CHAPTER 2

LITERATURE REVIEW

2.1 Introduction to Serpentes

2.1.1 Evolution and Phylogeny of Serpentes

Snakes (order: Squamata, suborder: Serpentes) is a diverse group of reptiles (Order: Squamata) that comprise approximately 2,800 species and can be classified into 20 families (O'Shea, 2011). Snakes are found in every continent except Antarctica (Vitt and Caldwell, 2009). The first fossil records of snakes dated back to the Cretaceous period (Durand, 2004).

The suborder Serpentes is divided into two major monophyletic groups: the fossorial Scolecophidia (blindsnakes) which are small snakes with limited gape size and feed on small prey (mainly ants and termites); and the Alethinophidia (true snakes) that are more ecologically diverse and feed on larger prey (Cundall and Greene, 2000; Rieppel, 1988a and 1988b). The monophyly of Scolecophidia and Alethinophidia is supported by osteological, soft anatomy, ecological characters (Lee and Scanlon, 2002) and molecular phylogenetic based on mitochondrial genes (Slowinski and Lawson, 2002). Alethinophidia is further classified into taxa Anomochilus, Macrostomata, and Caenophidia. The families Colubroidea which include Viperidae, Elapidae, and Atractaspdidae form a single, monophyletic lineage within the taxon Caenophidia (Vidal and Hedges, 2004).

2.1.2 Venomous snakes

Venomous snakes lie within the taxon Caenophidia (Vidal *et al*., 2007). Caenophidia consists of the superfamilies Acrochorididae and Colubroidea which are sister subfamilies to each other. Colubroids comprise primarily non-venomous Colubridae, the venomous Atractaspididae, Viperidae and Elapidae. The older phylogeny relationships of Caenophidia are shown in Figure 2.1.

Recently, a new phylogeny for the Colubroidea was constructed based on molecular analysis inferred from five genes, and taxonomic guidelines from previous phylogeny and classifications as illustrated in Figure 2.2 (Pyron *et al*., 2011). Elapids, one of the medically significant venomous snakes, are classified under the subfamily Elapidae (Greene *et al*., 2000 and Vitt and Caldwell, 2009).

All elapids have been characterized by the unique presence of a pair of permanently erect canaliculate, proteroglyphous front fangs that are used to inject venom from their venom glands located at the rear of the upper jaws (Greene *et al*., 2000 and McCarthy, 1985). To date, there are 312 species from 65 genera of elapids recognized and they are widely distributed across tropical and subtropical regions around the world, including the Americas, Africa, Asia, Australia, Malanesia and Pacific oceans (Golay *et al*., 1993). The appearance of most terrestrial elapids resembles the colubrids, where the head is rounded and possess similar width to the neck with long and slender bodies with smooth scales although there are a few elapids with keeled scales (O'Shea, 2011). Most elapids are oviparous (egg-laying) but southern species, like the South African ringhals and Australian tigersnakes are viviparous (live-bearers) (Thorpe *et al*., 1997). They are generally recognized as active foragers that capture active prey

(Shea *et al*., 1993). Kardong (1982) reported that cobras (*Naja* sp.) are less active in capturing prey than vipers.

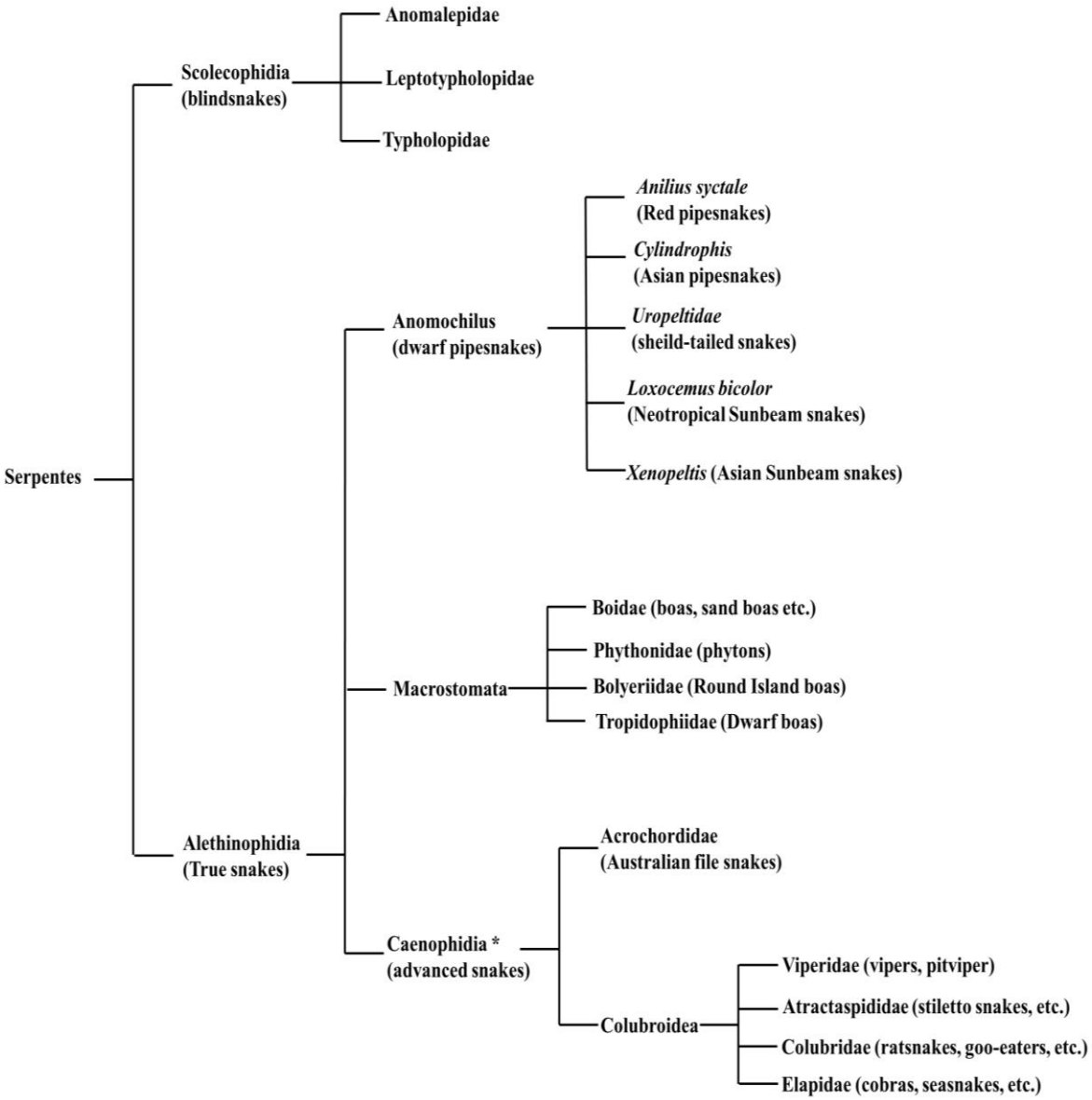

Figure 2.1: Classifications and divergence of the higher snake taxa.

The arrangement of major snake groups reflects well-supported evolutionary divergence and monophyletic relationships (Greene, 2000).

Adapted from Greene (2000). Snakes: The Evolution of Mystery in Nature, p 17, University of California Press, London, England.

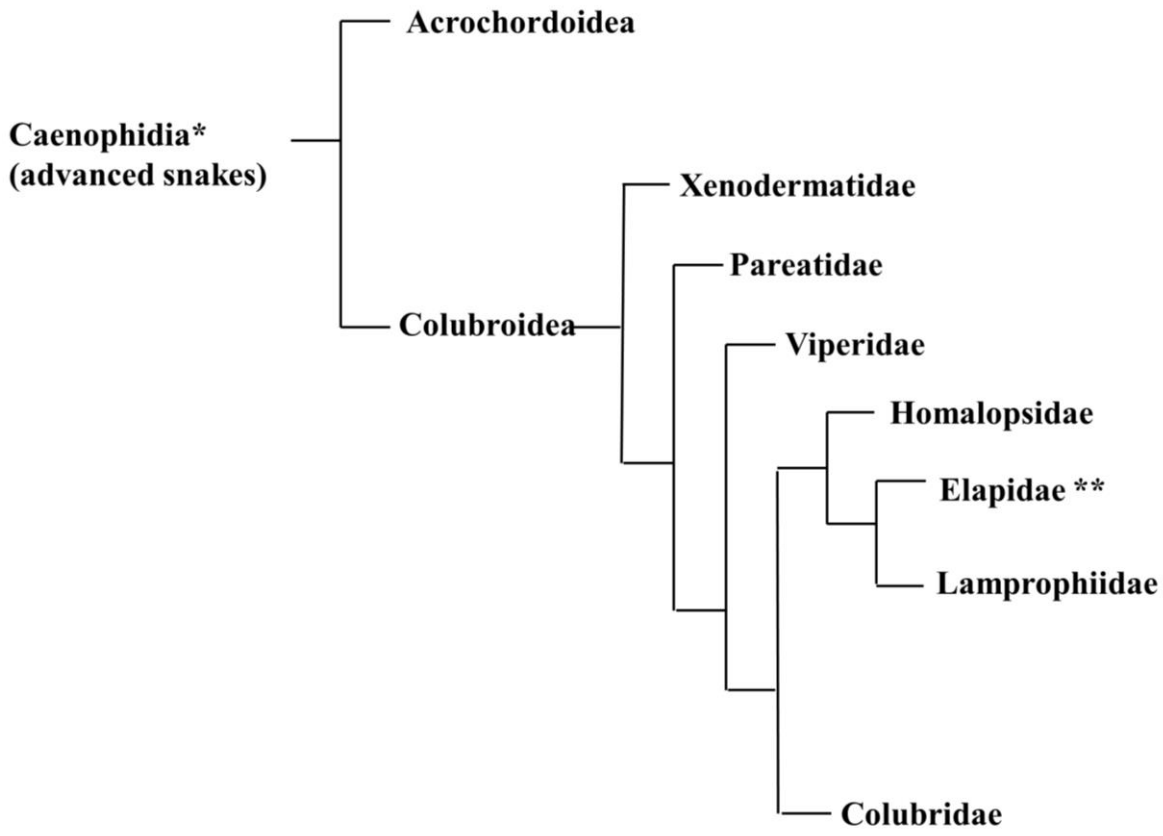

Figure 2.2: A summary of the phylogeny of advanced snakes (Colubroidea).

The phylogeny of Colubroid snakes was constructed based on molecular analysis of five genes. In this new classification, the subfamilies Elapinae, Hydrophiinae, and Laticaudinae that were previously classified in Elapidae are not recognized, as these subfamilies do not form well-supported monophyletic groups in the phylogeny (Pyron *et al*., 2011).

2.1.3 Venom delivery systems: Evolution and Anatomy

All venomous snakes possess a similar venom delivering system comprising the venom glands, ducts with accessory gland and fangs at the upper jaw for venom delivery or injection into prey (Kochva, 1987). These fangs contain a venom delivery channel (resembling a hypodermic needle) or groove to introduce the venom into the tissues of victims, often via intramuscular or subcutaneous route.

Venom delivery systems have been divided into front-fanged and rear-fanged system (Jackson, 2003; Kardong, 1982 and Vidal, 2002). There are two types of front-fanged systems: 1) fangs which are positioned at the anterior end of maxilla known as proeroglyph system (in elapids); 2) fangs which there are no maxillary teeth known as solenoglyph system (in vipers and atracraspidines). Fangs are always located on the maxilla that occupies various positions at upper jaw (Young and Kardong, 1995).

The front-fanged venom delivery systems of advanced snakes (some Atractaspididae, all Elapidae and all Viperidae) evolved several times independently from common ancestors via two pathways: 1) evolved from ancestors with enlarged anterior rear maxillary teeth in elapids; 2) evolved from ancestors with enlarged posterior rear maxillary teeth in vipers (Vidal, 2002).

The front-fanged venom delivery systems are highly specialized and consist of large and complex venom glands derived from Duvernoy's glands (located along the upper jaw), external venom gland compressor muscle and tubular fangs for efficient delivering of venom into prey (Jackson, 2003). Duvernoy's gland is a term for venom gland that is present in rear-fanged snakes (Taub, 1967 and Weinstein *et al*., 2011), but its structure is primitive and homolog to the venom

glands in advanced snakes, and the two glands are believed to evolve from the same origin (Fry *et al*., 2003a and 2006).

The development of front-fanged systems or venom glands with extensive evolution and recruitment of toxin gene families, lead to tremendous diversification of venomous snakes seen nowadays (Fry *et al*., 2009a; Fry *et al*., 2009b and Vonk *et al*., 2008). The evolution of venom toxins involves the duplication of toxin genes expressed in venom glands, resulting in the diversity in the venom toxin families (Casewell *et al*., 2012; Fry *et al.,* 2012a and Fry *et al*., 2012b).

2.2 Snake envenomation

2.2.1 The epidemiology of snake envenomation: Mortality and Morbidity

Snakebite is a global burden for public health particularly in tropical and subtropical area. It has been known as the 'disease of poverty' that affects poorer rural population and is considered as an occupational disease (Harrison *et al*., 2009 and World Health Organization, 2007a). The World Health Organization (WHO) estimated that there are 2.5 million snakebites each year with 125,000 deaths and approximately 100,000 of these cases occurred in Asia (World Health Organization, 2007b). In Asia, the number of deaths resulting from envenomation may be as high as 100,000 (Chippaux, 1998). Kasturiratne *et al*. (2008) reported that largest number of envenomation cases occurred in South Asia, with mainy in India, Sri Lanka and and Nepal. In India, the majority of the envenomation cases is attributed to the 'Big Four' (Russell's viper, Indian cobra, saw-scaled viper, and common krait) and most of the envenomation occurred in rural areas (Mohapatra *et al*., 2011). In Sri Lanka, high morbidity and mortality due to snake envenomation are mainly attributed to Russell's viper (*Daboia russelii*) and hump-nosed pit vipers (*Hypnale hypnale*) (Ariaratnam *et al*., 2009 and Kasturiratne *et al.,* 2008). Kasturiratne *et al*. (2008) reported that a conservative estimate indicated for the year 2007, the number of snake envenomation in Southeast Asia was as high as 111,000 cases, with approximately 20000 deaths. It was reported that in Thailand, Malayan pit viper (*Calloselasma rhodostoma*) accounts for the the majority of envenomation cases (38%), followed by Russell's viper (*Daboia russelii*,14%), Indo-Chinese spitting cobra (*Naja simensis*, 10%) and monocellate cobra (*Naja kaouthia*, 7%) (Warrell,1999). In Malaysia, the majority of the snakebites in Peninsula

Malaysia were inflicted by cobras and Malayan pit viper (Jamaiah *et al*., 2004; Jamaiah *et al*., 2006; Muthusamy, 1988 and Tan *et al*., 1990).

On the other hand, snake envenomations in East Asia are not as prevalent as in South Asia and Southeast Asia. For example, in Taiwan, approximately 600 snakebite cases were reported anually, with 20-30 mortality (Ong *et al*., 2004). While in Japan, only 1.67 snakebites per 100,000 were reported and the main biting species was the mamushi pit viper (*Gloydius blomhoffi*) (Yasunaga *et al*., 2011).

Victims that survived from snakebites are most likely to suffer from permanent physical disability due to local necrosis and surgical amputation (Gras *et al*., 2012a and Warrell, 2010a). Often, severe complications resulting from snakebites are acute renal failure (Kohli and Sakhuja, 2003), coagulopathy, uremic encephalopathy (Patil *et al*., 2012), neuroparalytic symptoms and brain stem dysfunction (Garg *et al*., 2012 and Srivastava *et al*., 2010).

The actual number of snake envenomations remains unknown as most of the statistics of envenomations are estmated figures (Chippaux, 2008). The actual number of envenomation cases is likely to be higher than number reported by hospitals, as often victims do not seek medical aids from hospital (Ahmed *et al*., 2008).

2.2.2 The management of snakebites

Different species of snakes (or even the same species but from different geographical locations) possess different venom compositions. As a result, they cause vastly different pathophysiological symptoms in the envenomed patients. As such, the correct management of snakebite depends on the correct diagnosis of the biting species (Johnson *et al*., 2013). World Health Organization/ South East Asian Organization (WHO/SEARO) has developed guidelines for the clinical management of snakebites for Southeast Asia region and highlighted the importance of rapid clinical assessments and subsequent appropriate treatments to patients (Warrell, 1999). Diagnosis of the biting species is usually based on the clinical manifestations of the envenomation and laboratory evaluation of the blood samples as well as detection of serum venom antigen by immunoassay (Brunda *et al*., 2006 and Dong Le *et al*., 2004).

To date, antivenom is the only specific and pivotal treatment for snake envenomation (Warrell, 2010b). Most of the victims that develop systemic poisoning symptoms require the parenteral administrations of antivenom after the initial evaluations and observations (Gras *et al*., 2012b; Isbister *et al*., 2011; Ogunbanjo, 2009 and Warrell, 2010a). The patients have to be closely monitored during and after antivenom administration (Ogunbanjo, 2009).

2.3 Antivenom as effective therapeutics for snake envenomations

Antivenom was first introduced and developed by Albert Calmette in 1895 (Hawgood, 1999). Antivenoms are immunoglobulins produced from hyper-immunization of horses or goats using relevant snake venoms (World Health Organization, 2010).

Venoms used for the production of antivenoms should meet the quality requirements with known taxonomic identity and geographical origins (Chippaux and Goyffon, 1991). Freund's complete adjuvant (FCA, contain mineral oil, emulsifier and inactivated *Myobacterium tuberculosis*) and Freund's incomplete adjuvant (FIA contain mineral oil and emulsifier) are both commonly used adjuvants that have been used in production of highly potent antivenoms (World Health Organization, 2010). Freund's complete adjuvant often causes granulomas at the site of injections in horses if large volume of adjuvant-venom mixture is injected at a single site. Therefore to minimize local adverse reactions, small volume of emulsified adjuvant-venom mixture is usually injected at multiple sites (Raw *et al*., 1991), and often in subsequent immunization the incomplete adjuvant is used. This immunization scheme also increases the total surface area for immunogens to elicit a more effective immune response (Sriprapat *et al*., 2003).

There are three different forms of antivenoms produced depending on the fractionation method(s): (1) Whole immunoglobulin molecule (IgG) prepared by caprylic acid or ammonium sulfate precipitation of the plasma (Lalloo and Theakston, 2003 and Rojas *et al*., 1994); (2) $F(ab')_2$ bivalent fragments which are prepared by pepsin digestion of the IgG to remove the highly reactive yet nonspecific Fc part of the molecule, followed by caprylic acid or ammonium

sulfate precipitation (Grandgeorge *et al*., 1996 and Simsiriwong *et al*., 2012) and (3) Fab monovalent fragments prepared by papain digestion of the IgG followed by ammonium sulphate fractionation (Al-Abdulla *et al*., 2003). Commercial antivenoms may be monovalent (raised against venom of single species) or polyvalent (raised against mixture of venoms from different species). The use of monovalent antivenom requires identification of biting species (Warrell, 1999), while polyvalent antivenom which possesses paraspecific neutralization potency (Archundia *et al*., 2011 and Casasola *et al*., 2009), is more useful in cases where the biting species could not be ascertained.

2.4 Biochemistry and toxinology of snake venom

Snake venoms are complex mixtures of proteins and polypeptides with diverse array of pharmacological activities both on prey and human victims. Typically, the proteins and polypeptides constitute about 90-95% of the dry weight of the venom (Russell, 1983 and Tu, 1988). Significant differences in venom composition have been reported between closely related species or even between the same species from different geographical origins (Magro *et al*., 2001; Queiroz *et al*., 2008; Salazar *et al*., 2009 and Saravia *et al*, 2002). Throughout the courses of evolution, many of the genes encoding venom toxin are recruited and undergo gene duplication and allelomorphism, leading to inter- and intra-specific variation of venom toxins in all lineage (Fry *et al*., 2008; Fry *et al*., 2009a and Fry *et al*., 2009b). The variations in venom toxin composition have significant impacts on the clinical managements of snakebites (Fry *et al*., 2003b).

2.4.1 Snake venom composition and the pathophysiology of snake envenomation with special emphasis on cobra venoms

Many snake venom proteins and polypeptides have been identified and characterized. The roles of many of these proteins/polypeptides in the pathophysiological action of the venom have been elucidated (Kini and Doley, 2010; Matsui *et al*., 2010 and Morita, 2005).

2.4.1.1 Phospholipase A_2

Phospholipase A_2 (PLA_2) is one of the most extensively investigated venom toxins (Mackessy, 2002). Snake venoms are the major source of Group I and Group II PLA_2. The PLA_2 are small proteins (~13-14 kDa) and generally require Ca^{2+} for their actions (Doley *et al*., 2010). The enzyme catalyzes the hydrolysis of phospholipids at Sn_2 positions and liberates lysophospholipids and fatty acids (Kini, 1997). Snake venom PLA_2 may possess presynaptic or postsynaptic neurotoxicity (Praznikar *et al*., 2009 and Rouault *et al*., 2006), systemic or local myotoxicity (Andrião-Escarso *et al*., 2000 and Gutiérrez *et al*., 2008a), cardiotoxicity (Zhang *et al*., 2002), platelet aggregation inhibition (Satish *et al*., 2004), anticoagulant (Zhao *et al*., 2000) and edema-inducing activities (Yamaguchi *et al*., 2001).

Cobra (*Naja* sp.) venom PLA_2s belong to Group 1 PLA_2 enzymes which typically contain 115-120 amino acid residues with seven disulphide linkages (Kang *et al*., 2011). Phospholiapses A_2 from cobra venoms exist in multiple forms and exhibit a wide spectrum of pharmacological effects despite their structural similarity (Kini, 2003).

2.4.1.2 Three-finger toxins

Three-finger toxins (3FTxs) are the major toxins in elapid venoms. These toxins belong to a superfamily of non-enzymatic proteins that contains 60-74 amino acid residues (Hedge *et al*., 2010). They exhibit distinct protein fold of three β-stranded loops (I-III that form a finger shape) protruding from a globular and hydrophobic core containing four conserved disulfide linkages (Ménez, 1998 and Tsetlin, 1999). They also consist of conserved aromatic residues *i.e.* Tyr25 or Phe27 for proper folding and to stabilize the anti-parallel β-sheet (Antil *et al*., 1999 and Torres *et al*., 2001). Some 3FTxs have additional disulphide bonds, for example candoxin from venom of *Bungarus candidus* (Parvathy *et al*., 2006).

Despite the overall common features of 3FTXs, they bind selectively to different biological targets and as a result exert different pharmacological effects (Harvey, 2013 and Kini, 2002).

Neurotoxins

Many 3FTxs are neurotoxins (Kini, 2002). All postsynaptic neurotoxin genes contain three exons that are interrupted by two introns, with a highly conserved promoter region (Siew *et al*., 2004). Neurotoxins are broadly classified into α-neurotoxins, weak neurotoxins, κ-neurotoxins and muscarinic toxins according to their selectivity in binding to various receptors.

Alpha-neurotoxins or curaremimetic toxins bind to the nicotinic acetylcholine receptors. It has been shown that α-neurotoxins inhibit the binding of acetylcholine to their receptor, disrupting the permeability response of post-synaptic membranes (Changeux, 1990). The majority of α-neurotoxins are potent in their action and their potencies depend on their binding affinity towards the nicotinic acetylcholine receptors (Barber *et al*., 2013). All α-

neurotoxins are closely related to each structurally, indicating that they probably evolved from a common ancestral polypeptide via gene duplication and subsequent multiple gene mutations (Hung *et al*., 1998). Alpha-neurotoxins can be classified into short chain α-neurotoxins and long chain α-neurotoxins. Short chain α-neurotoxins consist of 60-62 amino acid residues with four disulfide bonds, and bind selectively with high affinity to muscular acetylcholine receptors (Endo and Tamiya, 1991). Long chain α-neurotoxins have 66-75 amino acids and an additional disulfide bond between Cys30 and Cys34 (Mordvintsev *et al*., 2006). Long chain α-neurotoxins exhibit stronger binding affinity towards neuronal α7 nicotinic acetylcholine receptors due to the presence of fifth disulfide bond (Antil-Delbeke *et al*., 2000 and Servent *et al*., 1997).

Weak neurotoxins also contain five disulfide bonds, with the fifth disulfide bridge located at the N-terminal of loop I (Nirthanan *et al*., 2003). In general, they exhibit low toxicity and weak binding affinity toward acetylcholine receptors (Servent and Ménez, 2002). They are also known as non-conventional toxins as they belong to a poorly characterized class of 3FTXs (Nirthanan *et al*., 2003). Weak neurotoxins have been discovered in a few cobra venoms such as *Naja kaouthia* and *Naja sputatrix* venoms. Weak neurotoxins bind to both nicotinic and muscarinic acetylcholine receptors (Mordvintsev *et al*., 2007 and Ogay *et al*., 2005).

Kappa neurotoxins (κ-neurotoxins) share homologous nucleotide sequence with α-neurotoxins and have the fifth disulfide bond in loop II (Chang *et al.,* 2002). Typical κ-neurotoxins bind specifically to neuronal α3β4 nicotinic acetylcholine receptors (McLane *et al*., 1993). In general, they consist of 66 amino acids (Chiappinelli *et al*., 1996). There are two regions on N-terminal of κ-neurotoxins

that are responsible for antagonist interaction with neuronal α3 nicotinic acetylcholine receptors (Chiappinelli *et al*., 1996).

Muscarinic toxins are also structurally related to α-neurotoxins (Ducancel *et al*., 1991), and the genes encoding muscarinic toxins are similar to short-chain α-neurotoxins (Ducancel *et al*., 1991). They consist of 63-66 amino acids with four disulfide bonds (Karlsson *et al*., 2000) and form two- and three-stranded β-sheets (Ségalas *et al*., 1995). Muscarinic toxins bind selectively and specifically to M1, M2 and M4 muscarinic acetylcholine receptors (Karlsson *et al*., 1991 and Kornisiuk *et al*., 1995).

Cardiotoxins

Cardiotoxins are the second largest group of 3FTXs and only present in cobra venoms (Kini, 2002). Cardiotoxins are highly basic polypeptides consisting of 60-63 amino acid residues in single polypeptide chains that are cross-linked by four disulfide bonds (Hodges *et al*., 1987). The secondary structure of cardiotoxins consists of antiparallel β-pleated sheets while the tertiary structure consists of unique asymmetric distribution of non-polar and polar amino acid residues (Kumar *et al*., 1997).

Similar to other 3FTXs, cardiotoxin encoding genes have three exons interrupted by two introns and preferential mutations are observed in specific segments in exon 2 and 3 which contribute to diverse functions of cardiotoxins (Chang *et al*., 2000 and Lachumanan *et al*., 1998). The mRNA turnover and synthesis rates of cardiotoxins are high and therefore the toxins usually constitute 40-60% of dry weight of *Naja* venoms (Lachumanan *et al*., 1999).

Cardiotoxins in cobra venom exhibit diverse biological functions. Cardiotoxins exhibit direct and Ca^{2+}- dependent haemolysis on human erythrocytes (Jiang *et*

al., 1989 and Tan, 1982a), which presumably involved the synergistic interactions between phospholipase A_2 and cardiotoxins in hemolytic activities (Bougis *et al*., 1987 and Zusman *et al*., 1981). Cobra cardiotoxins also cause complete cardiac arrest (Tan, 1982a), which is likely caused by depolarization on ventricular cell membrane that leads to influx of Ca^{2+} (Harvey *et al*., 1982; Sun and Walker, 1986). The toxins also exhibit *in vitro* dose- and time-dependent cytotoxicity on human cancer cell lines (Chen *et al*., 2008 and Chien *et al*., 2010) and cause myonecrosis on skeletal muscles, damaging cell membranes and mitochondria (Ownby *et al*., 1993).

2.4.2 Other proteins and enzymes

Cobra venom also contains cysteine-rich secretory proteins (CRISPs) which interact with ion channels. The CRISPs are generally single chain proteins with molecular weight of 23-25 kDa (Osipov *et al*., 2001 and Osipov *et al*., 2005a). Phylogenetic analysis and sequence alignment showed that elapids' CRISPs share highly similarity with CRISPs from vipers (Jin *et al*., 2003).

Hyaluronidase is a ubiquitous enzyme in snake venoms. Hyaluronidase isolated from from *Naja naja* has molecular weight of 70 kDa. It indirectly enhances myotoxicity and hemorrhagic activities by increasing the dissemination of phospholipolytic myotoxin and hemorrhagic complex-I *in vivo* (Girish *et al*., 2004). The presence of hyaluronidase in venoms facilitates diffusion of toxins to reach their target tissues and thus modulate the systemic toxicity of venoms (Kemparaju *et al*., 2009) as well as local tissue damages at the biting site (Kemparaju and Girish, 2006). The enzyme is therefore known as the 'spreading factor' of snake venom.

Proteases from snake venoms have been categorized into serine proteases and metalloproteinases. Serine proteases are widely distributed in viper venoms, but only small amount exists in elapid venoms. Serine proteases generally exhibit fibrinogenolytic activities and may activate coagulation factor V, protein C, plasminogen or platelets. Metalloproteinases, on the other hand, usually exhibit haemorrhagic activities (Matsui *et al*., 2000). A metalloproteinase isolated from *Naja atra* venom exhibits edema-inducing activity, inhibits activation of complement system by degrading complement components factor B, C6, C7 and C8 but does not induce hemorrhages (Sun and Bao, 2010).

L- amino acid oxidase (LAAO) is a flavoenzyme which catalyzes the oxidative deamination of L-amino acid to α-keto acid, with liberation of ammonia and hydrogen peroxide. This enzyme is widely distributed in venoms of most snake families including Viperidae, Crotalidae and Elapidae (Tan and Ponnudurai, 1992). Snake venom LAAOs are usually homodimers with molecular weight of each subunit around 50-70 kDa; whereas under native conditions, snake venom LAAOs possess molecular weight of 110-150 kDa (Ali *et al*., 2000; Du and Clemetson, 2002). X-ray crystallography shows that each dimer of snake venom LAAOs consist of FAD binding domain, substrate binding domain and helical domain (Pawelek *et al*., 2000). Snake venom LAAOs exhibit a wide range of isoelectric point (pI) from 4.4 to 8.12 (Souza *et al*., 1999; Tan and Swaminathan, 1992; Ueda *et al*., 1988). Snake venom LAAOs exhibit various pharmacological activities. Some studies reported potent platelet inhibitory activity by snake venom LAAOs (Samel *et al*., 2008) whereas some LAAOs induce platelet aggregation (Stábeli *et al*., 2004). Snake venom LAAOs also possess anti-microbial activities (Rodrigues *et al*., 2004 and 2009). Snake venom LAAOs bind to bacterial cell surface and produce H_2O_2, which could inhibit bacterial

growth significantly even at low concentrations, as observed for *Agkistrodon halys* LAAO (Zhang *et al*., 2004). Furthermore, snake venom LAAOs are able to induce apoptosis in vascular endothelial cells (Araki *et al*., 1993), human embryonic kidney cells (Torii *et al*., 2000), human promyelocytic leukemia cells (Torii *et al*., 1997), human monocytic cells (Ali *et al*., 2000), mouse lymphocytic leukemia and human T-cell leukemia (Suhr and Kim, 1996), due to the production of H_2O_2 by LAAOs (Suhara *et al*., 1998). In addition to the above mentioned activities, snake venom LAAOs also induce edema (Wei *et al*., 2009), hemolysis (Ciscotto et al., 2009) and hemorrhage (Souza *et al*., 1999).

Phosphodiesterase acts as exonucleases that catalyze the hydrolysis of phosphodiester bonds on ribonuecleotide and deoxyribonucleotide sequentially from 3'terminus of polynucleotides producing 5' mononucleotides (Mackessy, 1998). Snake venom phosphodiesterases are generally basic enzymes with molecular weight ranging from 98-140 kDa, and usually inhibited by EDTA (Dhananjaya and D'Souza, 2010b). Phosphodiesterase from *Crotalus adamanteus* venom is a Mg^{2+}-activated zinc metalloezyme (Pollack *et al*., 1983). Higher levels of phosphodiesterase activities are observed in the snake venoms from *Bothrops*, *Crotalus* and *Lachesis* genera (Sales *et al*., 1983). Santoro *et al*. 2009 reported a phosphodiesterase isolated from *Bothrops jararaca* venom which inhibited ADP-induced platelet aggregation. Snake venom phosphodiesterases may produce purine nucleosides such as adenosine which involve in smooth muscle relaxation, vasodilation and cardiovascular effects (Müller and Jacobson, 2011).

Snake venom 5'-nucleotidases are widely distributed among various venomous snakes and hydrolyzes nucleotides into nucleosides. Snake venom 5'-nucleotidases act synergistically with phospholipases and disintegrins to exert

anti-coagulant activities (Dhananjaya *et al*., 2006; Dhananjaya and D'Souza, 2010a). It has been suggested that 5'-nucleotidases are involve in the production of adenosine which contributes to prey immobilization (Aird, 2002). Ouyang and Huang (1983) reported a purified 5'-nucleotidase from *Trimeresurus gramineus* venom as inhibitors for platelet aggregation, it is a thermostable glycoprotein with estimated molecular weight of 74 kDa.

Acetylcholinesterase is abundant in Elapid snake venoms and plays important role in cholinergic transmission (Cousin and Bon, 1999). Elapids acetylcholinesterase is present as highly active non-amphiphilic monomer and catalyzes the hydrolysis of acetylthiocholine (Frobert *et al*., 1997).

Cobra venom factor is a three-chain protein consists of α-chain, β-chain and γ-chain. The protein is highly homologous to complement component C3 (Vogel and Müller-Eberhard, 1984). Cobra venom factor has been found in the venoms of *Naja naja*, *Naja kaouthia* and *Naja atra* (Eggertsen *et al*., 1981; Takahashi and Hayashi, 1982; Vogel and Müller-Eberhard, 1984), *N. melanoleuca* (Osipov *et al*., 2005b) and Australian Elapid snake (Rehana and Kini, 2007). Cobra venom factor is involved in the activation of complement proteins (Van den Berg *et al*., 1991). The activation of complement proteins is responsible for the local inflammation and tissue damages in cobra bites (Vogel and Fritzinger, 2010).

Snake venom is a rich reservoir of nerve growth factor (NGF). In general, snake venom NGFs are non-covalent dimers made up of two identical subunits (Smith *et al*., 1992). Many NGFs have been isolated from *Naja* sp. such as *Naja siamensis* (Inoue *et al*., 1991) and *Naja atra* (Oda *et al*., 1989). Nerve growth factor is a protein that stimulates the differentiation and maintenance of

sympathetic and embryonic sensory neurons (Harper and Thoenen, 1980). However, its role in the toxic action of snake venoms remains unknown (Kostiza and Meier, 1996).

2.4.3 Immunological cross-reactivity of snake venoms

It is well-established that venoms from unrelated snake species contain homologous proteins and therefore exhibit immunological cross-reactivity (Lipps and Khan, 2000 and Stábeli *et al*., 2005). Because of the antigenic similarities of certain venom proteins, antivenoms produced from a particular species often are capable of cross-neutralizing venoms from closely-related species (Furtado *et al*., 2010 and Isbister *et al*., 2010).

2.5 Pharmacokinetics of snake venoms and antivenoms

2.5.1 Pharmacokinetics of snake venoms and venom components

The pathophysiological and pharmacological effects of snake envenomation are related to the absorption and distribution of the venom toxins into systemic circulation and tissues. It has been reported that the serum concentrations of venom antigen in snakebite victims are well correlated with the severity of systemic and local symptoms after envenomation (Hung *et al*., 2003). Pharmacokinetics provides mathematical models to help researchers understand the time course of absorption and distribution of a drug in the body, and allows mathematical quantification of absorption, distribution, metabolism and excretion of the drug (ADME) (Dhillon and Gill, 2006; Ratain and Plunkett, 2003). The model can be applied to the study of ADME of venom or venom toxins. Enzyme-linked immunosorbent assay (ELISA) has been used to measure serum levels of venom antigen and venom toxins, and thus can be used in the study of pharmacokinetics of venom and venom toxins in the body (Amuy *et al*., 1997; Mello *et al*., 2010 and O'Leary *et al*., 2006). Various authors have reported the investigations of the pharmacokinetics of snake venom and occasionally, purified venom toxins using animal models such as mice, rats, rabbits or even dogs (Audebert *et al*., 1994; Guo *et al.,* 1993; Ismail *et al.,* 1998; Jacome *et al.,* 2002; Mello *et al*., 2010; Nakamura *et al.,* 1995; Rocha *et al.,* 2008 and Zhao *et al*., 2001). However, there are limited pharmacokinetic data on cobra venom and venom toxins, in particular the bioavailability of the venom/venom toxins in the circulation following injection of cobra venom. Tseng and his colleagues (1968) first reported absorption and distribution of *Naja naja atra* venom and its purified neurotoxin and cardiotoxin using radioisotope labeling with 131-I in mice. Following subcutaneous injection, it

was shown that the absorption of neurotoxin was faster compared to cardiotoxin and other venom components in whole venom. Furthermore, cardiotoxin was found to deposit in various organs, especially kidneys, liver, spleen and lung whereas neurotoxin accumulated in kidneys. In another study of pharmacokinetics of cytotoxin from *Naja naja atra*, Guo *et al.* (1993) reported a plasma concentration-time profile which follows a two-compartment open model following intravenous administration of the cytotoxin, and the authors reported a rapid absorption of cytotoxin following intramuscular administration of the cytotoxin into rabbits. Ismail *et al*. (1996) described a three-compartment open pharmacokinetic model of African cobra venoms and their α-neurotoxins. The venoms and α-neurotoxins exhibited stronger binding affinity towards 'deep' tissue compartment where their biological actions were exerted.

2.5.2 Pharmacokinetics of antivenom: IgG, Fab and F(ab')$_2$ fragments and their effects on the pharmacokinetics of snake venoms

Antivenoms consist of either IgG, F(ab')$_2$ fragments or Fab fragments, which has molecular weight of 150 kDa, 100 kDa and 50 kDa, respectively. As such, these different forms of antivenom possess different pharmacokinetics properties. Pharmacokinetics of antivenoms generally fit the two-compartment model with bi-exponential curve on semi-logarithm plot (Azuma *et al*., 1991). Typically, the volumes of distribution, elimination half-life and systemic clearance of antivenoms are inversely proportional to the size of the antivenom molecules (Bazin-Redureau *et al*., 1997 and Covell *et al*., 1986). Thus, IgG molecules, which exhibit a longer terminal half-life and slower clearance, are retained longer in the circulation (Quesada *et al*., 2006). The Fab fragments, on the other hand, possess larger volume of distribution but faster clearance and shorter elimination half-life (Theakston and Smith, 1997). The pharmacokinetics characteristics of F(ab')$_2$ lies in between IgG and Fab (Covell *et al*., 1986). The F(ab')$_2$ fragments possess large volume of distribution, and distribute like IgG molecule but exhibit a faster clearance in human (Bazin-Redureau *et al*., 1998).

There are only a few studies that investigated the effects of snake antivenom on the pharmacokinetics of venom in animal models. In an experimental envenomation with *Vipera aspis* venom in rabbit, it was reported that Fab antivenom was less effective in immunoneutralization of the venom compared to F(ab')$_2$. Maung-Maung-Thwin *et al.* (1988) reported a two-compartment open pharmacokinetic model of monovalent antivenom F(ab)'$_2$ fragments in which the distribution of antivenom was closely similar to *Vipera russelli* venom but the elimination rate was almost double. In an experimental envenomation of *Bothrops asper* using a mouse model, intramuscular administration of ovine Fab antivenom

was more effective in neutralization of the myonecrosis and hemorrhage probably due to rapid diffusion of Fab fragments as compared to the larger IgG molecule. However, intramuscular administration of both molecules was ineffective in neutralization of the lethality of the venom in mice due to the poor bioavailability and slow absorption of antivenom by the intramuscular route of administration (Pepin *et al*., 1995 and Pépin-Covatta *et al*., 1996). In another study, $F(ab')_2$ antivenom was shown to be effective in the neutralization of the lethality of *Walterinnesia aegyptia* venom and its toxin in rats, whereas IgG antivenom was only partially effective, and Fab antivenom was not effective at all in neutralizing the lethality of the venom (Ismail *et al*., 1998).

There are numerous reports on the pharmacokinetics of commercial antivenoms during the treatment of envenomed patients. Ho *et al*. (1990) reported that the serum concentrations of monovalent antivenom in patients treated with C*alloselasma rhodostoma* antivenom declined bi-exponentially, with large volume of distribution. Ariaratnam *et al*. (2001) suggested that in *Daboia russelii russelii* envenomed patients, antivenom should be administered in repeated doses in order to completely restore blood coagulability. Ariaratnam e*t al.* (1999) also reported that in envenomation by the Sri Lankan Russell's viper (*Daboia Russelii Russelii*), local and systemic recurrences occurred after administration of the antivenom, suggesting pharmacokinetics and pharmacodynamics mismatch between administered antivenom and the venom.

2.6 Snake venomics

Recent advances in protein identification using mass spectrometry and separation technology make it possible to study the global protein composition of snake venom. The investigation of the proteome of snake venom is also known as snake venomics (Calvete, 2009), which in broader term also includes investigation of the transcriptomics of the venom glands. Snake venomics will make possible an overall understanding of the function and diversity of the venomous system and the genes associated with the venom proteins. Knowledge on the complete (global) protein compositions of the venom provides a strong basis for in-depth comprehension of pathophysiology of envenomation and hence contributes to the improvement of management of envenomation (Ménez *et al*., 2006). Proteomic analysis of snake venoms could also be used in quality control of antivenoms productions (Gutiérrez *et al*., 2009).

2.6.1 Proteomic tools to unravel the protein compositions of venom

Venom proteomics usually includes concurrent identification, quantification, determination of post-translational modifications, biological and functional properties of venom proteins (Fox and Serrano, 2008).

To date, proteomic characterization of venom proteins of a considerable number of medically important venomous snakes have been reported (Ching *et al*., 2012; Makran *et al*., 2012; Petras *et al*., 2011 and Rodrigues *et al*., 2012).

(1) Mass spectrometry identification of venom proteins

The fundamental of snake venom proteomics is the whole venom protein profiling which is possible with the advances in protein identification using mass spectrometry or N-terminal sequencing, as there now exists a rather comprehensive venom protein library (Escoubas *et al*., 2008).

Mass spectrometer typically consists of an ion source, a mass analyzer that measures mass-to-charge ratio (*m/z*) of the ionized samples and a detector that capture the number of ions at each *m/z* values (Aebersold and Mann, 2003). Matrix-assisted laser desorption/ionization (MALDI) and Electrospray ionization (ESI) are the two common techniques applied in mass spectrometry. MALDI is usually coupled to time-of-flight (TOF) analyzers that measures the mass of intact peptides, whereas ESI has been coupled to ion traps and triple quadrupole instruments and used to generate collision-induced (CID) spectra of selected precursor ions (Aebersold and Goodlett, 2001).

(2) Separation techniques

Snake venom proteins can be effectively separated using multifaceted strategies, including multidimensional chromatographic methods such as reverse-phase high-performance chromatography (Fernández *et al*., 2011; Kohlhoff *et al*., 2012 and Nawarak *et al*., 2003), ion exchange liquid chromatography (Binh *et al*., 2010) and electrophoretic methods such as sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Gutiérrez *et al*., 2008b) and more importantly, two-dimensional electrophoresis (Birrell *et al*., 2006; Nawarak *et al*., 2003 and Serrano *et al*., 2005).

Shotgun analysis

In this approach, the venom proteins were digested with trypsin and the mixture was fractionated by reverse-phase HPLC (Li *et al*., 2004) and followed by protein mass profiling using MALDI-TOF (Yanes *et al*., 2007) or CID tandem mass spectrometry (CID-MS/MS) (Sanz *et al*., 2008 and Wagstaff *et al*., 2009). The high quality mass spectra acquired from mass spectrometry provides useful peptide sequences that can be compared to annotated protein database for identification of the protein family (Ménez *et al*., 2006; Fox and Serrano, 2008).

Li *et al*. (2004) reported the proteomic profiles on venom of Chinese cobra, *Naja naja atra* using combination strategy such as shotgun LC-MS/MS, SDS-PAGE coupled to HPLC-MS/MS, gel filtration coupled to HPLC-MS/MS and gel filtration-2DE-MS/MS. A total of 124 novel proteins were identified by combination of all approaches in which high abundance of cardiotoxin were detected corresponds to 56% (or 68 proteins) of total venom proteins.

Petras *et al.* (2011) investigated the proteome of venoms from African spitting cobras using reverse-phase HPLC separation followed by characterization of the venom fractions by N-terminal sequencing, ESI mass spectrometry and SDS-PAGE. Proteomic analysis indicated that venoms from the five species of African spitting cobra have similar toxin compositions, being dominated by three-finger toxins (mainly cytotoxin, 70% of the venom protein) and phospholipase A_2 (22-30%). Snake venom metalloproteinases (SVMPs), Cysteine-rich secretory proteins (CRISPs), endonucleases and nawaprin (present only in certain *N. nigricollis* venom) are also present, but in minute amount. It is interesting to note that generally the spitting cobra venoms contain low content of neurotoxin (from < 0.1% to 4.4%), except for *N. nubiae* which has a neurotoxin content of 12.6%.

Ali *et al*. (2013) reported the proteome of Pakistani *Naja naja* venom using 1DE (SDS-PAGE), 2DE followed by LC-MS/MS and shotgun LC-MS/MS. The proteomic analyses revealed a high abundance of three-finger toxins (mostly of cytotoxins) in *Naja naja* venom. Acidic phospholipase A_2, CRISP, L-amino acid oxidase, SVMP and cobra venom factor were also identified. The authors suggested that the large amount of cytotoxin could be responsible for the myotoxicity, cytotoxicity and local tissue destruction activity of the venom.

The detailed knowledge of the venom compositions as unraveled by proteomics will contribute not only to in-depth understanding on the pathogenesis of envenomation and development of highly effective antivenom but could also lead to discovery of novel drugs as well as biomedical tools for clinical diagnostic (Georgieva *et al*., 2008 and Peichoto *et al*, 2012).