparation (Figure 5.3; n = 3-4; $p < 0.05$). The time required for the twitches to be reduced by 90% (i.e. t_{90}) was: 30 ± 2 min (5 µg/ml), 41 ± 7 min (3 µg/ml), 112 ± 6 min (1 µg/ml) for NK-M venom; 10 ± 1 min (5 µg/ml), 15 ± 1 min (3 µg/ml), 23 ± 1 min (1 µg/ml) for NK-T venom and 15 ± 1 min (5 µg/ml), 23 ± 1 min (3 µg/ml) 70 ± 3 min (1 µg/ml) for NK-V venom, respectively (mean ± SEM, n = 3-4). Also, all three venoms caused remarkable muscle contractures at the venom concentrations of 3 and 5 µg/ml. At 5 µg/ml (n = 3-4), the muscle contracture effects were observed and baseline tensions were increased 0.80 ± 0.10 gram tension (gt), 0.08 ± 0.03 gt and 0.48 ± 0.09 gt for NK-M, NK-T and NK-V venoms, respectively (Figure 5.2(b)).

Incubation with the three venoms (NK-M, NK-T or NK-V) at all concentrations (1, 3 and 5 µg/ml) completely abolished the tissue contractile response to exogenous nicotinic agonists (ACh and CCh) (Figure 5.4). However, the tissue response to potassium chloride (KCl) was altered to varying degree as compared to the pre-venom incubation level. All venoms significantly reduced the KCl-induced tissue contractile response (Figure 5.4; n = 3-4; $p < 0.05$) when incubated with venom at 3 µg/ml, while the inhibitory effect was negligible at 1 µg/ml. At 5 µg/ml venom concentration, KCl added after the abolishment of twitches was able to produce a full tissue contractile response in the preparations of NK-T and NK-V but not NK-M. However, it should be noted that the NK-T and NK-V preparations exhibited a time-dependent attenuation of

KCl response, where it was found significantly reduced following a prolonged exposure to the venoms at 180 min incubation (Figure 5.5; n = 3-4; $p < 0.05$).

5.3.1.2 Effects on Muscle-evoked Direct Muscle Twitches

In this study, direct electrostimulation was applied on the CBC muscle (Barfaraz & Harvey, 1994) over 180 min of the incubation period. All venoms at the concentration of 5 µg/ml showed time-dependent attenuation of the direct muscle-evoked twitches, with significant inhibition up to 60-80% at the end of the incubation period (180 min). Among the three, the inhibitory effect was most prominent in the NK-M preparation, followed by NK-T and NK-V (Figure 5.6; n = 3-4; $p < 0.05$).

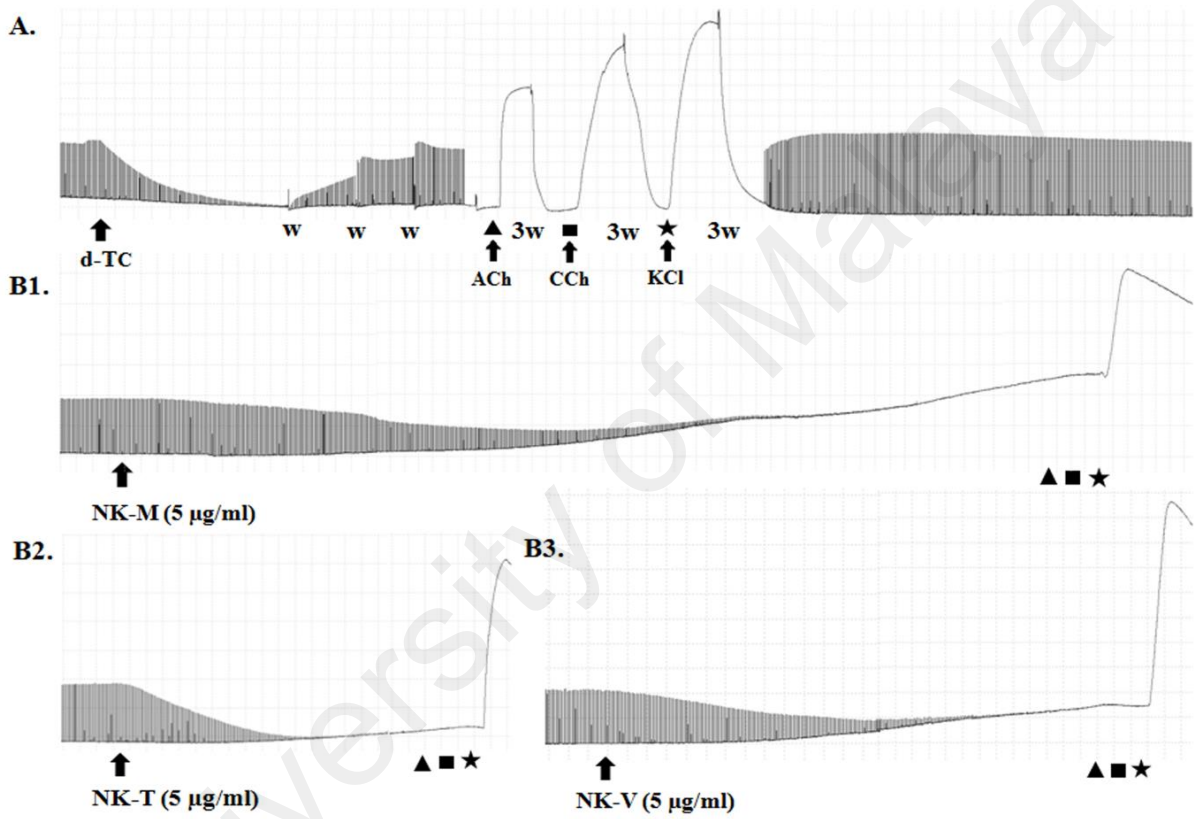


Figure 5.2 Representative tracings of chick biventer cervicis contractile responses to the inhibitor, agonists and *N. kaouthia* venoms of three geographical regions. (A) Nerve-evoked indirect stimulation of the muscle was confirmed by the abolishment of twitches by d-tubocurarine (d-TC; 10 µM) and the restoring of responses by nicotinic agonists (ACh; 1 mM, CCh; 20 µM) and KCl (40 mM). The responses to exogenous agonists were obtained in the absence of electrical stimulation prior to the addition of venom (5µg/ml). (B) Nerve-evoked indirect twitches of CBC upon addition of *N. kaouthia* venoms (B1; NK-M, B2; NK-T, B3; NK-V) and the responses to exogenous agonists were obtained after incubation with venoms (5 µg/ml). W: Washing; ▲: ACh; ■: CCh; ★: KCl.

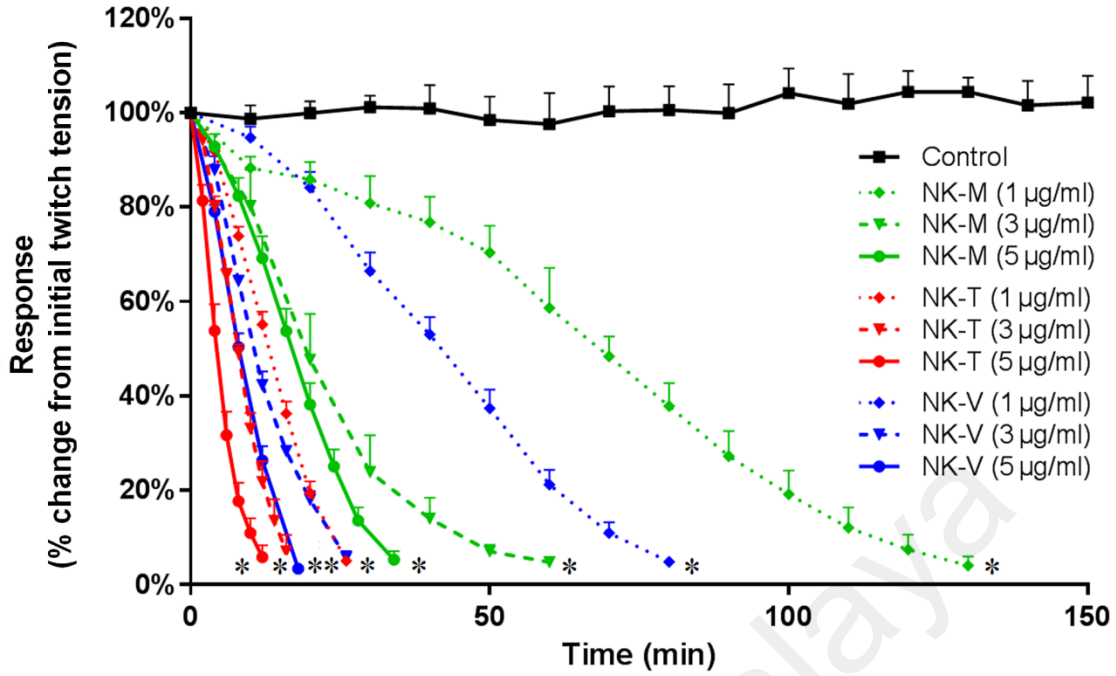


Figure 5.3 The effect of *N. kaouthia* venoms (NK-M, NK-T and NK-V) (1, 3 and 5 µg/ml) on the nerve-evoked indirect twitches of chick biventer cervicis nerve-muscle (CBCNM) preparation. Data are expressed as the mean ± SEM. **p* < 0.05, significantly different from control (physiological salt solution) (n = 3-4, one-way ANOVA).

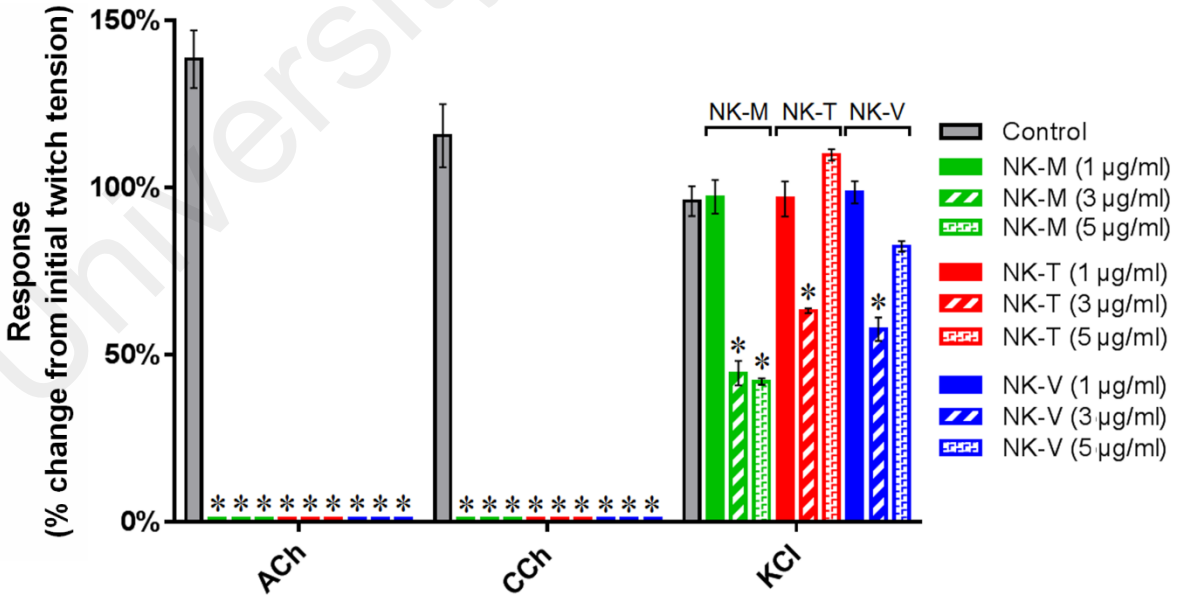


Figure 5.4 The effect of *N. kaouthia* venoms (NK-M, NK-T and NK-V) (1, 3 and 5 µg/ml) on the responses to exogenous agonists (ACh, CCh and KCl) right after the abolishment of nerve-evoked indirect twitches. Data are expressed as the mean ± SEM. **p* < 0.05, significantly different from control (physiological salt solution) (n = 3-4, one-way ANOVA).

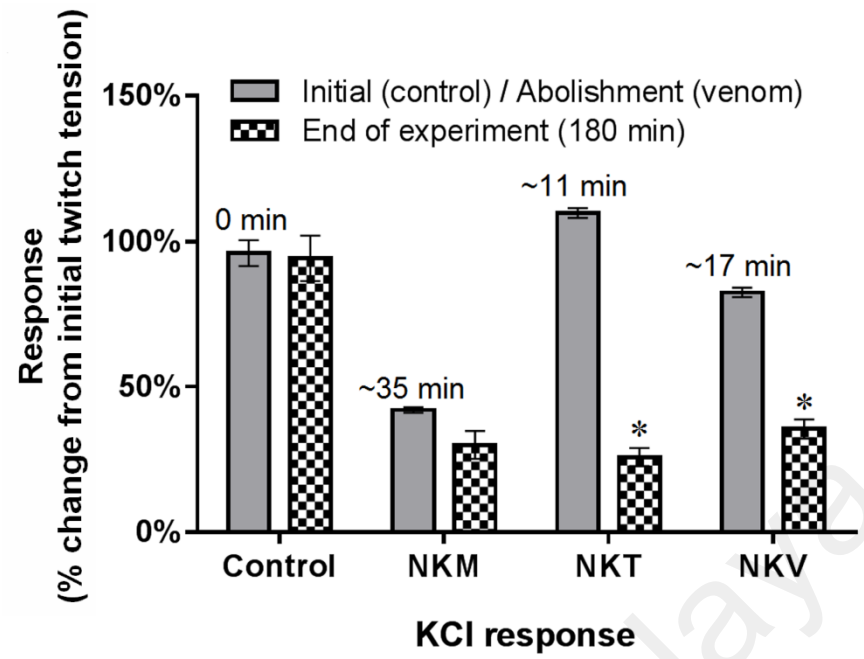


Figure 5.5 The effect of *N. kaouthia* venoms (NK-M, NK-T and NK-V) (5 µg/ml) on tissue response to the exogenous agonist KCl observed immediately after the abolishment of nerve-evoked indirect twitches and after a maximum incubation period (180 min). The time-dependent inhibitory effect was observed in both the NK-T and NK-V preparation. Data are expressed as the mean ± SEM. *$p < 0.05$, significantly different from control (abolishment point) (n = 3-4, one-way ANOVA)

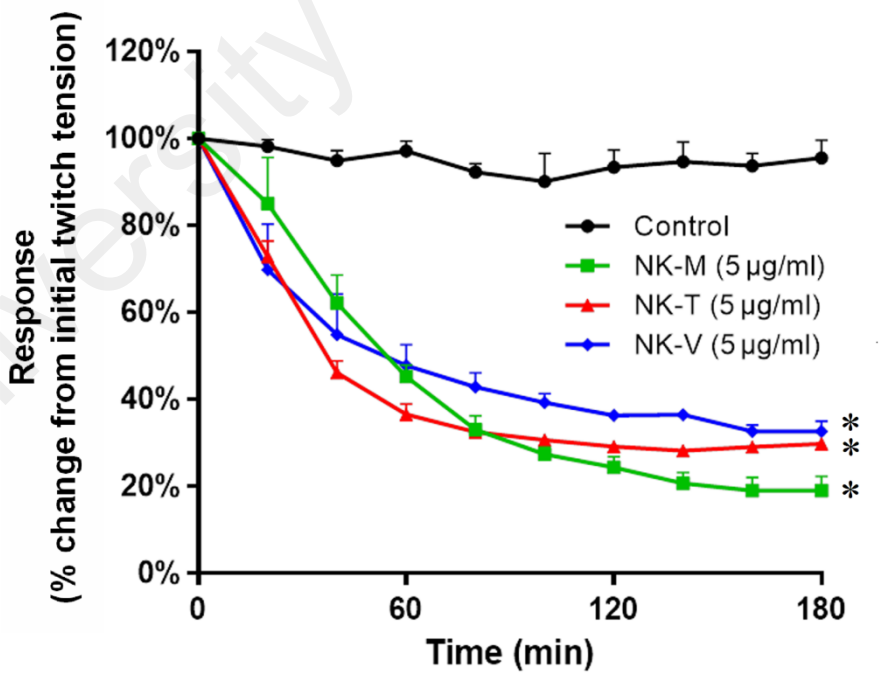


Figure 5.6 The effect of *N. kaouthia* venoms (NK-M, NK-T and NK-V) (5 µg/ml) on the muscle-evoked direct twitches of chick biventer cervicis nerve-muscle preparation. Data are expressed as the mean ± SEM. *$p < 0.05$, significantly different from control (physiological salt solution) (n = 3-4, one-way ANOVA)

5.3.2 Antivenom Neutralization

5.3.2.1 *In vitro* Pre-incubation with *N. kaouthia* Monovalent Antivenom (NKMAV) prior to Venom Challenge (T_{-10})

The pre-incubation of NKMAV 10 min prior (T_{-10}) to venom addition had either prevented (0.5x and 1x potency for NK-M; 2x and 4x potency for NK-T; 1x and 2x potency for NK-V) or delayed (0.25x potency for NK-M, 1x potency for NK-T and 0.5x potency for NK-V) the onset of neuromuscular blockade induced by these venoms at 5 µg/ml (Figure 5.7(a), (b) and (c); n = 3-4; $p < 0.05$). The NKMAV demonstrated a spectrum of efficacy: more effective against NK-M venom (ED_{100} = 1x potency), followed by NK-V (ED_{100} = 2x potency) and least effective against NK-T (ED_{100} = 4x potency) (Figure 5.7(a), (b) and (c)).

The CBCNM preparations pre-incubated with moderate to high doses of NKMAV (0.5x and 1x potency for NK-M; 2x and 4x potency for NK-T; 1x and 2x potency for NK-V) spared the tissue from post-synaptic receptor blockade or direct muscle damage by venom toxins. This is reflected in the significant improvement in tissue responses to ACh, CCh and KCl as compared to that incubated with venom alone (Figure 5.8; n = 3-4; $p < 0.05$). However, the lowest NKMAV doses used (0.25x potency for NK-M, 1x potency for NK-T and 0.5x potency for NK-V) were unable to prevent the neuromuscular blockade exerted by the venoms and the responses to both exogenous agonists (ACh and CCh) were abolished.

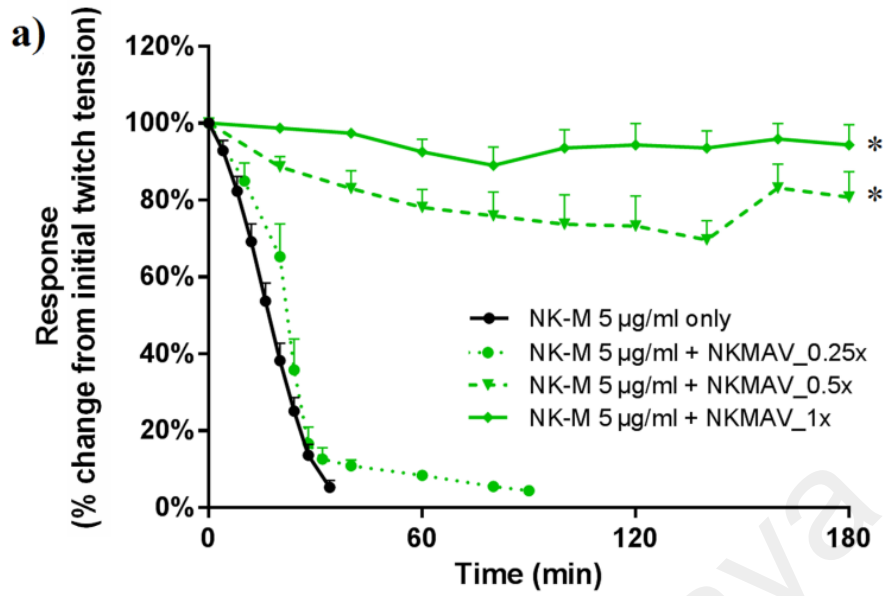


Figure 5.7(a) The effect of prior addition (T_{-10}) of different doses of *N. kaouthia* Monovalent Antivenom (NKMAV) on the neurotoxic activity of *N. kaouthia* venom (5 µg/ml) sourced from Malaysia (NK-M) in a nerve-evoked CBCNM preparation. The NKMAV doses (0.25x, 0.5x and 1x potency) were determined using the *in vitro* immunocomplexation neutralization potency in mice (Chapter 4, Table 4.5) according to Section 5.2.1.3 (a), 1x potency is the ED_{100}. Data are expressed as the mean ± SEM. *p < 0.05, significantly different from group with venom only (n = 3-4, one-way ANOVA).

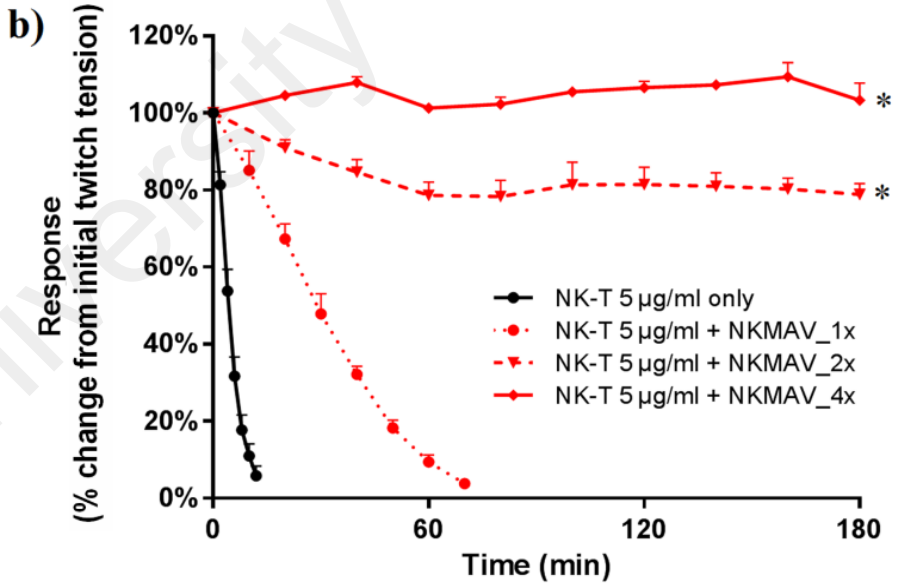


Figure 5.7(b) The effect of prior addition (T_{-10}) of different doses of *N. kaouthia* Monovalent Antivenom (NKMAV) on the neurotoxic activity of *N. kaouthia* venom (5 µg/ml) sourced from Thailand (NK-T) in a nerve-evoked CBCNM preparation. The NKMAV doses (1x, 2x and 4x potency) were determined using the *in vitro* immunocomplexation neutralization potency in mice (Chapter 4, Table 4.5) according to Section 5.2.1.3 (a), 4x potency is the ED_{100}. Data are expressed as the mean ± SEM. *p < 0.05, significantly different from group with venom only (n = 3-4, one-way ANOVA).

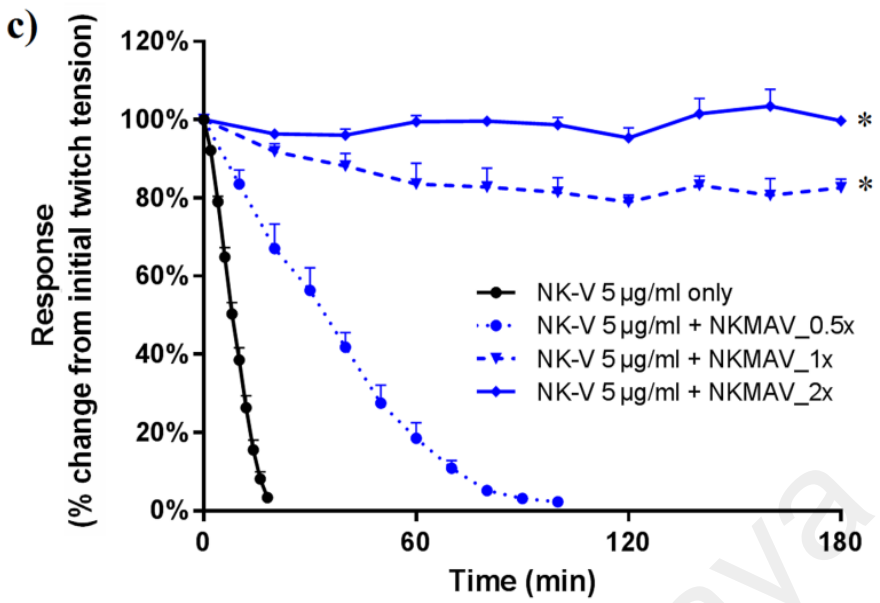


Figure 5.7(c) The effect of prior addition (T_{-10}) of different doses of *N. kaouthia* Monovalent Antivenom (NKMAV) on the neurotoxic activity of *N. kaouthia* venom (5 µg/ml) sourced from Vietnam (NK-V) in a nerve-evoked CBCNM preparation. The NKMAV doses (0.5x, 1x and 2x potency) were determined using the *in vitro* immunocomplexation neutralization potency in mice (Chapter 4, Table 4.5) according to Section 5.2.1.3 (a), 2x potency is the ED_{100}. Data are expressed as the mean ± SEM. *p < 0.05, significantly different from group with venom only (n = 3-4, one-way ANOVA).

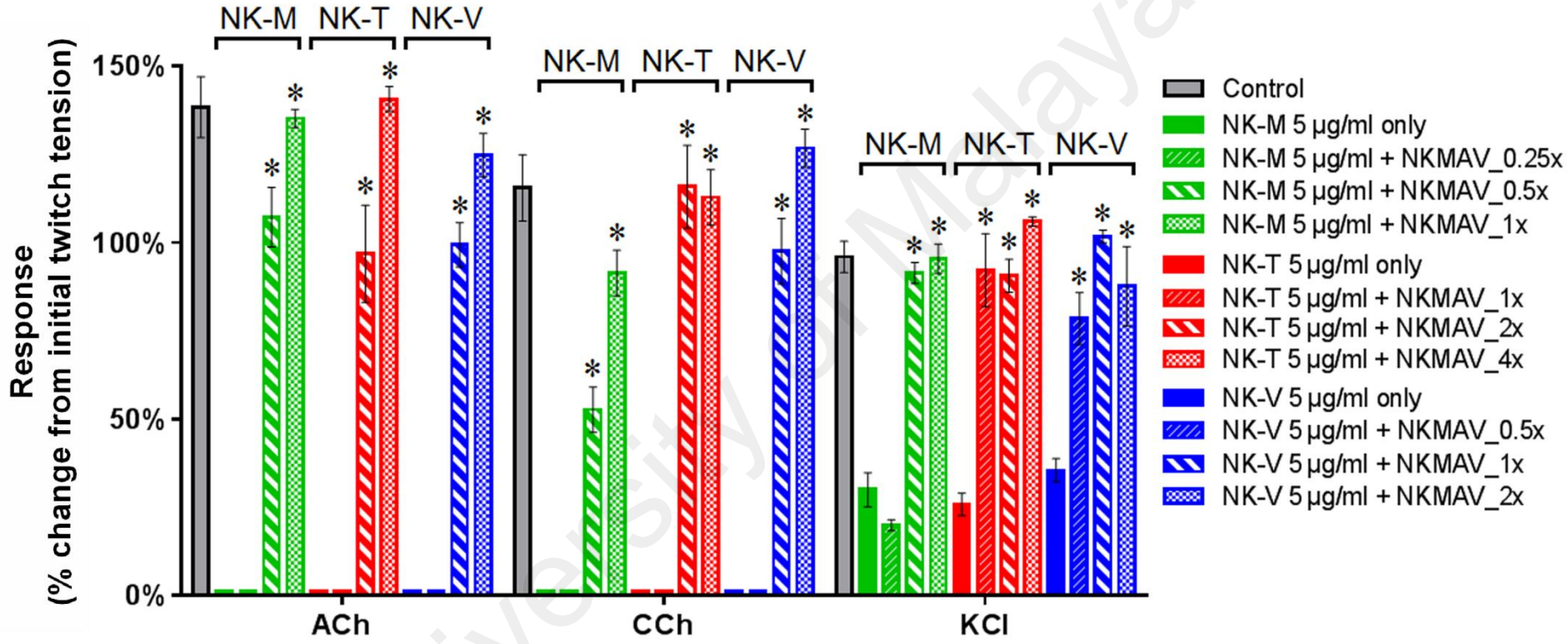


Figure 5.8 Chick biventer cervicis contractile responses to exogenous agonists (ACh, CCh and KCl) at the end of the experiment where *N. kaouthia* Monovalent Antivenom (NKMAV) at various doses was added to the tissue, 10 min prior (T_{-10}) to venom (5 µg/ml) challenge. The NKMAV doses (0.5x, 1x and 2x potency) were determined using the *in vitro* immunocomplexation neutralization potency in mice (Chapter 4, Table 4.5) according to Section 5.2.1.3 (a) (ED_{100}: NK-M, 1x potency; NK-T, 4x potency; and NK-V, 2x potency). Data are expressed as the mean ± SEM. *$p < 0.05$, significantly different from group with venom only (n = 3-4, one-way ANOVA).

5.3.2.2 *In vitro* Venom Challenge followed by *Naja kaouthia* Monovalent Antivenom (NKMAV) Rescue Treatment at Different Time Points (t_{10}, t_{50} and t_{90})

The highest effective NKMAV titers (doses retained 100% twitches, ED_{100}: NK-M, 1x potency; NK-T, 4x potency; and NK-V, 2x potency) used could not fully reverse the twitches depression that had already occurred, but were able to halt further neuromuscular depression. NKMAV (ED_{100}) addition at t_{10} prevented further venom-induced depression of indirect twitches caused by NK-V (retained at ~80% twitches) and NK-T (~70%), but to a lesser extent in NK-M (30-40%) (Figure 5.9(a), (b) and (c); n = 3-4; $p < 0.05$). Besides, the indirect twitches of NK-T and NK-V preparations were retained at a level of 30-40% when NKMAV (ED_{100}) was added at t_{50}, but was unable to halt the depletion in the case of NK-M where the twitches depression continued further (much reduced to ~10%). The delayed NKMAV (ED_{100}) treatments applied at t_{90} to all preparations could not reverse but were able to retain the twitches of both NK-M and NK-V preparations at 10% level; whereas twitches in the NK-T preparation progressed to complete abolishment.

Likewise, the addition of NKMAV (ED_{100}) preserved the tissue contractile responses to exogenous agonists (ACh, CCh and KCl) in a time-dependent manner. Figure 5.10 showed that the responses were further attenuated when the "rescue" was delayed. Responses to exogenous agonists greatly reduced at the end of the experiment (end point of 180 min) when NKMAV was applied late at t_{90}, except for NK-T preparation where the responses to ACh and KCl remained high even though the indirect twitches were depleted in the early stage of venom exposure (Figure 5.9(b) and Figure 5.10(b); n = 3-4; $p < 0.05$).

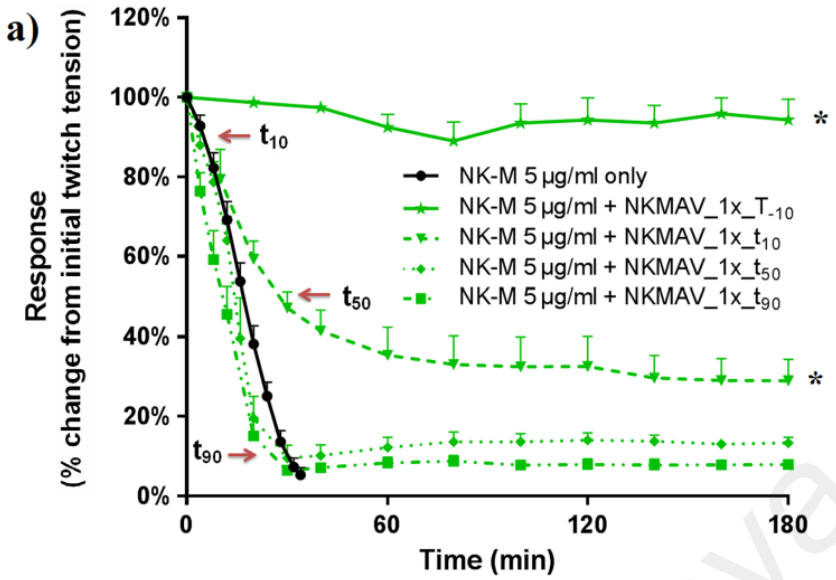


Figure 5.9(a) The effect of the highest effective titer or ED_{100} (NK-M, 1x potency) of *N. kaouthia* monovalent antivenom (NKMAV) added at different time points of twitch depression induced by *N. kaouthia* venom (5 µg/ml) sourced from Malaysia (NK-M). T_{-10} as in 10 min prior to venom addition; t_{10}, t_{50} and t_{90} as in time points where twitch tensions were reduced to 10%, 50% and 90% of initial tension, respectively. Data are expressed as the mean ± SEM. *$p < 0.05$, significantly different from group with venom only (n = 3-4, one-way ANOVA).

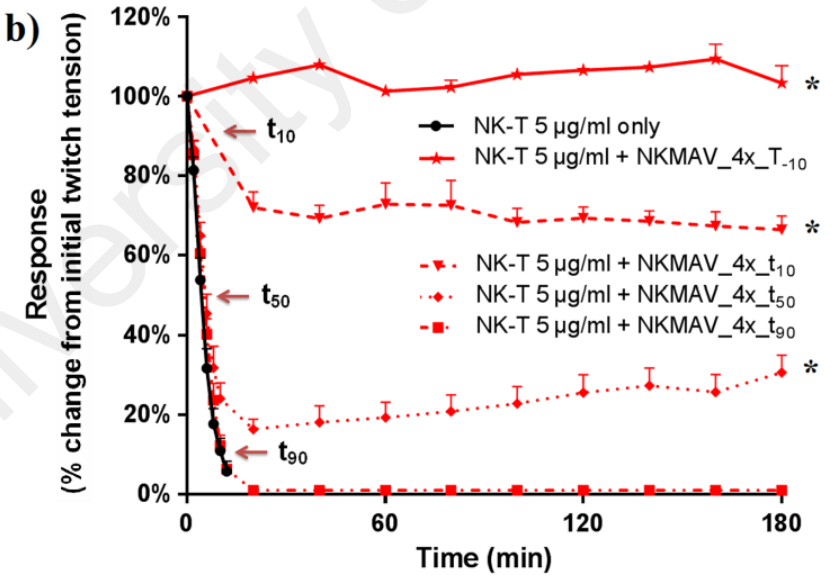


Figure 5.9(b) The effect of the highest effective titer or ED_{100} (NK-T, 4x potency) of *N. kaouthia* monovalent antivenom (NKMAV) added at different time points of twitch depression induced by *N. kaouthia* venom (5 µg/ml) sourced from Thailand (NK-T). T_{-10} as in 10 min prior to venom addition; t_{10}, t_{50} and t_{90} as in time points where twitch tensions were reduced to 10%, 50% and 90% of initial tension, respectively. Data are expressed as the mean ± SEM. *$p < 0.05$, significantly different from group with venom only (n = 3-4, one-way ANOVA).

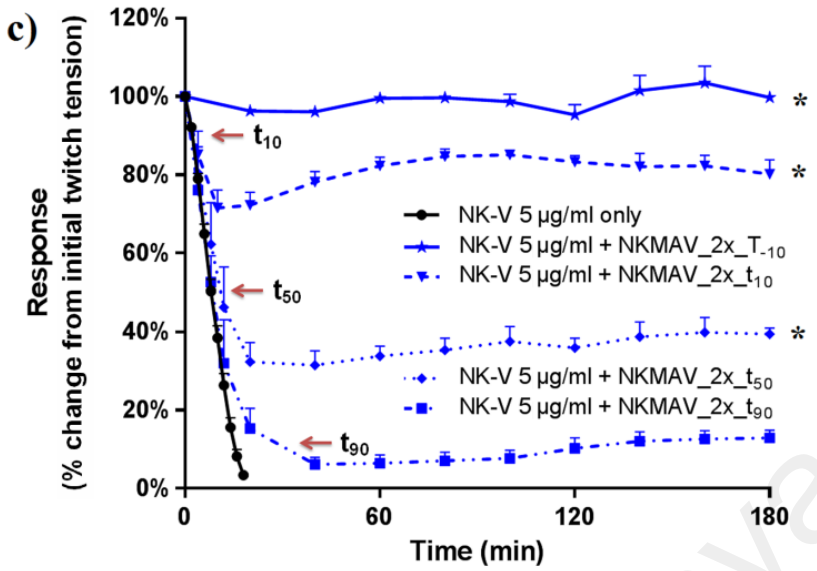


Figure 5.9(c) The effect of the highest effective titer or ED_{100} (NK-V, 2x potency) of *N. kaouthia* monovalent antivenom (NKMAV) added at different time points of twitch depression induced by *N. kaouthia* venom (5 µg/ml) sourced from Vietnam (NK-V). T_{-10} as in 10 min prior to venom addition; t_{10}, t_{50} and t_{90} as in time points where twitch tensions were reduced to 10%, 50% and 90% of initial tension, respectively. Data are expressed as the mean ± SEM. *$p < 0.05$, significantly different from group with venom only (n = 3-4, one-way ANOVA).

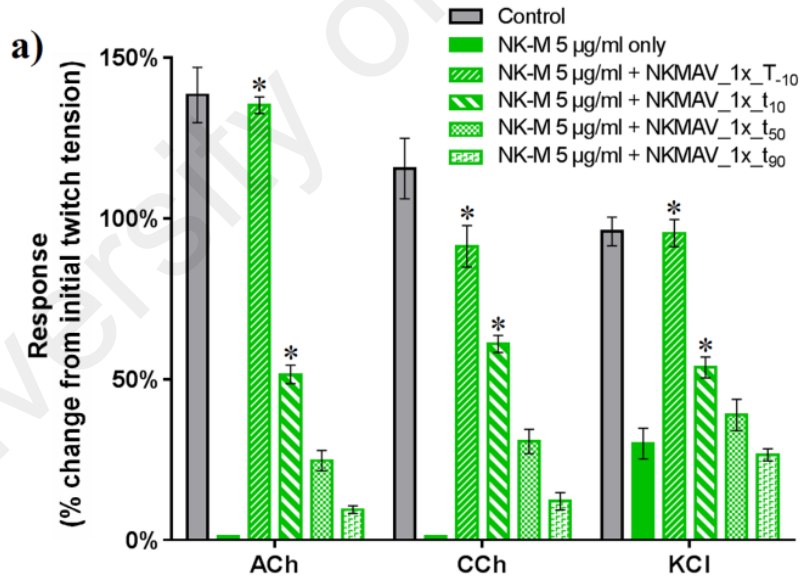


Figure 5.10(a) Tissue contractile responses to exogenous agonists (ACh, CCh and KCl) elicited at 180 min in the CBCNM preparation exposed to *N. kaouthia* venom sourced from Malaysia (NK-M) at 5µg/ml. In the tissue preparation, the highest effective titer (ED_{100}) of *N. kaouthia* Monovalent Antivenom (NKMAV: 1x Potency) was added at different time points (T_{-10} as in 10 min prior to venom addition, t_{10}, t_{50} and t_{90} as in time points where twitches were reduced to 10%, 50% and 90% of initial tension, respectively). Data are expressed as the mean ± SEM. *$p < 0.05$, significantly different from group with venom only (n = 3-4, one-way ANOVA).

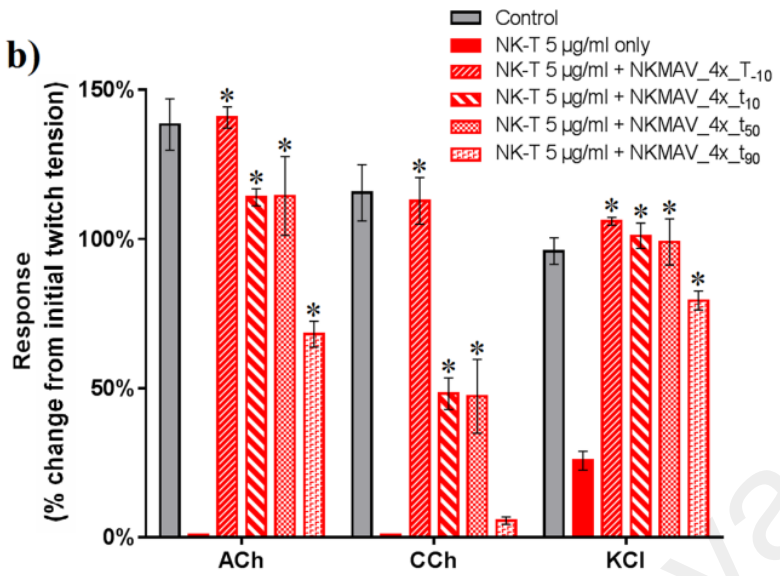


Figure 5.10(b) Tissue contractile responses to exogenous agonists (ACh, CCh and KCl) elicited at 180 min in the CBCNM preparation exposed to *N. kaouthia* venom sourced from Thailand (NK-T) at 5µg/ml. In the tissue preparation, the highest effective titer (ED_{100}) of *N. kaouthia* Monovalent Antivenom (NKMAV: 4x Potency) was added at different time points (T_{-10} as in 10 min prior to venom addition, t_{10}, t_{50} and t_{90} as in time points where twitches were reduced to 10%, 50% and 90% of initial tension, respectively). Data are expressed as the mean ± SEM. *$p < 0.05$, significantly different from group with venom only (n = 3-4, one-way ANOVA).

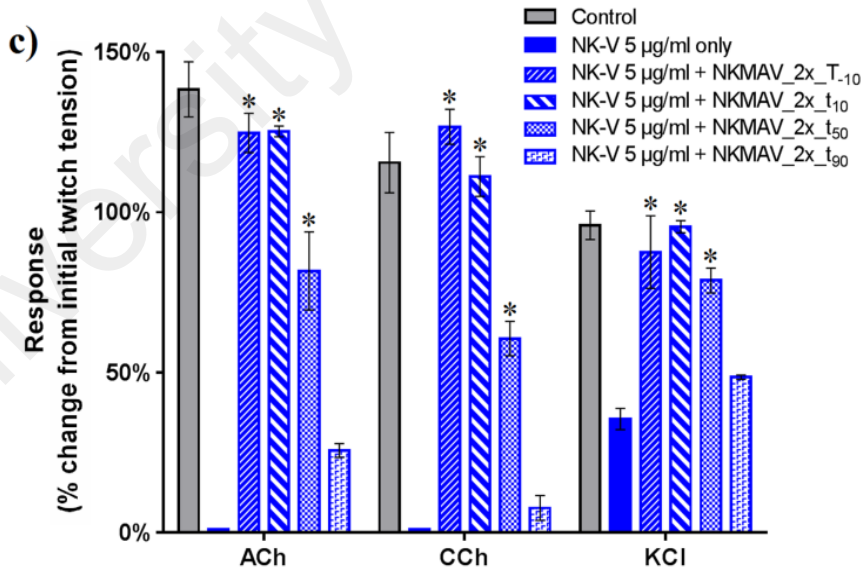


Figure 5.10(c) Tissue contractile responses to exogenous agonists (ACh, CCh and KCl) elicited at 180 min in the CBCNM preparation exposed to *N. kaouthia* venom sourced from Vietnam (NK-V) at 5µg/ml. In the tissue preparation, the highest effective titer (ED_{100}) of *N. kaouthia* Monovalent Antivenom (NKMAV: 2x Potency) was added at different time points (T_{-10} as in 10 min prior to venom addition, t_{10}, t_{50} and t_{90} as in time points where twitches were reduced to 10%, 50% and 90% of initial tension, respectively). Data are expressed as the mean ± SEM. *$p < 0.05$, significantly different from group with venom only (n = 3-4, one-way ANOVA).

5.3.3 *In vivo* Neurotoxic Activity Study in Mice

The neurological manifestations of mice experimentally envenomed by *N. kaouthia* venoms (NK-M, NK-T and NK-V) were summarized in Table 5.1. In general, the neurotoxic signs developed most rapidly in the mice injected with NK-V, followed by NK-T and NK-M. The death occurred in a range of 15–45 min, 60–120 min and 90–150 min for NK-V, NK-T and NK-M, respectively.

5.3.4 *In vivo* Challenge-rescue Study in Mice

In the *in vivo* challenge-rescue experiment, NKMAV administered intravenously was able to reverse the neurotoxic signs and prevented death caused by all three *N. kaouthia* venoms (NK-M, NK-T and NK-V) in mice (Table 5.2). However, the recovery speed from neurotoxicity varied among the three venoms. The time taken for full recovery was compatible (in the range of 240–270 min post-envenomation) in mice inoculated with both NK-M and NK-V venom, but the recovery was substantially longer in the case of NK-T venom (~800 min post-envenomation). NK-T venom-inoculated mice generally showed a much slower recovery upon rescue despite the use of the antivenom at the highest effective titer (ED_{100}).

Table 5.1: The *in vivo* neurotoxic effects and the time of onset in mice subcutaneously inoculated with *N. kaouthia* venoms (20-25 g, n = 5) from different geographical regions (NK-M, NK-T and NK-V).

Time (min)	Grooming behavior	Posterior limb paralysis	Dyspnea	Flaccid paralysis	Urinary sphincter relaxation	Death
2	1*v*, 2*v*, 3*v*, 4*v*, 5*v* 1*t*, 2*t*, 3*t*, 4*t*, 5*t* 1*m*, 2*m*, 3*m*, 4*m*, 5*m*					
10		1*v*	1*v*	1*v*		
15		2*v*	2*v*	2*v*	1*v*	1*v*
30		3*v*, 4*v*, 5*v* 5*t*	4*v*, 5*v*	4*v*, 5*v*	2*v*	2*v*
45		 1*t*, 2*t*, 4*t* 3*m*, 4*m*	3*v* 1*t*, 2*t*, 5*t* 3*m*	3*v* 1*t*, 2*t*, 5*t* 3*m*	4*v*, 5*v*	4*v*, 5*v*
60		5*m*, 2*m*	4*t* 4*m*, 2*m*	4*t* 4*m*, 2*m*	3*v* 5*t*	3*v* 5*t*
75		3*t* 1*m*	3*t* 1*m*, 5*m*	3*t* 1*m*, 5*m*	1*t*, 4*t*	1*t*, 4*t*
90					2*t* 3*m*, 4*m*	2*t* 3*m*, 4*m*
120					5*m*, 2*m* 3*t*	5*m*, 2*m*
135						3*t*
150					1*m*	1*m*

***v*: represents the mouse specimen inoculated with NK-V venom**

***t*: represents the mouse specimen inoculated with NK-T venom**

***m*: represents the mouse specimen inoculated with NK-M venom**

Table 5.2: The *in vivo* challenge-rescue in mice subcutaneously inoculated with *N. kaouthia* venoms (20-25 g, n = 6) from different geographical regions (NK-M, NK-T and NK-V) following the onset of early posterior limb paralysis. The time of recovery upon the intravenous injection of antivenom (NKMAV) was recorded.

Time (min)	Early posterior limb paralysis	Half recovery (move slowly)	Full recovery (move freely)
20	1*v*, 2*v*, 3*v*		
30	4*v* 1*t*, 2*t*, 3*t*, 4*t*, 5*t*, 6*t*		
40	5*v*, 6*v*		
50	1*m*, 2*m*, 3*m*		
70	4*m*, 5*m*, 6*m*		
105		1*v*, 2*v*, 3*v*	
130		4*v*, 5*v*, 6*v* 1*m*, 2*m*, 3*m*	
170		4*m*, 5*m*, 6*m*	
240			1*v*, 2*v*, 3*v*, 4*v* 1*m*, 2*m*, 3*m*
270			5*v*, 6*v* 4*m*, 5*m*, 6*m*
600		5*t*, 6*t*	
700		1*t*, 2*t*, 3*t*, 4*t*	
800			5*t*, 6*t*
850			1*t*, 2*t*, 3*t*, 4*t*

***v*: represents the mouse specimen inoculated with NK-V venom**

***t*: represents the mouse specimen inoculated with NK-T venom**

***m*: represents the mouse specimen inoculated with NK-M venom**

5.4 Discussion

5.4.1 Neuromuscular Depressant Effect of *Naja kaouthia* Venoms in CBCNM

The results obtained showed the inhibition of the indirect nerve-evoked muscle twitches by *N. kaouthia* venoms is dependent on the duration of venom exposure and the concentration of venom used. Nevertheless, the observation in this study also showed that the indirect twitch blockade induced by *N. kaouthia* venoms could not be reversed even after three consecutive washing with physiological salt solution. This finding is in agreement with the previous studies showing that the binding of the nicotinic acetylcholine receptors (nAChRs) by cobra venom alpha-neurotoxins is likely to be irreversible/pseudo-reversible (Barber et al., 2013). Besides, it was also consistent with the previous studies that showed *N. naja kaouthia* venom (unspecified source of locality) at concentrations of 1-10 µg/ml caused neuromuscular blockade in avian or rodent neuromuscular preparations, accompanied by skeletal muscle damages (Harvey et al., 1994; Reali et al., 2003).

The comparison of t_{90} values evidently showed that the NK-T venom required the lowest dose to achieve the fastest blockade. The differences in the *in vitro* neurotoxic potency of the three *N. kaouthia* venoms from different geographical regions are supported by the differences in the venom content of neurotoxins (particularly the content of alpha-neurotoxins), as discussed in **Chapter 4** in the present study. The proteomic study of NK-T venom showed that the main bulk of its neurotoxin (~50%) (Figure 4.2) was composed mainly of long neurotoxin (LNTX, ~33%) and substantial amount of short neurotoxin (SNTX, ~8%), which is also consistent with the recently published data (Laustsen et al., 2015). On the other hand, both the NK-M and NK-V venoms had a lower content of neurotoxin (NK-M, ~18%; NK-V, ~30%), and hence the substantially lower neurotoxicity observed when compared to that of NK-T venom.

Interestingly, despite the lack of LNTX, NK-V venom produced a faster blockade effect than NK-M venom at the same dose. It is presumably due to its higher content of SNTX (~9%) in NK-V as compared to NK-M (~4%) (**Chapter 4**). Besides, it was observed that at higher venom concentrations (3 and 5 µg/ml) of *N. kaouthia* venoms, the rapid neuromuscular blockade was accompanied by muscle contracture effect (increased in baseline tension) which reflected the action of cytotoxin (cardiotoxin) in the venoms. It has been suggested that this phenomenon was due to the release of calcium ions from the sarcoplasmic reticulum of skeletal muscle, as a result of the cytolytic action of snake venom (Fletcher et al., 1991).

In all the three *N. kaouthia* venoms, venom incubation with tissues at 3 µg/ml concentration caused a significant reduction (40-50% lower than control) of the tissue contractile response to KCl following the complete blockade of indirect twitches. However, the contractile response to KCl was unaffected at 1 µg/ml venom concentration, indicating that the direct tissue damage was not extensive at low venom dose. Interestingly, the tissue response to KCl following blockade at the highest venom concentration (5 µg/ml) was varied when compared with 3 µg/ml venom concentration. At the 5 µg/ml concentration, the KCl response was attenuated further in the NK-M preparation, slightly improved in the NK-V preparation, but remained about 100% in the case of NK-T. The 100% KCl response retained in NK-T was presumably due to the abolishment of twitches occurred too rapidly, leaving insufficient time for the venom to cause significant tissue damage at the time of KCl addition. However, in a separate experiment, a prolonged exposure to 5 µg/ml venom that ended at 180 min of the experimental time demonstrated a significant reduction (> 50%) of tissue response to KCl in all venoms, indicating that the muscle damage caused by *N. kaouthia* venom in the CBCNM preparation is both concentration- and time-dependent.

5.4.2 Myotoxic Effect of *Naja kaouthia* Venoms in CBCNM

In support of the above speculation that *N. kaouthia* venoms could cause muscle damage (**Section 5.4.1**), the direct muscle-evoked twitches of CBC (where electrical stimulation was applied at the muscle's belly) was significantly reduced by all *N. kaouthia* venoms (5 µg/ml) at the end of 180 min (60-80% reduction compared to control). This confirms that the cobra venom was capable of inducing myotoxicity, and the findings are in agreement with the previous *in vitro* studies using rodent and chick preparations (Harvey et al., 1994; Stringer et al., 1971). The abundant cytotoxin/cardiotoxin (CTX) in *N. kaouthia* venoms (~28-45%) (as shown in **Chapter 4** and another study (Laustsen et al., 2015)) and other *Naja* species (~40-70%) (Huang et al., 2015; Petras et al., 2011) apparently represents the principal family of myotoxic toxins that cause severe necrotic effect (dermonecrosis, myonecrosis) during an envenomation (Reid, 1964; Wongtongkam et al., 2005). In this study, tissues treated with NK-M venom showed the most significant reduction in muscle-evoked contraction as well as the tissue contractile response to KCl. This is presumably because NK-M venom has the highest CTX content (~46%), almost twice their abundance in NK-T venom as shown in the proteomic study (**Chapter 4**).

Other than CTX, it has been suggested that the snake venom phospholipase A_2 (PLA_2) is also associated with the cytotoxic and necrotic effects of venom (Condrea et al., 1981; Kini & Evans, 1989). Earlier, a study suggested that the phospholipase A_2 (PLA_2) isolated from the Indian *N. kaouthia* venom exhibited cell membrane-specific cytotoxic action (Mukherjee, 2007), while some other studies reported that the PLA_2 can act synergistically with CTX in enhancing myonecrosis (Fletcher & Lizzo, 1987; Gasanov et al., 1997; Gasanov et al., 2014; Harvey et al., 1983; Rakhimov et al., 1981). The same phenomenon was also reported in the weak neurotoxin (WNTX) isolated from Indian *N. kaouthia*, where synergism was observed between PLA_2 and WNTX to

display cell-specific cytotoxicity (Mukherjee, 2008, 2010). The role of the several acidic PLA_2s from the Southeast Asian *N. kaouthia* venoms (NK-T, NK-M and NK-V) in the induction of cell death, however, has not been thoroughly investigated.

5.4.3 *In vitro* Neutralization of *N. kaouthia* Venoms by Antivenoms in CBCNM

5.4.3.1 *N. kaouthia* Monovalent Antivenom (NKMAV) Pre-incubation prior to Venom Challenge (T_{-10})

Prior addition (T_{-10}) of NKMAV to the CBC successfully prevented the neuromuscular blockade induced by all the three *N. kaouthia* venoms albeit with varying degree of effectiveness (as indicated by the different ED_{100} values). The findings suggest that the venoms with rather diverse neurotoxin profiles responded variably to the antivenom that was raised from a single geographical source. During the pre-incubation, circulating antivenom bound to the free venom toxins that were added in later, thus protecting the neuromuscular junction from the binding of neurotoxin and the muscle from cytotoxic damage. The findings highlight the importance of early administration of adequate antivenom to stop the progress of pathogenesis.

This *in vitro* finding correlates well with the neutralization of the lethal effect of these venoms by NKMAV in mice (**Chapter 4**), supporting that the neutralization of neuromuscular depressant effect is the key to prevent death. The variable effectiveness of NKMAV on the three venoms may be attributed to the differences in neurotoxin composition of the venoms. Both NK-M and NK-V venoms have a lower content of the lethal LNTX and SNTX, hence requiring a lower amount of NKMAV for neutralization to sustain the muscle contraction. Therefore, the alpha-neurotoxin is the key target toxin in order for the antivenom to achieve effective neutralization of lethality.

5.4.3.2 *In vitro* Venom Challenge followed by *N. kaouthia* Monovalent Antivenom (NKMAV) Rescue Treatment at Different Time Points (t_{10}, t_{50} and t_{90})

In most developing or under-developed nations, considerable delay in obtaining the antivenom treatment is common, as many victims seek treatment from traditional healers at first (Bénard-Valle et al., 2015). In view of circumstances where antivenom is likely to be administrated at various stages of severity of envenomation, the time-sensitive reversibility of neurotoxicity by antivenom was investigated in this study, where NKMAV was added at different time points following the onset of the neuromuscular depression. This *in vitro* rescue study revealed the consequence of tissue exposed directly to venom and showed the relative efficacy of the antivenom in protecting the nerve-muscle from the neurotoxic effect.

The result generally showed that antivenom was unable to restore the muscle contraction response to the pre-venom level once the neuromuscular blockade had taken place. This indicates that the antibody ($F(ab)_2$) did not induce the displacement of neurotoxins bound to the post-synaptic nicotinic receptors in the *in vitro* setting. However, the antivenom was able to halt further neuromuscular depression induced by all three *N. kaouthia* venoms. Presumably, the antivenom added after the venom addition managed to bind to the yet unbound venom antigen (including neurotoxins) presents in the tissue bath, forming immunocomplexes that hinder further binding of toxins to the remaining nicotinic receptors on the CBCNM preparation. It is clearly shown that the percentage of muscle contraction retained was dependent on the time of antivenom "rescue" took place. This indicates that the earlier the antivenom "rescue", the more neuromuscular contractile function was preserved. The clinical implication is obvious as the results confirm that the antivenom therapy should commence as early as possible, although in real situation, the antivenom is only indicated upon the

development of the very first sign of neurotoxicity (typically ptosis), since not all snakebites result in substantial toxin absorption and systemic toxicity.

On the other hand, the tissue responses to exogenous agonists (ACh, CCh and KCl) varied with the time of antivenom "rescue". In general, the magnitude of responses to these agonists was near proportional to the percentage of muscle twitches preserved. This condition implies the relative sparing of post-synaptic nicotinic receptors (responses to ACh and CCh) and muscle cell viability (response to KCl) when venom toxins were sequestered by NKMAV in the tissue preparation.

5.4.4 *In vivo* Neurotoxic Activity Study in Mice

In human envenomed by *N. kaouthia*, neurological signs e.g. ptosis, dysarthria, dysphagia, ophthalmoplegia can begin within 3-4 hours or even earlier following the bite (Reid, 1964; Viravan et al., 1986). Mimicking human envenomation and treatment, the *in vivo* neurotoxicity scoring was carried out in this study using mice envenomed experimentally by the three *N. kaouthia* venoms to gauge the syndrome evolution pattern. The early neurotoxic sign was used to indicate antivenom treatment for "rescue" in the later study. The development of posterior limb paralysis, followed by dyspnea, full flaccid paralysis and death in mice indicated compatible syndrome progression of systemic cobra envenomation in human patients (Wongtongkam et al., 2005). Although NK-T venom appeared to be the most potent in inhibiting CBC's indirect twitches among the three geographical samples, the NK-V venom surprisingly induced a more rapid neurological manifestation in the mice subcutaneously envenomed. Probably, at the high dose of venom used (5x LD_{50}), the role of other neurotoxin subtypes (such as atypical weak toxins that are abundant in the NK-V venom) became remarkable as they interacted synergistically with the alpha-neurotoxin in the venom. On the other hand, the onset of neurological signs was the slowest in mice injected with NK-M venom, in

agreement with the proteomic findings that showed a lower content of neurotoxin in the venom (**Chapter 4**).

5.4.5 *In vivo* Challenge-rescue Study in Mice

Despite the variable effectiveness of NKMAV in the *in vitro* CBC studies, the in *vivo* challenge-rescue experiment demonstrated that NKMAV was indeed able to reverse the neurotoxicity that had manifested in all mice inoculated with *N. kaouthia* venoms (NK-M, NK-T and NK-V). The findings imply that the reversibility of venom toxicity is not direct, but likely involves complex *in vivo* mechanisms. Although the antivenom was unable to displace the toxins bound to receptors *in vitro*, it has been postulated that the *in vivo* reversibility is achieved through the hastened elimination of bound-circulating toxins (immunocomplexes in the blood) that produces an inter-compartmental equilibrium shift between the vascular compartment and the tissue compartment. The equilibrium shift promotes tissue-to-blood transfer of toxins in a continuous manner where the toxins will be further sequestered by the circulating antivenom (Gutierrez et al., 2003). However, it is important to note that mismatch of venom and antivenom pharmacokinetics (Boyer et al., 2015; Boyer et al., 2001; Seifert & Boyer, 2001) can result in the recurrence of the toxic manifestations (Isbister, 2010). In this *in vivo* assay, the mice were treated with an optimal dose of NKMAV presumably adequate to sustain the circulating antibody concentration beyond the absorption and re-distribution of neurotoxins into the blood. In human envenomation, the pharmacokinetic profiles of the venom and antivenom are likely much more complex than that in mice. It therefore requires close monitoring of post-antivenom treatment for "rebound phenomenon" to guide the need for repeated antivenom doses.

It was noted that the full recovery of neurotoxicity in mice envenomed with NK-T venom took far longer than envenomation by NK-M or NK-V when treated with the same optimal dose of NKMAV. This is likely due to the remarkably high abundance of neurotoxin in the NK-T venom, resulting in a slower diminishing of residual weakness in the mice. Clinically, the antivenom dose required for complete recovery from NK-T envenomation may be higher than that for NK-M and NK-V envenomation.This is also indicated by the higher antivenom dose (4x potency) required to sustain the CBC twitches in the *in vitro* antivenom pre-incubation, as well as *in vivo* challenge-rescue antivenom protection study.

5.5 Conclusion

This study unveiled the variable neurotoxic mechanisms and potencies of the venoms of *N. kaouthia* from Malaysia, Thailand and Vietnam. The variations can be correlated with the neurotoxin profiles of the three venoms as shown in **Chapter 4**. Besides, all three *N. kaouthia* venoms exhibited myotoxic activity as expected from the high content of cytotoxin in the venoms. The neutralization effectiveness of the Thai *N. kaouthia* Monovalent Antivenom (NKMAV) varied accordingly against the three venom samples. Early intervention with antivenom at optimum dose is of utmost importance as the delayed antivenom administration resulted in limited neutralizing effectiveness against the neurotoxic effect of the venoms. From the clinical standpoint, the envenomation by NK-T and NK-V is of high concern for its remarkably stronger paralyzing effect (NK-T) and the faster onset of neurotoxicity (NK-V). Prompt antivenom administration and meticulous post-treatment monitoring are among the various strategies, to further optimize the use of antivenom for *N. kaouthia* envenomation in the region.

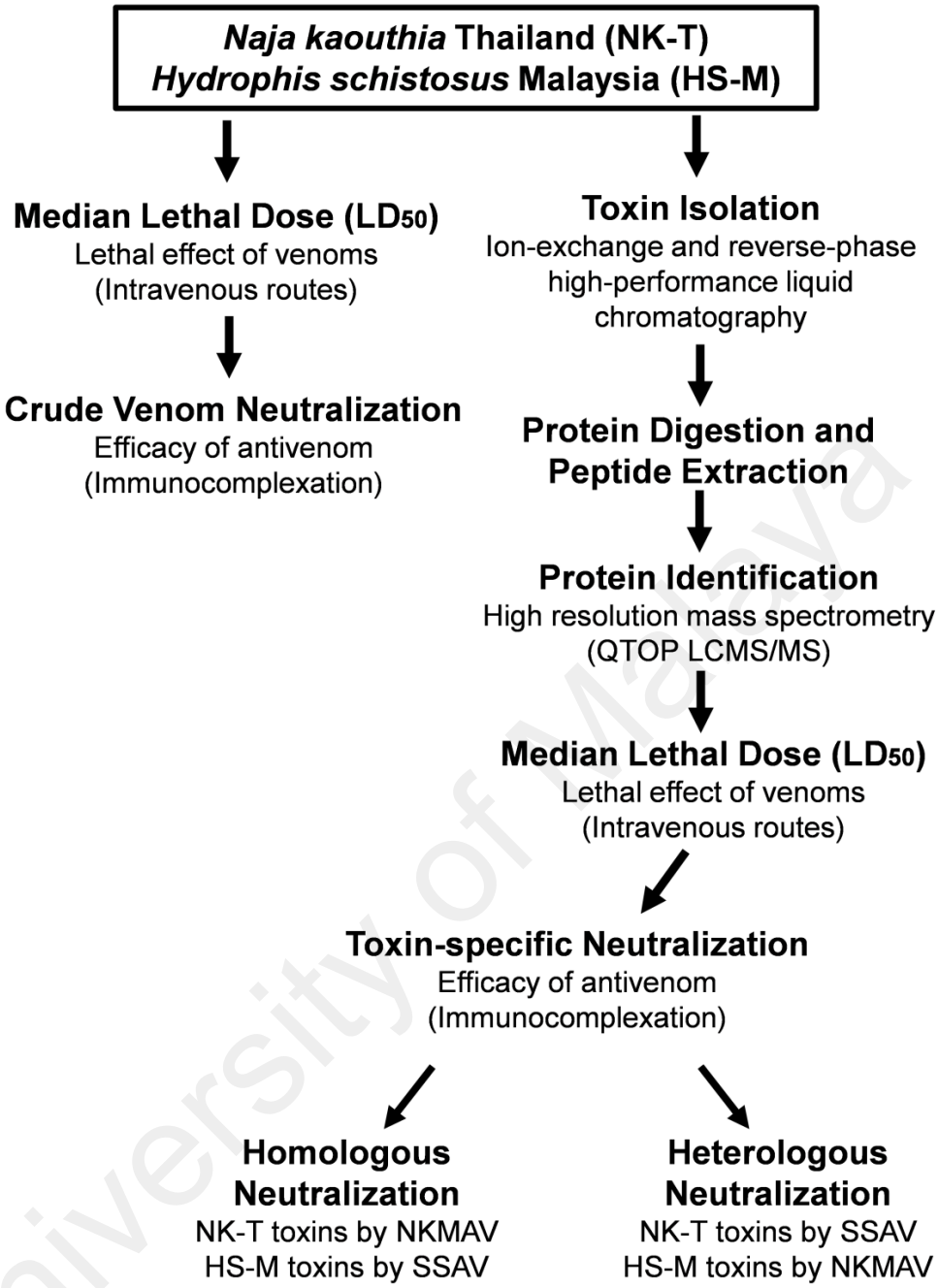
CHAPTER 6: PRINCIPAL TOXINS ISOLATED FROM *Naja kaouthia* VENOM AND THEIR SPECIFIC NEUTRALIZATION

6.1 Introduction

The appropriate use of good quality antivenom can effectively reduce the mortality and morbidity associated with snakebite envenomation (WHO, 2010c). However, very often, commercial antivenoms are of low potency and, therefore, a large amount must be used in treating severe envenomation. Because of this, immunotherapy of snakebite envenomation can be expensive because of the high cost of antivenom. Also, the administration of a large amount of antivenom (foreign proteins) poses a greater risk of hypersensitive reaction that can be fatal (Malasit et al., 1986). Therefore, there is an urgent need to improve the efficacy of snake antivenom. Many factors are involved in determining the efficacy of an antivenom. One limiting factor is the capability of an antivenom to neutralize various principal toxins of a venom. In order to improve the efficacy of an antivenom, it is necessary to elucidate the neutralization profile of the antivenoms against various principal toxins of the respective venom it reacting with, and this will shed light to overcoming various limitations.

In **Chapter 4**, it has been shown that the neutralization potency values (P) of commercial antivenoms against *N. kaouthia* venom were generally low (< 2 mg/ml, milligram of venom neutralized by per millilitre of antivenom), consistent with several other studies which reported similar findings tested on other elapids such as *N. sumatrana* (Leong et al., 2015; Leong et al., 2012) and *Hydrophis schistosus* (Tan et al., 2015b). On the other hand, the P values for viperid antivenoms were generally much higher (up to 10 mg/ml). It has been suggested that the low efficacy of elapid antivenom is due to the presence of a large amount of low molecular mass toxins in the elapid venoms. These low molecular mass toxins are generally less immunogenic and are

poorly neutralized by antivenom (Leong et al., 2015). In this study, the effectiveness of neutralization of the principal toxins isolated from the monocled cobra (*N. kaouthia*, Thailand) and the beaked sea snake (*H. schistosus*, Malaysia) was investigated using two commercial antivenoms: *N. kaouthia* Monovalent Antivenom (NKMAV) and CSL Sea Snake Antivenom (SSAV). The beaked sea snake venom was included in the study for comparative purpose as cross-neutralization of its venom by cobra antivenom had been reported earlier (Khow et al., 2001; Minton, 1967), presumably due to the cross-neutralization of its alpha-neurotoxins that are immunologically similar to the principal toxins of *N. kaouthia* venom. It is hoped that the current toxin-specific neutralization study could reveal the limitation of antivenom neutralization against the elapid toxins and hence contribute to the formulation and production of antivenom with greater neutralization potency. The experimental design of this study was summarized and shown in the following flow chart (next page):



NKMAV : Thai *Naja kaouthia* monovalent antivenom
SSAV : Australian CSL sea snake antivenom

6.2 Methods

6.2.1 Protein Concentration Determination

The protein concentration of antivenoms (NKMAV and SSAV) and purified toxins were determined according to the protocol described in **Section 3.2.1** and were expressed as means ± SEM of triplicates.

6.2.2 Isolation and Purification of Major Toxins

6.2.2.1 *Naja kaouthia* Venom

The isolation and purification of the major toxins from Thai *N. kaouthia* venom (NK-T) were conducted as described previously (Leong et al., 2015) through sequential chromatographic fractionations using high-performance ion-exchange chromatography, followed by reverse-phase HPLC (RP-HPLC) on a Shimadzu LC-20AD High-Performance Liquid Chromatography system (Japan). The ion-exchange chromatography was carried out using Resource® S cation-exchange column according to the protocol described in the **Section 3.2.2.2**. The protein fractions from cation-exchange were subjected to RP-HPLC for further purification using LiChroCART® 250-4 LiChrospher® WP 300 RP-18, according to the protocol described in **Section 3.2.2.1**.

6.2.2.2 *Hydrophis schistosus* Venom

The isolation of toxins from Malaysian *H. schistosus* (HS-M) venom was carried out using RP-HPLC, according to Tan et al. (Tan et al., 2015b) **(Section 3.2.2.1)**.

6.2.3 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Ten micrograms of purified toxins from the venoms of Thai *N. kaouthia* (NK-T) and Malaysian *H. schistosus* (HS-M) were subjected to 15% SDS-PAGE (Laemmli, 1970) according to **Section 3.2.3**.

6.2.4 In-solution Tryptic Digestion of Purified Toxins

Ten micrograms of purified toxins from the venoms of Thai *N. kaouthia* (NK-T) and Malaysian *H. schistosus* (HS-M) were subjected to "in-solution" trypsin protease digestion as according to the protocol described in **Section 3.2.4.1**. The tryptic digested peptides were then extracted and desalted as described in **Section 3.2.5**.

6.2.5 Protein Identification by Liquid Chromatography-Tandem Mass Spectrometry

The tryptic digested samples were subjected to Agilent 6550 Accurate-Mass Q-TOF LC/MS system (nano-electrospray ionization) as described in **Section 3.2.6.3**.

6.2.6 Estimation of the Relative Abundance of Purified Toxins

The relative abundance of individual protein fraction from ion-exchange or reverse-phase HPLC was estimated as according to the method described in **Section 3.2.7**.

6.2.7 Determination of the Median Lethal Dose (LD_{50})

The median lethal doses (LD_{50}) of purified toxins from the venoms of Thai *N. kaouthia* (NK-T) and Malaysian *H. schistosus* (HS-M) were determined by intravenous (*i.v.*) route as described in **Section 3.2.8**. The median lethal dose (LD_{50}) was calculated using Probit analysis (Finney, 1952) as described in **Section 3.2.10.1**.

6.2.8 Determination of Toxicity Score of the Purified Toxins

The Toxicity Score (TS) was calculated according to Laustsen et al. (Laustsen et al., 2015). It serves as an indicator of the significance of a toxin in contributing to the venom lethality, and is defined as the ratio of protein abundance of a toxin (%) in venom divided by its median lethal dose (LD_{50}) using the equation below:

$$\text{Toxicity Score, TS} = \frac{\text{Protein abundance of a toxin in venom (\%)}}{\text{Median lethal dose } (LD_{50})}$$

6.2.9 Antivenom Neutralization of Venom and Purified Toxins by *In vitro* Immunocomplexation

In vitro immunocomplexation of the venom (NK-T) and the purified toxins from NK-T or *H. schistosus* (HS-M) by *N. kaouthia* Monovalent Antivenom (NKMAV) and CSL Sea Snake Antivenom (SSAV) was conducted according to **Section 3.2.9.1**. The median effective dose (ED_{50}), median effective ratio (ER_{50}), neutralizing potency (P) and normalized Potency (n-P) were determined using Probit analysis (Finney, 1952) described in **Section 3.2.10.1**.

6.3 Results

6.3.1 Isolation of Major Toxins from the Venom of *N. kaouthia*

The venom of Thai *N. kaouthia* (NK-T) was fractionated by Resource® S cation-exchange chromatography into 8 peaks as shown the in Figure 6.1(a). Among these, 5 peaks constituted 85% of the total peak area of the chromatogram. These 5 major peaks were assigned as fractions F1, F2 (containing two peaks), F3 and F4 as indicated in Figure 6.1(a). Fractions F1-F4 were further fractionated individually by RP-HPLC (Figures 6.1(b)-(e)). Fraction F1 contained acidic proteins unbound by the cation-exchange column. It yielded only one major peak (F1a) in RP-HPLC. Fraction F2, the most abundant fraction comprising > 35% of the total peak area of Resource® S chromatogram, was separated by RP-HPLC into 2 main fractions (F2a and F2b) eluted at 55 min and 75 min, respectively. On cation-exchange HPLC, more basic proteins were collected in fraction F3 and F4. RP-HPLC also successfully yielded the purified F3a and F4a, from F3 and F4, respectively.

The purity of the purified proteins F1a to F4a was verified by reducing SDS-PAGE, all yielded a single homogenous band (7-14 kDa), indicating the proteins were homogeneous (Figure 6.1(f)). These purified proteins were then identified using Q-TOF LCMS/MS. All proteins obtained were homologous to the proteins previously identified from the cobra genus *Naja* (Table 6.1). From the results, F1a was identified as an acidic phospholipase A_2 (PLA_2); F2a is a short neurotoxin (SNTX), whereas F2b is a long neurotoxin (LNTX). On the other hand, F3a and F4a are two different cytotoxin (CTX) homologs, as indicated by the differences in their elution times as well as the differences in peptide sequences.

In total, these five purified toxins were estimated to account for about 75% of the total abundance of venom proteins (Table 6.1). The acidic PLA_2 (F1a) was termed NK-T PLA_2 and constitutes ~17.0% of the total protein abundances. The two alpha-neurotoxins, SNTX (F2a) and LNTX (F2b) were named NK-T SNTX and NK-T LNTX respectively, each accounts for 4.6% and 30.9% of the total abundance. The CTX homologs were labeled as NK-T CTX-I (F3a) and NK-T CTX-II (F4a), and their relative abundance was 19.3% and 4.6% of total venom protein, respectively (Table 6.1). The protein abundances, intravenous median lethal doses (*i.v.* LD_{50}) and Toxicity Scores of these purified toxins were shown in Table 6.3.

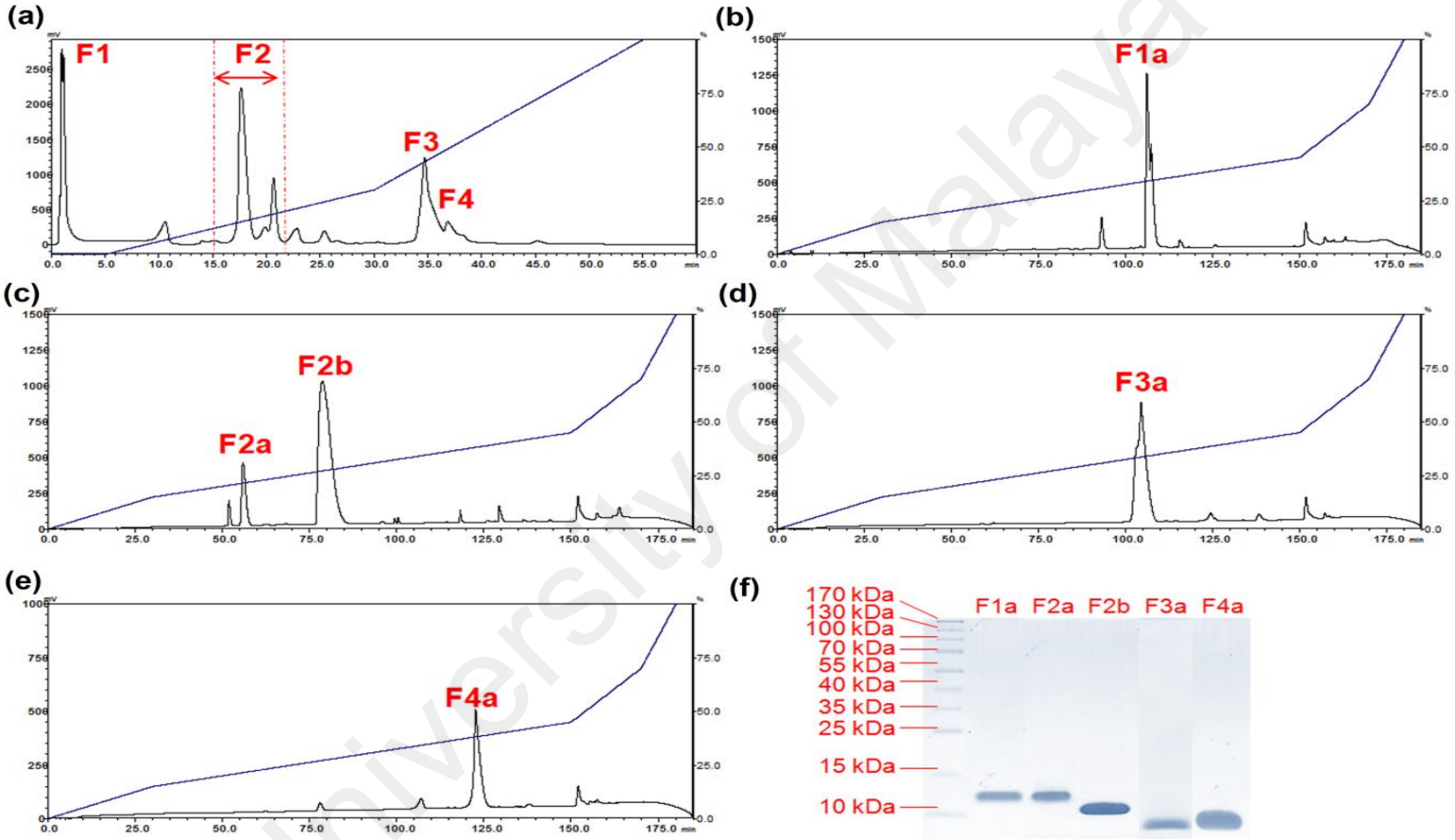


Figure 6.1 Purification of major toxins from the venom of Thai *N. kaouthia* (NK-T) through sequential fractionations using ion-exchange chromatography followed by reverse-phase RP-HPLC. (a) Resource® S cation-exchange HPLC of 5 mg Thai *N. kaouthia* (NK-T) venom. Concentrated venom fractions were subjected to C_{18} RP-HPLC for further purification: (b) Fraction F1; (c) Fraction F2; (d) Fraction F3; (e) Fraction F4; (f) Reducing SDS-PAGE of the purified venom toxins.

Table 6.1: Protein identification of the toxins purified from Thai *N. kaouthia* (NK-T) venom by nano-ESI-LCMS/MS and their respective protein abundances.

Protein fraction	%	Protein ID	MS/MS derived sequence	Matched peptide	Matched MH+	MH+ error (ppm)	Accession no. (Species)	Protein score
F1a	17.0	Acidic PLA_2 1	CCQVHDNCYNEAEK	1	1828.70	-0.3	P00596 (*N. kaouthia*)	187
			CCQVHDNCYNEAEK	1	1827.69	-2.1		
			CWPYFK	1	901.42	0.9		
			CWPYFKTYSYECSQGTLTCK	1	2581.12	-1.0		
			CWPYFKTYSYECSQGTLTCK	1	2580.11	0.0		
			GDNDACAAAVCDCDR	1	1670.61	0.5		
			GDNDACAAAVCDCDR	1	1671.62	1.0		
			LAAICFAGAPYNNNNYNIDLK	3	2357.14	-1.3		
			LAAICFAGAPYNNNNYNIDLK	1	2358.15	-1.4		
			LAAICFAGAPYNNNNYNIDLK	4	2358.15	0.4		
			LAAICFAGAPYNNNNYNIDLK	1	2358.15	-0.6		
			LAAICFAGAPYNNNNYNIDLK	1	2359.16	1.0		
			LAAICFAGAPYNNNNYNIDLKAR	1	2585.29	-0.2		
			NMIQCTVPNR	1	1249.59	-0.8		
			NMIQCTVPNR	1	1234.61	5.4		
			SWWDFADYGCYCGR	1	1843.71	0.6		
			SWWDFADYGCYCGR	2	1844.72	0.5		
			SWWDFADYGCYCGR	1	1843.71	-0.6		
			SWWDFADYGCYCGR	1	1843.71	-0.4		
			TYSYECSQGTLTCK	1	1698.72	0.6		
			TYSYECSQGTLTCK	1	1699.73	2.2		
			TYSYECSQGTLTCK	1	1698.72	0.1		
F2a	4.6	Cobrotoxin-c	LECHNQQSSQAPTTK	1	1730.81	1.1	P59276 (*N. kaouthia*)	64
			LECHNQQSSQAPTTKTCSGETNCYK	1	2932.27	-1.9		
			LECHNQQSSQAPTTKTCSGETNCYK	1	2931.26	-2.1		
			VKPGVNLNCCR	1	1317.66	0.6		

Table 6.1, continued.

Protein fraction	%	Protein ID	MS/MS derived sequence	Matched peptide	Matched MH+	MH+ error (ppm)	Accession no. (Species)	Protein score
F2b	30.9	Alpha-elapitoxin-Nk2a	CFITPDITSK	5	1182.60	1.6	P01391 (*N. kaouthia*)	101
			RVDLGCAATCPTVK	1	1549.81	15.3		
			TGVDIQCCSTDNCNPFPTR	1	2242.94	-0.2		
			TGVDIQCCSTDNCNPFPTR	1	2243.95	2.1		
			TGVDIQCCSTDNCNPFPTR	1	2244.96	2.7		
			TGVDIQCCSTDNCNPFPTRK	1	2372.04	0.9		
			TGVDIQCCSTDNCNPFPTRK	1	2371.03	-2.0		
			TWCDAFCSIR	2	1316.57	1.5		
			TWCDAFCSIR	1	1317.57	2.7		
			TWCDAFCSIR	1	1316.56	1.0		
			VDLGCAATCPTVK	1	1393.68	2.2		
F3a1	19.2	Cytotoxin 2	GCIDVCPKNSLLVK	1	1603.84	0.1	P01445 (*N. kaouthia*)	82
			LIPLAYK	1	818.53	0.6		
			LIPLAYK	5	818.53	0.9		
			LIPLAYK	1	818.53	3.8		
			LIPLAYKTCPAGK	1	1433.82	3.5		
			NSLLVKYVCCNTDR	1	1742.85	1.7		
			YVCCNTDR	1	1088.44	0.4		
F4a	4.6	Cytotoxin	CNKLVPLFYKTCPAGK	1	1897.99	-7.6	Q02454 (*N. sputatrix*)	146
			MFMVATPKVPVK	1	1349.76	-4.5		
			LKCNKLVPLFYK	1	1523.88	-3.0		
			SSLLVKYVCCNTDR	1	1715.83	-2.2		
			YVCCNTDR	1	1088.44	-0.8		
			GCIDVCPKSSLLVK	1	1576.83	-0.5		
			NLCYKMFMVATPK	1	1603.79	0.4		
			SSLLVKYVCCNTDR	1	1716.84	0.6		
			LVPLFYKTCPAGK	1	1495.83	0.8		
			MFMVATPK	2	925.48	1.7		
			LVPLFYK	6	880.54	2.6		

6.3.2 Isolation of Major Toxins from the Venom of *H. schistosus*

The RP-HPLC profile of *H. schistosus* venom (HS-M) obtained here is very similar to the RP-HPLC profile published earlier by Tan et al. (Tan et al., 2015b). The three toxins of interest were isolated and the purified proteins obtained were termed H1, H2 and H3 (Figure 6.2). The purity of these fractions was verified by reducing SDS-PAGE and all three fractions showed a single homogenous band (7-14 kDa). Fraction H1 is a short neurotoxin as demonstrated by Q-TOF LCMS/MS. It is the most abundant protein of *H. schistosus* venom (52.5% of total venom protein) and was termed HS-M SNTX. Fraction H2 is shown to be a long neurotoxin (LNTX, 11.9% of total venom protein) and termed as HS-M LNTX, while fraction H3 is a basic PLA_2 (19.2% of total protein) and termed as HS-M basic PLA_2 (Table 6.3). The relative protein abundances, intravenous median lethal doses (*i.v.* LD_{50}) and Toxicity Scores of these purified toxins are shown in Table 6.3.

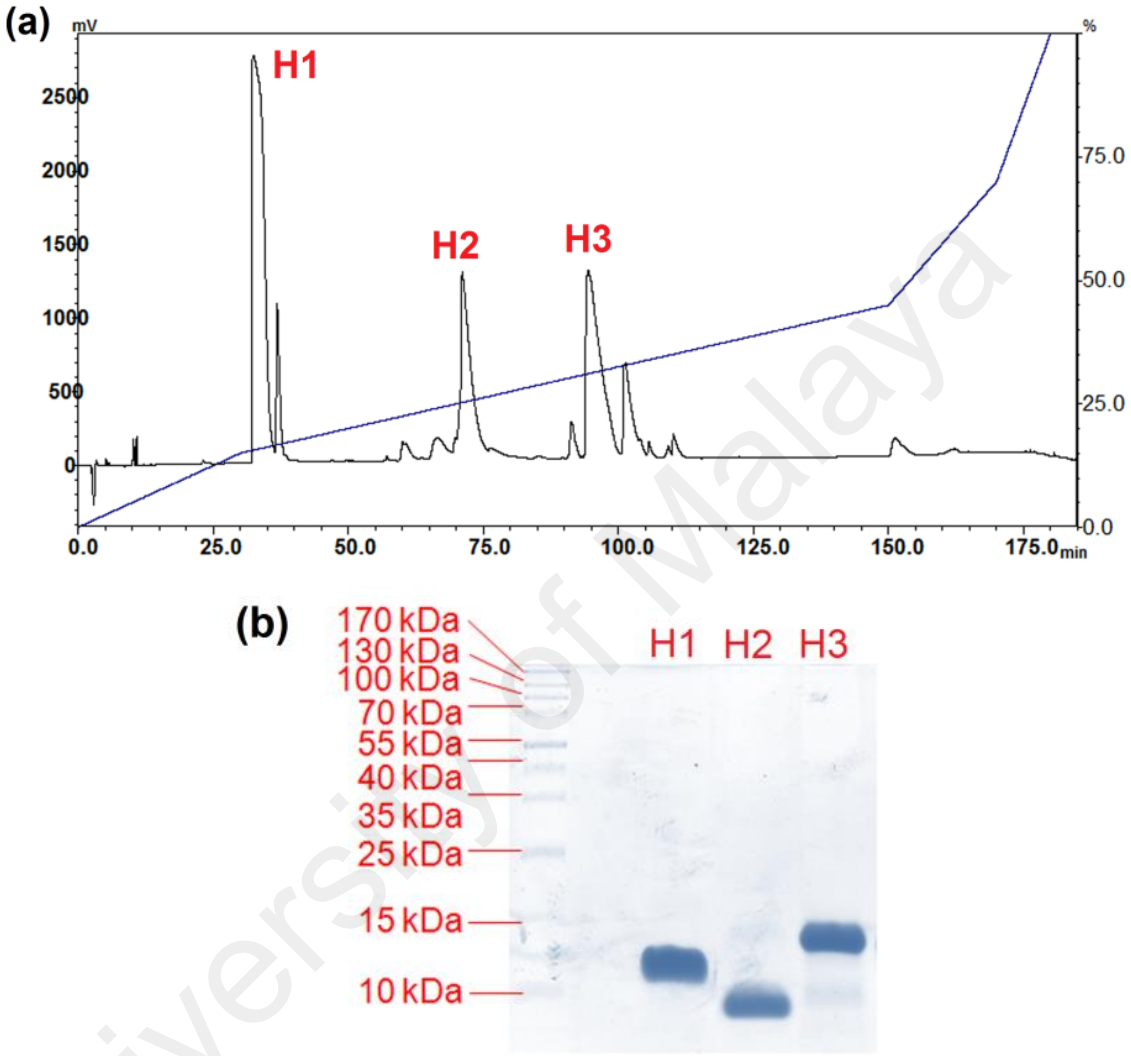


Figure 6.2 Fractionation of *H. schistosus* venom (HS-M) using C_{18} reverse-phase high-performance liquid chromatography (RP-HPLC). (a) RP-HPLC of 3 mg *H. schistosus* venom; (b) Reducing SDS-PAGE of the purified venom toxins.

6.3.3 Protein Concentration of Antivenoms and the Neutralization of Thai *N. kaouthia* Venom

The protein content of the SSAV is approximately 5 times higher than that of NKMAV (Table 6.2). By volume (of antivenom), the neutralization potencies (P) of NKMAV and SSAV against *N. kaouthia* venom appear to be comparable. However, a comparison of potency based on antivenom protein content (the normalized P, n-P) revealed that NKMAV is more effective than SSAV in neutralizing the NK-T venom (Table 6.2). The normalized P (n-P) is expressed as the amount of venom (mg) completely neutralized per unit amount of antivenom protein (g).

6.3.4 Median Lethal Dose (LD_{50}) of Purified Toxins and its Toxicity Score

The alpha-neurotoxins, NK-T LNTX and NK-T SNTX, isolated from NK-T venom are highly lethal with LD_{50} values of 0.09 and 0.12 µg/g, respectively (Table 6.3). On the other hand, the cytotoxins (NK-T CTX-I, NK-T CTX-II) possess much higher LD_{50} values of 1.41 and 1.75 µg/g, respectively. In contrast to the neurotoxins and cytotoxins, the NK-T acidic PLA_2 was non-lethal to mice up to 5 µg/g, a dose that is ~25 times the LD_{50} of the crude NK-T venom (0.18 µg/g).

Toxicity Score (TS) was previously proposed by Laustsen et al. (Laustsen et al., 2015) as an indicator of the relative contribution of a toxin to venom lethality. In general, most of the toxins purified from NK-T and HS-M venoms have TS that exceeded the value of 5, a threshold value proposed for significant toxicity (Laustsen et al., 2015). However, the TS values for the NK-T acidic PLA_2 (far below 5) and NK-T CTX-II (TS = 3) were exceptionally low. This is mainly due to their high LD_{50} value and low abundance in the venom (NK-T CTX-II) (Table 6.3).

Table 6.2: Protein concentrations of *N. kaouthia* Monovalent Antivenom (NKMAV) and CSL Sea Snake Antivenom (SSAV) and neutralization of *N. kaouthia* venom (NK-T) by the antivenoms.

Antivenom	Protein concentration (mg/ml)	Neutralization of *Naja kaouthia* venom (NK-T)[a, b]			
		ED_{50} (µl)[c]	ER_{50} (mg/ml)[d]	P (mg/ml)[e]	Normalized P (mg/g)[f]
NKMAV	45.0[@] ± 0.6	18.75[#]	1.15[#] (0.77-1.73)	0.92[#]	20.44
SSAV	217.2 ± 3.0	11.24	2.00 (1.33-3.00)	1.60	7.37

Results are presented as mean of value ± S.E.M

[a] Intravenous (*i.v.*) median lethal dose (LD_{50}) of *N. kaouthia* (NK-T) = 0.18 µg/g (Chapter 4, Table 4.3)

[b] Challenge dose used in the neutralization was 5x *i.v.* LD_{50} of the *N. kaouthia* (NK-T) venom and proven to be above 100% lethal dose (LD_{100})

[c] ED_{50} : median effective dose, the antivenom dose (µl) at which 50% of mice survived

[d] ER_{50} : median effective ratio, the ratio of the amount of venom (mg) to the volume dose of antivenom (ml) at which 50% of mice survived

[e] Potency, P : neutralization potency of the antivenom (mg/ml), the amount of venom (mg) completely neutralized by one ml antivenom (ml)

[f] Normalized P, n-P : neutralization potency of the antivenom (mg/g), the amount of venom (mg) completely neutralized per unit amount of antivenom protein (g)

[@] Reference value from Chapter 4, Table 4.4

[#] Reference value from Chapter 4, Table 4.5

Table 6.3: Intravenous median lethal doses (*i.v.* LD_{50}) of toxins purified from Thai *N. kaouthia* (NK-T) and Malaysian *H. schistosus* (HS-M) venoms and Toxicity Score (TS) for toxins.

Venom/toxin	Toxin abundance in venom (%)	*i.v.* LD_{50} (µg/g)[a]	TS (g/µg)[b]
***Naja kaouthia* (Thailand)**		0.18 (0.12–0.27)[#]	
F1a (NK-T acidic PLA_2)	17.0	> 5.00	< 5
F2a (NK-T SNTX)	4.6	0.12 (0.11-0.14)	38
F2b (NK-T LNTX)	30.9	0.09 (0.06-0.14)	343
F3a (NK-T CTX-I)	19.2	1.41 (1.08-1.85)	14
F4a (NK-T CTX-II)	4.6	1.75 (1.68-1.83)	3
***Hydrophis schistosus* (Malaysia)**		0.07 (0.05–0.09)[@]	
H1 (HS-M SNTX)	52.2*	0.07 (0.05-0.09)*	746
H2 (HS-M LNTX)	11.9*	0.18 (0.16-0.20)*	66
H3 (HS-M basic PLA_2)	19.2*	0.08 (0.06-0.10)*	240

Values of 95% C.I. were in parentheses

[a] LD_{50} : median lethal dose (µg/g)

[b] Toxicity score : the ratio of protein abundance of a toxin (%) divided by its median lethal dose (LD_{50}) (Laustsen et al., 2015).

[#] Reference values from Chapter 4, Table 4.3.

[@] Reference values from Tan et al. (Tan et al., 2015d)

* Reference values from Tan et al. (Tan et al., 2015b)

6.3.5 Neutralization of the Purified Toxins by Antivenoms – *In vitro* Immunocomplexation

The results of toxin neutralization by NKMAV and SSAV are shown in Table 6.4. For comparison, the normalized values of antivenom potency (P) that is expressed as normalized P (n-P) in mg/g unit (the amount of venom (mg) completely neutralized per unit amount of antivenom protein (g)) are also included in the table. The results showed the neutralization potency of NKMAV against short neurotoxins and basic PLA_2 was relatively low. The n-P value of NKMAV against the short neurotoxin from homologous and heterologous venoms was 1.33 mg/g (against NK-T SNTX) and 0.22 mg/g (against HS-M SNTX) respectively; while n-P value was only 0.22 mg/g against the sea snake venom basic PLA_2 (HS-M basic PLA_2). However, the NKMAV neutralized the long neurotoxin isolated from both species (NK-T and HS-M) more effectively; with an n-P value of 4.89 mg/g (NK-T LNTX) and 4.00 mg/g; (HS-M LNTX), respectively.

On the other hand, SSAV was approximately two times more potent in neutralizing the NK-T SNTX (n-P = 2.49 mg/g) as compared to NKMAV (n-P = 1.33 mg/g), but less effective against the NK-T LNTX (SSAV; n-P = 2.49 mg/g, NKMAV; n-P = 4.89 mg/g). As expected, SSAV failed to neutralize the isolated cytotoxins, whereas NKMAV was able to neutralize both NK-T CTX-I and NK-T CTX-II effectively with an n-P value of 6.44 mg/g and 2.89 mg/g, respectively.

Table 6.4: Neutralization of purified toxins by *N. kaouthia* Monovalent Antivenom (NKMAV) and CSL Sea Snake Antivenom (SSAV)

Venom toxin	*i.v.* LD_{50}[a] (µg/g)	NKMAV					SSAV				
		Challenge[b]	ED_{50} (µl)[c]	ER_{50}[d] (mg/ml)	P[e] (mg/ml)	Normalized P (mg/g)[f]	Challenge[b]	ED_{50}[c] (µl)	ER_{50}[d] (mg/ml)	P[e] (mg/ml)	Normalized P (mg/g)[f]
***Naja kaouthia* (Thailand)**											
F2a (NK-T SNTX)	0.12 (0.11-0.14)	2.5x LD_{50}	70.68	0.10 (0.09-0.12)	0.06	1.33	5x LD_{50}	21.37	0.67 (0.62-0.79)	0.54	2.49
F2b (NK-T LNTX)	0.09 (0.06-0.14)	5x LD_{50}	39.14	0.28 (0.18-0.43)	0.22	4.89	5x LD_{50}	16.05	0.67 (0.45-1.05)	0.54	2.49
F3a (NK-T CTX-I)	1.41 (1.08-1.85)	1.5x LD_{50}	53.16	0.875 (0.670-1.148)	0.29	6.44	1.5x LD_{50}	175.00	0.27 (0.20-0.35)	0.09	0.41
F4a (NK-T CTX-II)	1.75 (1.68-1.83)	1.5x LD_{50}	156.57	0.40 (0.39-0.42)	0.13	2.89	1.5x LD_{50}	N.E.[#]	N.E.[#]	N.E.[#]	N.E.[#]
***Hydrophis schistosus* (Malaysia)**											
F1 (HS-M SNTX)	0.07 (0.05-0.09)*	1.5x LD_{50}	128.41	0.02 (0.01-0.02)	0.01	0.22	5x LD_{50}	17.67	0.44 (0.31-0.56)	0.35	1.61
F2 (HS-M LNTX)	0.18 (0.16-0.20)*	5x LD_{50}	81.25	0.22 (0.20-0.25)	0.18	4.00	5x LD_{50}	11.98	1.73 (1.54-1.92)	1.38	6.35
F3 (HS-M basic PLA_2)	0.08 (0.06-0.10)*	1.5x LD_{50}	125.00	0.02 (0.01-0.02)	0.01	0.22	5x LD_{50}	5.62	1.57 (1.17-1.96)	1.25	5.76

Values of 95% C.I. were in parentheses

[a] LD_{50} : median lethal dose (µg/g)
[b] Challenge : all challenge doses were proven to be above 100% lethal dose (LD_{100}) when given intravenously
[c] ED_{50} : median effective dose, the antivenom dose (µl) at which 50% of mice survived
[d] ER_{50} : median effective ratio, the ratio of the amount of venom (mg) to the volume dose of antivenom (ml) at which 50% of mice survived
[e] Potency, P : neutralization potency of the antivenom (mg/ml), the amount of venom (mg) completely neutralized by one ml antivenom (ml)
[f] Normalized P, n-P : neutralization potency of the antivenom (mg/g), the amount of venom (mg) completely neutralized per unit amount of antivenom protein (g)

[#] N.E. : non-effective, the antivenom considered non-effective when a maximum volume (200µl) of antivenom used in the immunocomplexation does not protect the mice from the lethal effect of venom at a minimum challenge dose of 1.5x LD_{50}.

*Reference values from Tan et al. (Tan et al., 2015b).

6.4 Discussion

6.4.1 Venom Lethality and its Principal Toxins

Cobra envenomation is highly lethal due to the rapid development of neuromuscular paralysis and subsequent respiratory failure. In addition, the extensive tissue necrosis resulting from the bite can also contribute significantly to the morbidity of cobra envenomation (Campbell, 1979; WHO, 2010a). The toxic activities of cobra venoms that lead to these cardinal effects are mainly attributed to two main venom protein families, i.e. three-finger toxins (3FTx) and phospholipase A_2 (PLA_2) (Tan & Tan, 2015). This study demonstrates the correlation between high abundance of alpha-neurotoxins (belonging to the 3FTx family) and the lethality of the venom (**Chapter 4**). The alpha-neurotoxins are post-synaptic nicotinic blockers that are responsible for the rapid onset of flaccid paralysis clinically (Barber et al., 2013). They are typically the main lethal toxins with low LD_{50} and high Toxicity Score (TS). On the other hand, cytotoxins/cardiotoxins (CTXs), constitute another major 3FTx subgroup, are less lethal than alpha-neurotoxins (with higher LD_{50} and lower TS) but they play a major role in local tissue destruction (necrosis), presumably as a result of the *in situ* cytolytic activities (Osipov et al., 2008; Yap et al., 2014b).

Apart from this, the major phospholipase A_2 isolated from NK-T venom is an acidic isoform that exhibits negligible lethality to mice. This finding is consistent with the previous study showing that the acidic PLA_2 from *N. kaouthia* venom of Indian origin is non-toxic (Mukherjee, 2007). The role of the acidic PLA_2 in the pathogenesis of *N. kaouthia* envenomation remains to be elucidated. Among the snake venom PLA_2s, the neutral and basic types had been reported to play a significant role in the toxic action of snake venom. Myotoxic basic PLA_2s are rarely reported in cobra venoms, although they are important toxins in the venoms of some elapids such as sea snakes (Tan et al.,

2015b). In this study, the basic PLA_2 of *H. schistosus* (HS-M basic PLA_2) exhibits high TS comparable to those of the long- and short-neurotoxins in the venom.

It should be noted that although the toxicity scoring can serve as an indicator to measure the extent of toxin's contribution to the venom lethality (Laustsen et al., 2015), the application of this approach is limited to elapid venoms. In contrast to elapid venoms, viperid or crotalid venoms consist of moderate to high molecular mass proteins that are less lethal with higher LD_{50}, but these proteins generally act in synergism to cause toxic effects. The toxic scoring system is hence unlikely to reflect the true contribution of the individual toxin to the lethality of viperid or crotalid venoms.

6.4.2 Neutralization of *N. kaouthia* Venom by Antivenoms

Cobra antivenom (NKMAV) was reported to confer cross-neutralization against *H. schistosus* venom (HS-M) (Tan et al., 2015d) although the potency of neutralization is rather low (P = 0.03 mg/ml, or n-P = 0.67 mg/g) in comparison with its neutralization potency against the homologous NK-T venom (P = 0.92 mg/ml, or n-P = 20.44 mg/g) (**Chapter 4**). In this study, the capability of CSL Sea Snake Antivenom (SSAV) to effectively cross-neutralize NK-T venom (P = 1.60 mg/ml, or n-P = 7.37 mg/g) was also demonstrated. The cross-neutralization phenomenon indicates that the cobra and sea snake venom shares common toxin antigens or immunological determinants, as suggested in the earlier studies (Khow et al., 2001; Minton, 1967; Tan et al., 2015d). The examination of the venom proteome of *N. kaouthia* (**Chapter 4**) and *H. schistosus* (Tan et al., 2015b) indicates that the cross-reactivity is likely due to the presence of substantial amount of alpha-neurotoxins. In addition, the more potent protective effect observed in the cross-neutralization of cobra venom by SSAV could also be due to the inclusion of the Australian tiger snake venom (*Notechis scutatus*) as an immunogen during antivenom production of SSAV. In fact, SSAV is known to be a bivalent product

raised against the venoms of beaked sea snake (*H. schistosus*) and the tiger snake (*N. scutatus*). The efficacy of this antivenom against the *in vitro* neurotoxic effect of the two venoms (*H. schistosus* and *N. scutatus*) had been previously examined, and the results showed that SSAV is more effective against sea snake venoms as compared to *N. scutatus* venom (Chetty et al., 2004). Besides, another recent study also showed that the commercial Australian tiger snake antivenom (used for *N. scutatus* envenomation) was able to cross-neutralize the neurotoxic effect of Egyptian cobra (*Naja haje*) venom in mice (Kornhauser et al., 2013). Thus, it is likely that the *N. scutatus* venom which contains a large amount of PLA_2 as well as some alpha-neurotoxins (unpublished data), may play a role in enhancing the potency of SSAV, especially against the toxic PLA_2 of *H. schistosus*.

6.4.3 Neutralization of the Purified Toxins by Antivenoms

The capabilities of two antivenoms (NKMAV and SSAV) to neutralize the major toxins of NK-T and HS-M venoms were examined. It has been shown that the composition of neurotoxin subtypes of the two venoms differ substantially. NK-T venom contains a large amount of LNTX (33.3%) and a much smaller amount of SNTX (7.7%); while HS-M venom is predominated with SNTX (55.8%) and contains a much lower content of LNTX (14.7%) (Tan et al., 2015b) (Table 6.2). In this study, NKMAV consistently showed low neutralization potency against the SNTX of homologous (NK-T SNTX, n-P = 1.33 mg/g) and heterologous (HS-M SNTX, n-P = 0.22 mg/g) origins. The low efficacy may be partly due to the low content of SNTX in NK-T venom which was used as an immunogen for NKMAV production, yielding a relatively low titer of anti-SNTX in the antivenom. This observation is in agreement with previous reports on low immunoreactivity and weak neutralization capability of the cobra antivenom against neurotoxins of short chain isoforms (Leong et al., 2015; Tan et al., 2015d). The other

reason for low efficacy of NKMAV in the neutralization of short neurotoxins could be due to the poor antigenicity of short neurotoxins. Apart from this, NKMAV also exhibited poor cross-neutralization against HS-M basic PLA_2, which is presumably due to the absence of basic PLA_2 in *N. kaouthia* venom. On the other hand, the neutralization potencies of NKMAV against the LNTX from both venoms were high and comparable, suggesting that the antigenic site of the HS-M LNTX is likely to be similar to those of cobra's LNTX.

On the other hand, SSAV could neutralize HS-M LNTX (n-P = 6.35 mg/g) and HS-M basic PLA_2 (n-P = 5.76 mg/g) effectively but appeared to be less potent against HS-M SNTX (n-P = 2.49 mg/g). The results further suggest that SNTX is a relatively poor immunogen compared to the long neurotoxins. It is interesting to note that SSAV outperformed NKMAV in neutralizing the short neurotoxin of both venoms (NK-T SNTX and HS-M SNTX) by several folds. It is possible that the higher immunoreactivity of SSAV towards SNTXs (NK-T SNTX and HS-M SNTX) is attributed at least partly to the high SNTX content of the sea snake venom that used as an immunogen in the production of SSAV. Thus, one of the ways to improve the efficacy of cobra antivenom could be by enriching the SNTX content in the immunogen.

It is not surprising that SSAV was ineffective against the two CTXs (NK-T CTX-I and NK-T CTX-II) of *N. kaouthia* (n-P = 0.00-0.41 mg/g) as the sea snake venom is essentially devoid of cytotoxin. However, despite the fact that the cobra venom contains a large amount (> 20%) of the CTX, the neutralization potency of NKMAV against both CTXs is low, indicating that CTX also possesses weak immunogenicity. Hence, the low neutralization potency of NKMAV against CTX also contributes to the low neutralization potency of the antivenom against cobra venoms.

6.5 Conclusion

This study showed that poor neutralization of the low molecular mass toxins, especially SNTX is the main limiting factor on the neutralization potency of cobra antivenom. Also, the study demonstrated that SSAV exhibited higher potency in neutralizing SNTX compared to NKMAV, presumably partly due to the much higher SNTX content in sea snake venom used as an immunogen for SSAV production. These findings suggest enriching SNTX content could be a way to enhance the titer of anti-SNTX in cobra antivenom, thereby improving the neutralization potency of cobra antivenom.

CHAPTER 7: CONCLUSION AND FUTURE STUDIES

7.1 Conclusion

The present study revealed substantial geographical variations in the composition and pharmacological properties of the *Naja kaouthia* venoms sourced from three Southeast Asian regions (Malaysia, NK-M; Thailand, NK-T and Vietnam, NK-V). A comprehensive proteomic approach using reverse-phase HPLC coupled with nanoESI-LCMS/MS and data mining revealed remarkable compositional variation among the three venoms, particular on the subtypes of three-finger toxin (3FTx). These variations were well correlated to the differences in the lethality (tested on a murine model) and neurotoxic activities (test on a chick biventer cervicis nerve-muscle preparation, CBCNM) of the three venoms. Notably, NK-T venom exhibited the highest lethality and the most potent neurotoxic activity (in terms of the onset of neuromuscular paralysis) tested *in vitro* and *in vivo*. Despite the variations in their venom proteomes and toxicity profiles, the three venoms from different locales could be neutralized to varying degree by Thai *N. kaouthia* Monovalent Antivenom (NKMAV) and Neuro Polyvalent Antivenom (NPAV), both of which were prepared using Thai *N. kaouthia* venom as immunogen.

In the CBCNM preparation, the *in vitro* experiment revealed that the antivenom (NKMAV) was able to halt the progression of venom-induced neuromuscular depression but unable to completely overcome the neuromuscular blockade caused by the venom. Nevertheless, in experimentally envenomed mice, NKMAV administered at the onset of neurotoxicity was effective to reverse the neuromuscular depressant effect *in vivo* and thus, rescuing the mice from the lethal action of the three *N. kaouthia* venoms. Taken together, the findings indicate that the Thai cobra antivenoms (monovalent or polyvalent) are the appropriate choice of antidote useful in the treatment

of monocled cobra envenomation in Malaysia and Vietnam. Also, the findings support that the prompt administration of adequate antivenom in timely frequency is of utmost importance in the protocol of antivenom treatment for cobra envenomation.

Furthermore, the principal toxins of the Thai *N. kaouthia* (NK-T) and the Malaysian *H. schistosus* (HS-M) venoms were purified and investigated for their neutralization profiles by the antivenoms NKMAV and CSL Sea Snake Antivenom (SSAV). The results showed that the antivenoms were consistently poor in neutralizing low molecular mass toxins especially short neurotoxins (SNTXs) and cytotoxins (CTXs), and apparently these are the factors that limit the neutralization efficacy of the elapid antivenoms tested. The findings suggest a possibility to overcome the limitation of cobra antivenom potency through improving the immunogen formulation by toxin enrichment, where various essential low molecular mass toxins especially SNTXs, long neurotoxins (LNTXs) and CTXs should be included in the immunogen mixture in sufficient amount. Coupled with the existing low dose, low volume, multi-site immunization protocol (Chotwiwatthanakun et al., 2001; Sriprapat et al., 2003), it may be possible to enhance the immunogenicity of the toxins and increase the anti-toxin titer against the main toxins of the venom.

7.2 Limitation of the Present Study

The present study only examined *N. kaouthia* venom samples from three different geographical regions (Malaysia, Thailand and Vietnam), although this species is known to be prevalent in many other regions such as South China, Northern India and certain other regions of Indochina. Thus, to achieve a more holistic understanding of "pan-geographical venom variations" for this species, it would be necessary to also investigate *N. kaouthia* venoms sourced from those other regions mentioned. This study was conducted using pooled venoms from mainly adult snakes; hence the findings could not verify conclusively any ontogenic venom differences in snakes of different ages, although preliminarily screening by reverse-phase HPLC revealed that the venoms from Malaysian juvenile and subadult *N. kaouthia* did not exhibit marked variations from their adult counterpart as established in the current study.

7.3 Future Studies

The findings of this study pave interesting avenues for future research in toxinology. In view of the geographical variations in the 3FTx profiles of *N. kaouthia*, it would be highly relevant to do a comparative study of the venom gland transcriptomics of this species. This will provide deeper insights into the understanding of toxin gene's regulations that lead to the geographical diversification of the venom composition, in particular the 3FTx. Besides, there are many toxin families that have been newly identified from the venom proteome of *N. kaouthia*, and thus characterization of these novel proteins should be further explored in the future. Furthermore, with the availability of venom gland transcriptomic data, the expression of recombinant proteins can be achieved, especially of those with low abundance, to screen for their functional activities and medicinal values for drug discovery. On the other hand, the present study has also demonstrated that neutralization capacity of cobra antivenom is mainly limited by weak neutralization of SNTXs and CTXs. Future work should examine whether the proposed use of toxin-enriched immunogen (especially with a higher portion of SNTX) can result in improvement of the neutralization efficacy of cobra antivenom.

Other than *N. kaouthia* venom examined in this present study, the understanding of venom proteomes of other congeneric species (*Naja* genus) from different geographical regions are equally important for scientific discoveries in the evolution and biodiversity, as well as the medical importance of this genus across Asia and Africa. Findings from the current study should be correlated further at a wider pan-generic scale by studying the other cobra venoms in the future. From a clinical standpoint, fundamental study as such should also be correlated clinically through a serial and populational study on human envenomation cases so that the research yields positive impact to the society, where patients can benefit from the scientific advances in toxinology and the management of snakebite envenomation cases can be improved.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

Publications:

i. Tan, K. Y., Tan, C. H., Fung, S. Y., & Tan, N. H. (2015). Venomics, lethality and neutralization of *Naja kaouthia* (monocled cobra) venoms from three different geographical regions of Southeast Asia. *Journal of Proteomics*, 120, 105-125.

ii. Tan, K. Y., Tan, C. H., Fung, S. Y., & Tan, N. H. (2016). Geographical venom variations of the Southeast Asian monocled cobra (*Naja kaouthia*): venom-induced neuromuscular depression and antivenom neutralization. *Comparative Biochemistry and Physiology, Part C.*, 185-186, 77-86.

iii. Tan, K. Y., Tan, C. H., Fung, S. Y., & Tan, N. H. (2016). Neutralization of the principal toxins from the venoms of Thai *Naja kaouthia* and Malaysian *Hydrophis schistosus*: insights into toxin-specific. *Toxins*, 8 (4), 86.

Papers presented:

i. Poster : Venom variation and impact: insights into the proteome, mechanism and neutralization of the venom of monocled cobra (*Naja kaouthia*) from three geographical areas, Faculty of Medicine Research Week 2015, 11-15 May 2015 (University).

ii. Poster : Geographical variations of *Naja kaouthia* (monocled cobra) venom from Southeast Asia: a venomic and functional study, 18th World Congress of the International Society of Toxinology, University of Oxford, 25-30 September 2015 (International).

APPENDIX A: PUBLICATIONS

APPENDIX B: ETHICAL APPROVAL LETTERS

APPENDIX C: ANTIVENOM PRODUCT SHEETS

Polyvalent snake antivenin

Polyvalent snake antivenin is classified into 2 kinds: Hemato Polyvalent Snake Antivenin and Neuro Polyvalent Snake Antivenin.

Composition

In 1 ml solution contains polyvalent equine immunoglobulins with specific antibodies which can neutralize the quantities of snake venoms as follow:

Hemato Polyvalent Snake Antivenin :

Russell's Viper venom *(Daboia russelii siamensis)*	0.6 mg
Green Pit Viper venom *(Trimeresurus albolabris)*	0.7 mg
Malayan Pit Viper venom *(Calloselasma rhodostoma)*	1.6 mg

Neuro Polyvalent Snake Antivenin :

Malayan Krait venom *(Bungarus candidus)*	0.4 mg
Cobra venom *(Naja kaouthia)*	0.6 mg
Banded Krait venom *(Bungarus fasciatus)*	0.6 mg
King Cobra venom *(Ophiophagus hannah)*	0.8 mg

Both polyvalent snake antivenins contain other non-active ingredients as follow :

Aminoacetic acid (glycine)
Carbolic acid (phenol)
Sodium chloride
Sterile water for injection

Indication

Passive immunization against poisonous of a range of hematotoxic snakebites or neurotoxic snakebites, base on the type of snake identified.

Dosage

For initial dose, at least 20 mL of reconstituted serum should be given by slow intravenous infusion (not more than 1 ml/minute). If symptoms still persist, the second dose should be repeated 2 hours or even earlier after the initial dose. The further dose should be repeated every 6 hours according to the clinical symptoms.

Administration

Draw 10 ml of the sterile water for injection to the freeze-dried antivenin, shake well to dissolve the contents until the serum became clear colorless or pale yellow liquid, ready for administration.

Contra-indication

None, unless the patient is known to be hypersensitive to any of the constitution of the product, appropriate precautions must be taken (see below).

Side effects

Occasionally, transient tenderness at the injection site, cutaneous reaction and alterations in temperature may occur.

In some cases, nausea, vomiting and also circulatory reactions (e.g. tachycardia, bradycardia, hypotension, sweating, vertigo) and allergic reactions (e.g. with flush, urticaria, dyspnea) have been observed, extending in isolated cases as far as shock. The patient should be monitored for an extended period of time.

Therapeutic measures depend on the nature and severity of side effects. Antihistamines and if necessary adrenaline, high doses of corticosteroids, volume replacement, oxygen should be available when needed.

Special precautions

Since the antivenin is prepared from horse serum, sensitization to heterologous protein may occur in some individuals. To avoid serious allergic reactions, skin test should be performed prior to the administration by injection 0.02 ml of 1:100 antivenin dilutions intradermally. It should be noted that the skin test may not predict the anaphylaxis nor delay serum sickness reactions.

Storage and stability

Store below 25°C. The product shall have a shelf life of 5 years from the manufacturing date

Manufactured by
Queen Saovabha Memorial Institute. The Thai Red Cross Society.
1871 Rama IV Rd. Patumwan, Bangkok 10330, Thailand

เซรุ่มแก้พิษงูชนิดรวม

เซรุ่มแก้พิษงูหลายชนิดรวมอยู่ในขวดเดียวกัน แบ่งออกได้เป็น 2 ประเภท คือ เซรุ่มแก้พิษงูระบบโลหิต และเซรุ่มแก้พิษงูระบบประสาท

ส่วนประกอบ

ในผงยาที่ละลายเป็นน้ำยาแล้ว 1 มิลลิลิตร ประกอบด้วย อิมมูโนโกลบูลินที่ได้จากพลาสมาของม้าที่สามารถทำลายพิษงู ดังนี้

เซรุ่มแก้พิษงูระบบโลหิต

พิษงูแมวเซา *(Daboia russelii siamensis)*	0.6 มิลลิกรัม
พิษงูเขียวหางไหม้ *(Trimeresurus albolabris)*	0.7 มิลลิกรัม
พิษงูกะปะ *(Calloselasma rhodostoma)*	1.6 มิลลิกรัม

เซรุ่มแก้พิษงูระบบประสาท

พิษงูทับสมิงคลา *(Bungarus candidus)*	0.4 มิลลิกรัม
พิษงูเห่า *(Naja kaouthia)*	0.6 มิลลิกรัม
พิษงูสามเหลี่ยม *(Bungarus fasciatus)*	0.6 มิลลิกรัม
พิษงูจงอาง *(Ophiophagus hannah)*	0.8 มิลลิกรัม

เซรุ่มแก้พิษงูชนิดรวมทั้ง 2 ประเภท มีส่วนประกอบของสารไม่ออกฤทธิ์ที่เหมือนกันคือ

กรดอะมิโนอะเซติค (กลัยซิน)
กรดคาร์บอลิค (ฟีนอล)
โซเดียม คลอไรด์
น้ำกลั่นสำหรับฉีด

ข้อบ่งใช้

สำหรับทำลายพิษงูในกลุ่มระบบโลหิต หรือ พิษงูในกลุ่มระบบประสาท ทั้งนี้ขึ้นกับชนิดของงูที่กัด

ขนาดยา

เริ่มต้นด้วยการให้สารละลายเซรุ่มที่ละลายดีแล้วอย่างน้อย 20 มิลลิลิตร ทางหลอดโลหิตดำช้าๆ (ไม่เกิน 1 มิลลิลิตร/นาที) สามารถให้ซ้ำได้ภายในเวลา 2 ชั่วโมง ในกรณีที่ยังมีอาการ จากนั้นอาจให้ซ้ำได้ทุก 6 ชั่วโมง โดยประเมินจากสภาวะผู้ป่วย

การใช้ยา

ละลายเซรุ่มแก้พิษงูด้วยน้ำกลั่นสำหรับฉีด 10 มิลลิลิตร เขย่าให้เข้ากันเพื่อให้ผงยาละลายกลายเป็นของเหลวไม่มีสี หรือสีเหลืองอ่อน ก่อนนำไปฉีด

ข้อห้ามใช้

ไม่มีข้อห้ามใช้ นอกจากผู้ป่วยที่มีประวัติว่าแพ้ หรือไวต่อส่วนประกอบตัวใดตัวหนึ่งในน้ำยา

อาการข้างเคียง

บางครั้งอาจพบว่ามีอาการปวดบริเวณที่ฉีด มีปฏิกิริยาทางผิวหนัง และ อาจมีไข้

อาการต่อไปนี้อาจพบได้แต่น้อยมาก ได้แก่ คลื่นไส้ อาเจียน และมีปฏิกิริยาทางการไหลเวียนของเลือด (เช่น หัวใจเต้นเร็ว หรือ ช้ากว่าปกติ ความดันโลหิตต่ำ เหงื่อออก วิงเวียนศีรษะ) และ ปฏิกิริยาภูมิแพ้ (เช่น หน้าแดง คอแดง ลมพิษผื่นคัน หายใจลำบาก) บางรายอาจเป็นมากถึงช็อค ได้ ดังนั้นในผู้ป่วยทุกรายที่มีอาการ ควรเฝ้าดูอาการต่อไประยะหนึ่ง

การรักษาปฏิกิริยาช็อค ขึ้นกับอาการและความรุนแรง อาจให้ยาต้านฮิสตามีนหรือถ้าจำเป็นอาจต้องให้ อะดรีนาลีน คอร์ติโคสเตียรอยด์ ขนาดสูง สารน้ำทดแทนปริมาตรเลือด และออกซิเจน ซึ่งควรมีการเตรียมให้พร้อมเสมอ

ข้อควรระวัง

เนื่องจากเซรุ่มแก้พิษงูเตรียมจากพลาสมาของม้า จึงอาจก่อให้เกิดปฏิกิริยาแพ้ต่อโปรตีนม้าได้ เพื่อหลีกเลี่ยงการเกิดปฏิกิริยาดังกล่าว ก่อนฉีดเซรุ่มควรทดสอบอาการแพ้โดยฉีดเซรุ่มเจือจาง 1:100 ปริมาณ 0.02 มิลลิลิตร เข้าใต้ผิวหนังเพื่อดูปฏิกิริยา อย่างไรก็ตาม การทดสอบนี้ไม่สามารถคาดการณ์การเกิดอาการช็อค และหรืออาการแพ้ได้ทั้งหมด ในผู้ป่วยทุกราย

การเก็บรักษา

ควรเก็บเซรุ่มแก้พิษงูชนิดผงแห้งใน ที่อุณหภูมิต่ำกว่า 25 องศาเซลเซียส

ผลิตโดย
สถานเสาวภา สภากาชาดไทย
1871 ถนนพระราม 4 ปทุมวัน กรุงเทพฯ 10330 ประเทศไทย

No. 0030107

Cobra antivenin / King Cobra antivenin / Banded Krait antivenin / Malayan Krait antivenin
Russell's Viper antivenin / Malayan Pit Viper antivenin / Green Pit Viper antivenin

Composition

Snake antivenin (as stated on label)	Composition / mL
Cobra antivenin	Equine specific immunoglobulin with neutralizing activity against 0.6 mg of Cobra venom, Aminoacetic acid(glycine), Carbolic acid(phenol), Sodium chloride, Sterile water for injection
King Cobra antivenin	Equine specific immunoglobulin with neutralizing activity against 0.8 mg of King Cobra venom, Aminoacetic acid(glycine), Carbolic acid(phenol), Sodium chloride, Sterile water for injection
Banded Krait antivenin	Equine specific immunoglobulin with neutralizing activity against 0.6 mg of Banded Krait venom, Aminoacetic acid(glycine), Carbolic acid(phenol), Sodium chloride, Sterile water for injection
Malayan Krait antivenin	Equine specific immunoglobulin with neutralizing activity against 0.4 mg of Malayan Krait venom, Aminoacetic acid(glycine), Carbolic acid(phenol), Sodium chloride, Sterile water for injection
Russell's Viper antivenin	Equine specific immunoglobulin with neutralizing activity against 0.6 mg of Russell's Viper venom, Aminoacetic acid(glycine), Carbolic acid(phenol), Sodium chloride, Sterile water for injection
Malayan Pit Viper antivenin	Equine specific immunoglobulin with neutralizing activity against 1.6 mg of Malayan Pit Viper venom, Aminoacetic acid(glycine), Carbolic acid(phenol), Sodium chloride, Sterile water for injection
Green Pit Viper antivenin	Equine specific immunoglobulin with neutralizing activity against 0.7 mg of Green Pit Viper venom, Aminoacetic acid(glycine), Carbolic acid(phenol), Sodium chloride, Sterile water for injection

Indication

Passive immunization against specific poisonous snake bites as stated on the label.

Administration

The freeze-dried antivenin must be reconstituted with the solution supplied (or 10 ml of sterile water for injection) prior to the administration.

Dosage

Hematotoxic snake antivenin : Russell's Viper antivenin, Malayan Pit Viper antivenin, Green Pit Viper antivenin. Initial dose of 30 ml of reconstituted antivenin should be given by slow intravenous infusion (approx. 2 ml/min). Subsequent dose can be given every 6 hours according to the clinical symptoms.

Neurotoxic snake antivenin : King Cobra antivenin, Banded Krait antivenin and Malayan Krait antivenin initial dose 50 ml, **Cobra antivenin** initial dose 100 ml. The reconstituted antivenin should be given by slow intravenous infusion (approx. 2 ml/min). Subsequent dose can be given every 12 hours according to the clinical symptoms.

Storage and stability

Keep in tightly closed container and store below 25°C. The product shall have a shelf life of 5 years from the manufacturing date.

Contraindication

None, unless the patient is known to be hypersensitive to constitutions of the product, appropriate precautions must be taken (see below).

Side effects

Occasionally, transient tenderness at the injection site, cutaneous reaction and alterations in temperature may occur.

In some cases nausea, vomiting and also circulatory reactions (e.g. tachycardia, bradycardia, hypotension, sweating, vertigo) and allergic reactions (e.g. with flush, urticaria, dyspnea) have been observed, extending in isolated cases as far as shock. Therefore, the patient should be monitored for an extended period of time.

Therapeutic measures depend on the nature and severity of side effects. Antihistamines, if necessary adrenaline, high doses of corticosteroids, volume replacement, oxygen.

Special precautions

In cases of Cobra and Krait bites, respiratory support may be essential.

Since the antivenin is prepared from horse serum, sensitization to heterologous protein may occur in some individuals. To avoid serious allergic reactions, skin test should be performed prior to the administration by injection of 0.02 ml of 1:100 antivenin dilution intradermally. It should be noted that the skin test may not predict the anaphylaxis nor delay serum sickness reactions.

Manufactured by Queen Saovabha Memorial Institute. The Thai Red Cross Society.
1871 Rama IV Rd. Patumwan, Bangkok 10330, Thailand

เซรุ่มแก้พิษงูเห่า / เซรุ่มแก้พิษงูจงอาง / เซรุ่มแก้พิษงูสามเหลี่ยม / เซรุ่มแก้พิษงูทับสมิงคลา
เซรุ่มแก้พิษงูแมวเซา / เซรุ่มแก้พิษงูกะปะ / เซรุ่มแก้พิษงูเขียวหางไหม้

ส่วนประกอบ

ชนิดของเซรุ่มแก้พิษงู (ตามที่ระบุบนฉลาก)	ส่วนประกอบ / มล.
เซรุ่มแก้พิษงูเห่า	อิมมูโนโกลบูลินที่ได้จากพลาสมาของม้าที่ทำลายพิษงูเห่าได้ไม่น้อยกว่า 0.6 มิลลิกรัม กรดอะมิโนอะเซติค (กลัยซิน), กรดคาร์บอลิค (ฟีนอล), โซเดียม คลอไรด์, น้ำกลั่นสำหรับฉีด
เซรุ่มแก้พิษงูจงอาง	อิมมูโนโกลบูลินที่ได้จากพลาสมาของม้าที่ทำลายพิษงูจงอางได้ไม่น้อยกว่า 0.8 มิลลิกรัม กรดอะมิโนอะเซติค (กลัยซิน), กรดคาร์บอลิค (ฟีนอล), โซเดียม คลอไรด์, น้ำกลั่นสำหรับฉีด
เซรุ่มแก้พิษงูสามเหลี่ยม	อิมมูโนโกลบูลินที่ได้จากพลาสมาของม้าที่ทำลายพิษงูสามเหลี่ยมได้ไม่น้อยกว่า 0.6 มิลลิกรัม กรดอะมิโนอะเซติค (กลัยซิน), กรดคาร์บอลิค (ฟีนอล), โซเดียม คลอไรด์, น้ำกลั่นสำหรับฉีด
เซรุ่มแก้พิษงูทับสมิงคลา	อิมมูโนโกลบูลินที่ได้จากพลาสมาของม้าที่ทำลายพิษงูทับสมิงคลาได้ไม่น้อยกว่า 0.4 มิลลิกรัม กรดอะมิโนอะเซติค (กลัยซิน), กรดคาร์บอลิค (ฟีนอล), โซเดียม คลอไรด์, น้ำกลั่นสำหรับฉีด
เซรุ่มแก้พิษงูแมวเซา	อิมมูโนโกลบูลินที่ได้จากพลาสมาของม้าที่ทำลายพิษงูแมวเซาได้ไม่น้อยกว่า 0.6 มิลลิกรัม กรดอะมิโนอะเซติค (กลัยซิน), กรดคาร์บอลิค (ฟีนอล), โซเดียม คลอไรด์, น้ำกลั่นสำหรับฉีด
เซรุ่มแก้พิษงูกะปะ	อิมมูโนโกลบูลินที่ได้จากพลาสมาของม้าที่ทำลายพิษงูกะปะได้ไม่น้อยกว่า 1.6 มิลลิกรัม กรดอะมิโนอะเซติค (กลัยซิน), กรดคาร์บอลิค (ฟีนอล), โซเดียม คลอไรด์, น้ำกลั่นสำหรับฉีด
เซรุ่มแก้พิษงูเขียวหางไหม้	อิมมูโนโกลบูลินที่ได้จากพลาสมาของม้าที่ทำลายพิษงูเขียวหางไหม้ได้ไม่น้อยกว่า 0.7 มิลลิกรัม กรดอะมิโนอะเซติค (กลัยซิน), กรดคาร์บอลิค (ฟีนอล), โซเดียม คลอไรด์, น้ำกลั่นสำหรับฉีด

ข้อบ่งใช้

สำหรับทำลายพิษงูจำเพาะชนิด ตามที่ระบุบนฉลาก

การให้ยา

ก่อนใช้เซรุ่มแก้พิษงูชนิดผงแห้ง ต้องละลายด้วยสารละลายที่บรรจุมาในกล่อง (หรือละลายด้วยน้ำกลั่นสำหรับฉีดปริมาณ 10 มล.)

ขนาดยา

เซรุ่มแก้พิษงูกลุ่มพิษต่อระบบโลหิต คือ เซรุ่มแก้พิษงูแมวเซา เซรุ่มแก้พิษงูกะปะ และ เซรุ่มแก้พิษงูเขียวหางไหม้ เริ่มด้วยให้สารละลายเซรุ่มที่ละลายดีแล้วปริมาณ 30 มล. ทางหลอดโลหิตดำช้าๆ (ประมาณ 2 มล./นาที) และอาจให้ซ้ำได้ทุก 6 ชั่วโมง โดยประเมินจากสภาวะผู้ป่วย

เซรุ่มแก้พิษงูกลุ่มพิษต่อระบบประสาท คือ เซรุ่มแก้พิษงูจงอาง เซรุ่มแก้พิษงูสามเหลี่ยม และเซรุ่มแก้พิษงูทับสมิงคลา เริ่มด้วยให้สารละลายเซรุ่มที่ละลายดีแล้วปริมาณ 50 มล. **ส่วนเซรุ่มแก้พิษงูเห่า** เริ่มให้ด้วยสารละลายเซรุ่มที่ละลายดีแล้ว ปริมาณ 100 มล. โดยให้ ทางหลอดโลหิตดำช้าๆ (ประมาณ 2 มล./นาที) และ อาจให้ซ้ำได้ทุก 12 ชั่วโมง โดยประเมินจากสภาวะผู้ป่วย

การเก็บรักษา

ควรเก็บเซรุ่มแก้พิษงูชนิดผงแห้งในขวดปิดสนิทที่อุณหภูมิต่ำกว่า 25°ซ หากเก็บรักษาตามที่กำหนด จะมีอายุ 5 ปี นับจากวันผลิต

ข้อห้ามใช้

ไม่มีข้อห้ามใช้ เนื่องจากหากไม่ฉีดเซรุ่มแก้พิษงูเมื่อถูกงูพิษกัดผู้ป่วยอาจเป็นอันตรายถึงชีวิต นอกจากผู้ป่วยจะมีประวัติว่าแพ้ หรือไวต่อส่วนประกอบตัวใดตัวหนึ่งในน้ำยา จำเป็นต้องเตรียมการป้องกันล่วงหน้า (โปรดอ่านรายละเอียดด้านล่าง)

อาการข้างเคียง

บางครั้งอาจพบว่ามีอาการปวดบริเวณที่ฉีดยา มีปฏิกิริยาทางผิวหนัง และ อาจมีไข้

อาการต่อไปนี้อาจพบได้แต่น้อยมาก ได้แก่ คลื่นไส้ อาเจียน และมีปฏิกิริยาทางการไหลเวียนของเลือด (เช่น หัวใจเต้นเร็ว หรือช้ากว่าปกติ ความดันโลหิตต่ำ เหงื่อออก วิงเวียนศีรษะ) และ ปฏิกิริยาภูมิแพ้ (เช่น หน้าแดง คอแดง ลมพิษผื่นคัน หายใจลำบาก) บางรายอาจเป็นมากถึงช็อคได้ ดังนั้น ในผู้ป่วยทุกรายที่มีอาการ ควรเฝ้าดูอาการต่อไประยะหนึ่ง

การรักษาปฏิกิริยาช็อค ขึ้นกับอาการและความรุนแรง อาจให้ยาต้านฮิสตามีนหรือถ้าจำเป็นอาจต้องให้ อะดรีนาลีน คอร์ติโคสเตียรอยด์ขนาดสูง สารน้ำทดแทนปริมาตรเลือด และออกซิเจน

ข้อควรระวัง

ผู้ป่วยที่ถูกงูเห่า หรือ งูจงอางกัด อาจจำเป็นต้องใช้เครื่องช่วยหายใจ

เนื่องจากเซรุ่มแก้พิษงูเตรียมจากพลาสมาของม้า จึงอาจก่อให้เกิดปฏิกิริยาแพ้ต่อโปรตีนม้าได้ เพื่อหลีกเลี่ยงการเกิดปฏิกิริยาดังกล่าวก่อนฉีดเซรุ่มควรทดสอบความไวโดยฉีดเซรุ่มเจือจาง 1:100 ปริมาณ 0.02 มล. เข้าใต้ผิวหนังเพื่อดูปฏิกิริยา อย่างไรก็ตาม การทดสอบนี้ไม่สามารถคาดการณ์การเกิดอาการช็อค และ/หรืออาการแพ้ได้ทั้งหมด ในผู้ป่วยทุกราย

ผลิตโดย สถานเสาวภา สภากาชาดไทย
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No. 0261211

Product Information

NAME OF THE MEDICINE

SEA SNAKE ANTIVENOM

AUST R 74901

DESCRIPTION

SEA SNAKE ANTIVENOM is prepared from the plasma of horses immunised with the venom of the sea snake *Enhydrina schistosa*. Each vial contains 1,000 units of antivenom. The product also contains phenol, sodium chloride and other equine plasma proteins in an aqueous solution. SEA SNAKE ANTIVENOM has been shown to be effective not only against the venom of *Enhydrina schistosa* but, to a varying degree, against the venoms of a wide variety of sea snakes present in northern Australian waters.

PHARMACOLOGY

Sea snakes are abundant throughout the warmer waters of the Indian and Pacific oceans. They require a sea temperature of at least 20°C and are therefore common in Australian tropical waters although there has been one confirmed sea snake bite at a Sydney beach.

The venom of the sea snake is very potent but in many cases of human bites, little venom is released. In a study of 101 cases in Malaysia, only 22% of bites were considered to be serious. However, 6 of the 11 who were seriously envenomed died before antivenom was available and 2 of 11 after introduction of the antivenom.

Sea snake bites can occur from inadvertently standing on the snake or, more commonly, they occur as an occupational hazard to fishermen sorting fish in their nets. The venom of the sea snakes contains potent neurotoxins that can cause muscle paralysis and respiratory failure leading to death. The venom also has myolytic properties. The muscle destruction can cause myalgia and renal failure. Hyperkalaemia can be severe. Myolysis has been demonstrated in monkeys with considerable elevation of creatinine kinase levels. There is also elevation of serum glutamic-oxaloacetic transaminase (SGOT) levels in humans which can be used to determine or monitor the degree of envenoming.

As there is considerable similarity between the toxins of the sea snakes and the Australian elapids, TIGER SNAKE ANTIVENOM is often effective in cases of sea snake envenoming and may be used if SEA SNAKE ANTIVENOM is not available.

INDICATIONS

For the treatment of patients who exhibit manifestations of systemic envenoming following a bite by a sea snake.

CONTRAINDICATIONS

There are no absolute contraindications, but the product should not be used unless there is clear evidence of systemic envenoming with the potential for serious toxic effects.

(See PRECAUTIONS for use of SEA SNAKE ANTIVENOM in patients with a known allergy).

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PRECAUTIONS

When medicinal products prepared from animal plasma are administered, infectious diseases due to the transmission of infective agents cannot be totally excluded. This applies to pathogens of hitherto unknown origin. This possibility must always be considered and should be conveyed, whenever possible, to patients who may receive the product. Historically there have been no known recorded cases of transmission of viruses by this product.

Most cases of sea snake bites are painless with no local swelling. A row of small teeth marks may be seen. Intense pain from underwater trauma is more likely to be due to a fish than a sea snake.

Up to two thirds of those bitten by a sea snake have little or no effect from the bite. Severely envenomed patients often develop symptoms soon after the bite, but in some, the potentially dangerous effects may be delayed for several hours. It is therefore essential to observe all those who have been bitten by a sea snake for at least 4 hours.

If the limb has been immobilised and a firm bandage applied, removal of the bandage and splint may precipitate the systemic effects of the venom. The bandage and splint should not be removed until the patient is in hospital with appropriate antivenom treatment available. As immobilisation causes local retention of the venom, the requisite period of observation of the patient for a minimum of 4 hours commences when the splint and bandage are removed.

Severe cases of systemic envenoming should be managed in an intensive care unit, if possible.

As this product is prepared from animal plasma, severe **allergic reactions may follow, including anaphylactic shock. A syringe loaded with 1:1,000 adrenaline must be available during antivenom therapy.** Anaphylactic reactions may be more likely to occur in those who are atopic or have previously received equine serum. This would include patients who have previously received equine Tetanus Antitoxin (prior to 1974 in Australia). Some authorities have advocated premedication with subcutaneous adrenaline and intravenous antihistamine, particularly in those patients who are known to be at risk, but such use is controversial.

The results of skin testing to determine patients who may have an allergic reaction are not satisfactory and should not be undertaken.

Antivenoms may bind complement and produce an anaphylactoid reaction in patients who have had no previous contact with equine protein.

The risk of such a reaction can be reduced by adequate dilution of the antivenom (1:10 in adults and 1:5 in small children) prior to infusion (see also DOSAGE AND ADMINISTRATION).

Should anaphylaxis occur, cease administration of antivenom, administer oxygen and inject adrenaline 1:1,000 intramuscularly at the following dose rates: small adults (<50 kg) 0.25 mL, average adults (50-100 kg) 0.5 mL, large adults (>100 kg) 0.75 mL. For children (to age 12) use 1:10,000 and inject 0.25 mL per year of age. If there is little or no response to the initial intramuscular dose of adrenaline, administer the same dose (diluted to 1:10,000) slowly into an intravenous line. Repeat at 5 minute intervals depending on response. In severe cases, intravenous antihistamine and intravenous corticosteroids may also be given to reduce the chance of late reactions, but have a slower onset of action than adrenaline. Further administration of antivenom should be considered in the light of the relative problems of envenoming and anaphylaxis.

Delayed serum sickness can occur following the use of animal derived antivenoms. The most common manifestations include fever, cutaneous eruptions, arthralgia, lymphadenopathy and albuminuria. Less commonly, arthritis, nephritis, neuropathy and vasculitis can occur. The condition usually appears 8 to 13 days after the use of antivenom but can occur as soon as 12 hours after a second injection of a similar animal protein.

The incidence of serum sickness is greater with larger volumes of antivenom.

Use in pregnancy

There is no information on the safety of this product in pregnant women.

Use in lactation

No information is available on the use of this product during lactation.

ADVERSE REACTIONS

The following adverse reactions, presented below according to System Organ Class and frequency, have been identified during post-approval use of all CSL snake antivenoms. Adverse event frequencies are defined as follows:

Very common: ≥1/10; common: ≥1/100 and <1/10; uncommon: ≥1/1000 and <1/100; rare: ≥1/10,000 and <1/1000; and very rare: <1/10,000.

Immune system disorders

Common: Allergic reactions including anaphylactic shock and delayed serum sickness

Nervous system disorders

Common: Headache

Gastrointestinal disorders

Uncommon: Abdominal pain, vomiting, nausea and diarrhoea

Skin and subcutaneous tissue disorders

Common: Urticaria, rash

Musculoskeletal and connective tissue disorders

Uncommon: Myalgia

General disorders and administration site conditions

Common: Pyrexia, chills

Uncommon: Local injection site reactions, chest pain

DOSAGE AND ADMINISTRATION

A large proportion of people bitten by sea snakes have minimal or no effects from the bite and antivenom is unnecessary. When there is evidence of systemic envenoming from a sea snake, the contents of one vial (1,000 units) should be administered slowly by intravenous infusion after dilution with Hartmann's Solution or normal saline. The dose is the same for adults and children. The antivenom should be diluted 1 in 10, although in small children a dilution of 1 in 5 may be more appropriate to avoid fluid overload.

In cases of severe envenoming, when myalgia, muscle weakness, trismus, ptosis and ophthalmoplegia are present, an initial dose of 3,000 to 4,000 units should be given and up to 10,000 units may be required altogether. In less severe cases a total of 3,000 units will control most patients.

Some authorities have advocated premedication with 0.25 mL of 1:1000 adrenaline subcutaneously and intravenous antihistamine to reduce the chance of anaphylactic shock, particularly in those patients who are known to be at risk, but such use is controversial (see PRECAUTIONS).

If the patient has the affected limb immobilised, the splint and pressure bandage should not be removed until the patient is in a unit where full resuscitation measures and antivenom are available.

Severe cases of systemic envenoming should be managed in an intensive care unit if possible.

The patient must be monitored for at least 6 hours after the conclusion of the antivenom infusion.

Before starting the infusion of antivenom, a separate syringe should be loaded with 1:1,000 adrenaline, as anaphylactic reactions can occur rapidly (see PRECAUTIONS).

Should an anaphylactic reaction occur, cease administration of antivenom, administer oxygen and inject adrenaline 1:1,000 intramuscularly at the following dose rates: small adults (<50 kg) 0.25 mL, average adults (50-100 kg) 0.5 mL, large adults (>100 kg) 0.75 mL. For children (to age 12) use 1:10,000 and inject 0.25 mL per year of age. If there is little or no response to the initial intramuscular dose of adrenaline, administer the same dose (diluted to 1:10,000) slowly into an intravenous line. Repeat at 5 minute intervals depending on response.

As delayed serum sickness is relatively common following the use of large volumes of foreign protein, it is advisable to administer a corticosteroid either by a single intravenous injection or orally for 4 to 5 days to children and to those receiving multiple doses of antivenom.

It may occasionally be necessary to treat both envenoming and anaphylaxis simultaneously.

SEA SNAKE ANTIVENOM contains no antimicrobial preservative. Use once only and discard any residue.

OVERDOSAGE

No information is available on overdosage. Contact the Poisons Information Centre on 131 126 for further advice on overdosage management.

PRESENTATION

SEA SNAKE ANTIVENOM is available as vials containing 1,000 units in aqueous solution. The product volume is potency dependant thus it varies from batch to batch. Please refer to the product volume printed on the carton.

STORAGE

SEA SNAKE ANTIVENOM should be protected from light and stored between 2 and 8°C. Do not freeze.

NAME AND ADDRESS OF SPONSOR

CSL Limited
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Date of TGA Approval: 15 December 2009

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