CHAPTER I

INTRODUCTION

1. PROBLEMS AND RESEARCH RATIONALE

The mosquito *Aedes aegypti* is the major vector of dengue and dengue haemorrhagic fever (DHF) (Watts *et al.*, 1987). The global prevalence of dengue has grown dramatically in recent decades. The World Health Organization estimates 50 million cases of dengue infection occurring each year, with 500,000 cases of DHF and approximately 2.5 billion people living in dengue endemic countries (WHO, 2009). The disease is now endemic in more than 100 countries in Africa, America, Eastern Mediterranean, South East Asia and the Western Pacific.

In Thailand, dengue cases have been reported from all regions. In 2009, 25, 194 cases were reported all over the year which were about 72% decrease as compared to 2008 (WHO/SEARO, 2010). The reported cases in 2010 (between January and September) were increased to 57,948 cases. The patterns of dengue cases could be challenging to explain and predict. They were influenced by a large number of different factors including rainfalls, movement of mosquitoes, type of dengue viruses, environmental factors (such as temperature and humidity) and human behaviour.

Control of dengue transmission has been relied on vector control, while a dengue vaccine for public health use is in process. The only effective way to avoid

dengue infection is to avoid being bitten by infected mosquito. Vector control is implemented using environmental management methods through preventing or reducing vector propagation and chemical-based control methods (WHO, 1997). For emergency control to suppress a dengue virus epidemic or to prevent an imminent outbreak, a program of rapid and massive destruction of the *Ae. aegypti* population should be undertaken with insecticide.

After the first DHF outbreak in Thailand in 1958, DDT was widely used to control *Aedes* mosquitoes. The intensive uses of insecticides have led to development of resistance in many insect species. In Thailand, Ae. *aegypti* has been recognized to be resistant to DDT for decade (Neely, 1964). From 1986 to 1993, resistance to malathion, temephos and fenitrothion has also been reported from many regions of Thailand (Chareonviriyaphap *et al.*, 1999). Since 1992, synthetic pyrethroids have been used in agriculture and public health (Chareonviriyaphap *et al.*, 1999). Pyrethroids insecticides are very low toxicity in human and have rapid killing effect on the insect. Studies on insecticide susceptibility of *Ae. aegypti* revealed the occurrence of pyrethroids resistance in all regions of Thailand (Jirakanjanakit *et al.*, 2007, Paeporn *et al.*, 2004, Ponlawat *et al.*, 2005, Somboon *et al.*, 2003, Yaicharoen *et al.*, 2005). Effective vector control is essential to reduce the transmission of dengue, but this is hindered by the development of insecticide resistance in *Ae. aegypti*.

Pyrethroids resistance due to reduced neuronal sensitivity, known as knockdown resistance (*kdr*), is one of the most important mechanisms (Soderlund and Bloomquist, 1990). Voltage-gated sodium channel in nervous system is the primary target site for pyrethroids (Soderlund and Bloomquist, 1989). Insect voltage-

gated sodium channel contains a large (>260 kDa) and complex membrane proteins with the main body of the channel formed by a single polypeptide (the α-subunit). It comprises four homologous domains (I-IV), each containing six transmembrane segments (S1-S6) (Loughney *et al.*, 1989).

Research in the past 10 years provided clear evidence that sodium channel mutations are responsible for *kdr* in many insect species (Soderlund, 2005, Soderlund and Knipple, 2003). It appears that both common and unique sodium channel mutations are associated with pyrethroids resistance. Those amino acid mutations have been shown to reduce the insect sodium channel sensitivity to pyrethroids residing in different domains of the sodium channel, mostly within the sixth transmembrane segments or in the intracellular loops near the fifth and sixth segments (Soderlund, 2005).

Most studies have paid attention on M918T and L1014F mutations since the *kdr* and *super-kdr* strains of housefly were first proofed to have these point mutations in association with resistant phenotype (Williamson *et al.*, 1996). The most common amino acid substitution on the voltage-gated sodium channel protein is L1014F in IIS6. The L1014F mutation has now been identified in at least a dozen additional insect species but not in any *Ae. aegypti* -pyrethroids resistance (Davies *et al.*, 2007). In addition, more than 30 amino acid mutations in insect sodium channel protein have been identified to involve the reducing channel sensitivity to insecticides (Davies *et al.*, 2007).

Several mutations, i.e. G923V, L982T, S989P, I1011M, I1011V, V1016I, V1016G and D1794Y, have been identified in the voltage-gated sodium channel gene of *Ae. aegypti* from various countries and shown to confer pyrethroids resistance

(Brengues *et al.*, 2003, Chang *et al.*, 2009, Saavedra-Rodriguez *et al.*, 2007). Most of these amino acid mutations were located within domain II, whereas the D1794Y mutation was found within the domain IV. Two mutations, S989P and V1016G, have been observed in pyrethroids resistant-*Ae. aegypti* individuals from Thailand (Brengues *et al.*, 2003, Srisawat *et al.*, 2010).

In northern Thailand, a study on insecticide susceptibility in *Ae. aegypti* had indicated resistance to DDT and permethrin (Somboon *et al.*, 2003). DDT/permethrin resistant strain of *Ae. aegypti*, PMD-R, was established and selected from field caught insects in 1997. Biochemical and molecular studies demonstrated that elevated DDTase activity was the major mechanism involved in DDT resistance in this strain. Since pyrethroids resistance was not particularly characterized by alteration of metabolic parameters (Prapanthadara *et al.*, 2002, Prapanthadara *et al.*, 2005), pyrethroids resistance may mainly involve knockdown resistance.

2. LITERATURE REVIEW

2.1 Insecticide resistance mechanisms

Most insecticide resistance mechanisms can be divided into two groups, metabolic detoxification and target site resistance. Alone or in combination, these mechanisms confer resistance to all of the available classes of insecticides (Hemingway *et al.*, 2004).

Metabolic detoxification mechanisms

There are three major enzyme groups which are responsible for metabolically based resistance to organochlorines, organophosphates, carbamates and pyrethroids. The detoxification enzyme-based resistance occurs when enhanced levels or modified activities of carboxylesterases, cytochrome P450 monooxygenases or glutathione S-transferases (GST), preventing the insecticide from reaching its site of action. DDT-dehydrochlorinase was first recognized as a glutathione S-transferase in the housefly, *Musca domestica* (Clark and Shamaan, 1984). It has been shown to have this role commonly in *Anopheline* and *Aedes* mosquitoes (Grant and Matsumura, 1988, Prapanthadara *et al.*, 1995). Carboxylesterases are often involved in organophosphate and carbamate. Cytochrome P450 monooxygenases are involved in the metabolism of pyrethroids and the activation and/or detoxification of organophosphorus insecticides.

Target site mechanisms

Alterations of amino acids responsible for insecticide binding at its site of action cause the insecticide to be less effective or even ineffective. The target of organophosphorus and carbamate insecticides is acetylcholinesterase in nerve synapses, and the target of organochlorines (DDT) and synthetic pyrethroids is the sodium channel of the nerve sheath. DDT-pyrethroids cross-resistance may be produced by single amino acid changes (one or both of two known sites) in the axonal sodium channel insecticide-binding site (Miyazaki *et al.*, 1996, Williamson *et al.*, 1996). This cross-resistance appears to produce a shift in the sodium current activation curve and causes low sensitivity to pyrethroids (Vais *et al.*, 1997).

Similarly, cyclodiene (dieldrin) resistance is conferred by single nucleotide changes within the same codon of a gene for a γ-aminobutyric acid (GABA) receptor (Ffrench-Constant *et al.*, 1998). At least five point mutations in the acetylcholinesterase insecticide-binding site have been identified, singly or in concert causing various degrees of reduced sensitivity to organophosphorus and carbamate insecticides (Mutero *et al.*, 1994).

2.2 Mammalian voltage-gated sodium channels: Structure and function

Mammalian voltage-gated sodium channels contain one large α-subunit and one or two smaller β-subunits (*e.g.* β1and β2) (Catterall, 2000). The α-subunit is composed of four homologous domains (I–IV) connected by cytoplasmic linkers (Figure1.1) (Catterall, 2000, Morgan *et al.*, 2000, Yu and Catterall, 2003). Each of these domains contains six putative transmembrane segments (S1–S6). The four

domains fold together in a clockwise orientation, where domains I and IV are brought into close proximity, to form the outer pore vestibule and the selectivity filter. This is created by the S5–S6 linker loops from each domain that form re-entrant pore loops (P) that dip into the transmembrane region of the protein (Catterall, 2000, Liu *et al.*, 2001) (Figure 1.2). Each S4 segment, which is the most conserved segments, has positively charged amino acids (either arginine or lysine) at intervals of three residues and transport gating charges outward. Thus, these segments function as voltage sensors to initiate voltage dependent activation by moving outward under the influence of changes in the electric field (Cestele *et al.*, 2006, Chanda and Bezanilla, 2002).

Sodium channel inactivation is mediated by a short intracellular loop connecting domains III and IV, containing the key hydrophobic amino acid residues isoleucine, phenylalanine and methionine (IFM) (West *et al.*, 1992) (Figure 1.1). The α-subunit is also associated with one or two smaller auxiliary subunits (β1, β2, β3 and/or β4) of approximately 30 kDa that are required for normal kinetics and voltage-dependence of gating but are not required for ion flux, ionic selectivity and pharmacological modulation (Yu and Catterall, 2003) (Figure 1.1).

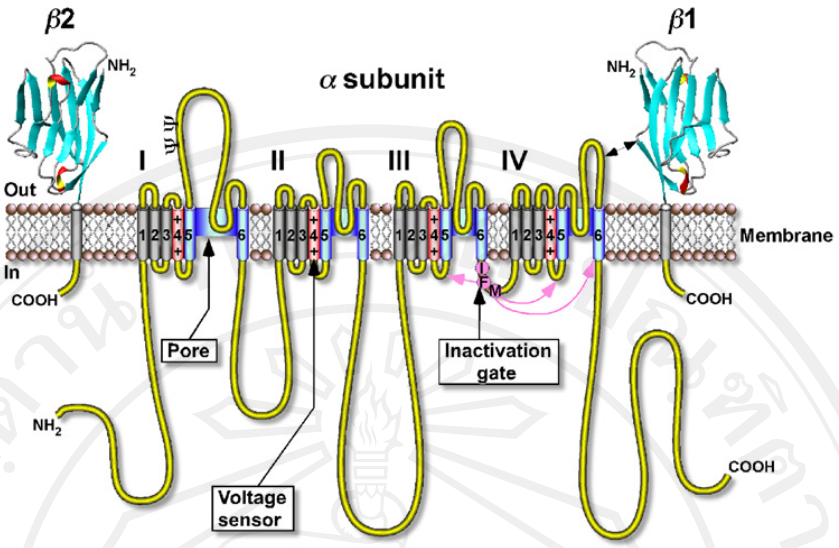


Figure 1.1 Schematic representation of the subunit structure of the voltage-gated sodium channel (Nicholson, 2007).

The structures showed the functional *α*-subunit (center) comprising four homologous domains (I–IV) and auxiliary *β*-subunits. Cylinders (S1–S6) represent putative transmembrane *α*-helical segments within each domain where the charged S4 segments (red) represent the voltage sensors. The polypeptide chain is represented by the yellow ribbon and is approximately proportional to the length of the amino acid chain. The inactivation gate (magenta) is represented by the inactivation particle (hydrophobic residues IFM with magenta arrows indicating the sites thought to form the inactivation gate receptor. The pore-lining segments S5 and S6, and intervening SS1/SS2 (*P* loop) that form the walls of the ion-conducting pathway are shown in blue. The extracellular domains of the *β*1 and *β*2 subunits are shown and represented as immunoglobulin-like folds similar to myelin protein *P*0. Ψ, sites of probable *N*-linked glycosylation.

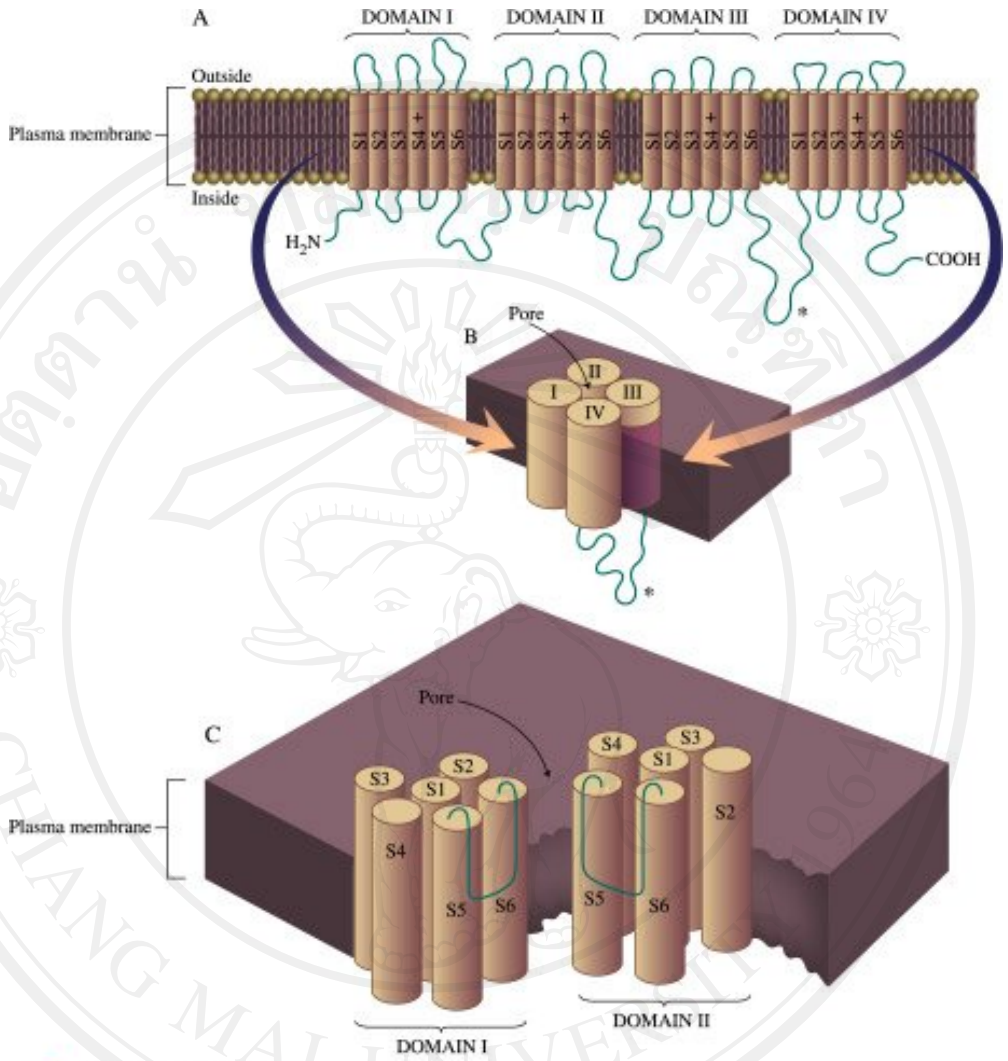


Figure 1.2 The transmemebrane topology of the voltage-gated sodium channel (Mathews, 2001).

The voltage-gated sodium channel α-subunit (A). The four homologous domains (I-IV) fold together in a clockwise orientation to form the outer pore vestibule (B). The selectivity filter and re-entrant pore loops (that dip into the transmembrane region of the protein) are formed by transmembrane segments S5 and S6 together with the membrane-reentrant segments that are part of loop connecting S5 and S6 of each domain (C).

Generation of an action potential

The extracellular fluid surrounding the insect axonal membrane contains a high concentration of sodium ions (Na^+) and a low concentration of potassium ions (K^+), whilst the reverse is true for the inside of the nerve cell. At the resting potential (Figure 1.3A) the axonal membrane is relatively permeable to K^+ but not Na^+. The sodium channel is closed because the activation gate (m-gate) blocks the channel pore. This makes the inside of the cell negative with respect to the outside. Membrane depolarization leads to conformational changes at the activation gate (m-gate) which this opens the channel for a few milliseconds, allowing influx of Na^+ (Figure 1.3B). This causes the inside of the axon to become transiently positive and generates the rising phase of the action potential.

This voltage-dependent activation leads to closing of the inactivation-gate (h-gate) within 1 – 2 milliseconds (fast inactivation). Now the channel is in an inactivation state due to block of the intracellular mouth of the pore (Figure 1.3C). This causes an efflux of K^+ as a result of opening of potassium channels and generates the falling phase of the action potential. Inactivation is coupled to activation and is faster from the open state but has little, or no, voltage dependence. Membrane repolarization leads to a reversal of the conformation change at the activation gate (m-gate) which closes the channel pore (deactivation) (Figure 1.3D). Both gates are now closed (since the inactivation (h) gate is still blocking the pore), but within a refractory period of 2 - 5 milliseconds the conformational changes of the activation gate (m-gate) force the inactivation gate (h-gate) to reopen bringing the channel back to the closed state.

An ATP driven Na^+- K^+ pump maintains the ion gradient across the axonal membrane and restores the resting potential. The generation of the action potential results in sequential depolarization of neighboring regions of the axon, resulting in a wave of depolarization along the axon.

Sodium channel as targets for neurotoxicants

Voltage-sensitive sodium channel is the site of action of a wide structure variety of naturally occurring neurotoxins that contribute to the chemical ecology of predation and defense (Cestele and Catterall, 2000, Wang and Wang, 2003). These sites, together with binding sites for synthetic neurotoxicants and drugs, identify at least 10 distinct binding domains associated with the voltage-sensitive sodium channel (Table 1.1).

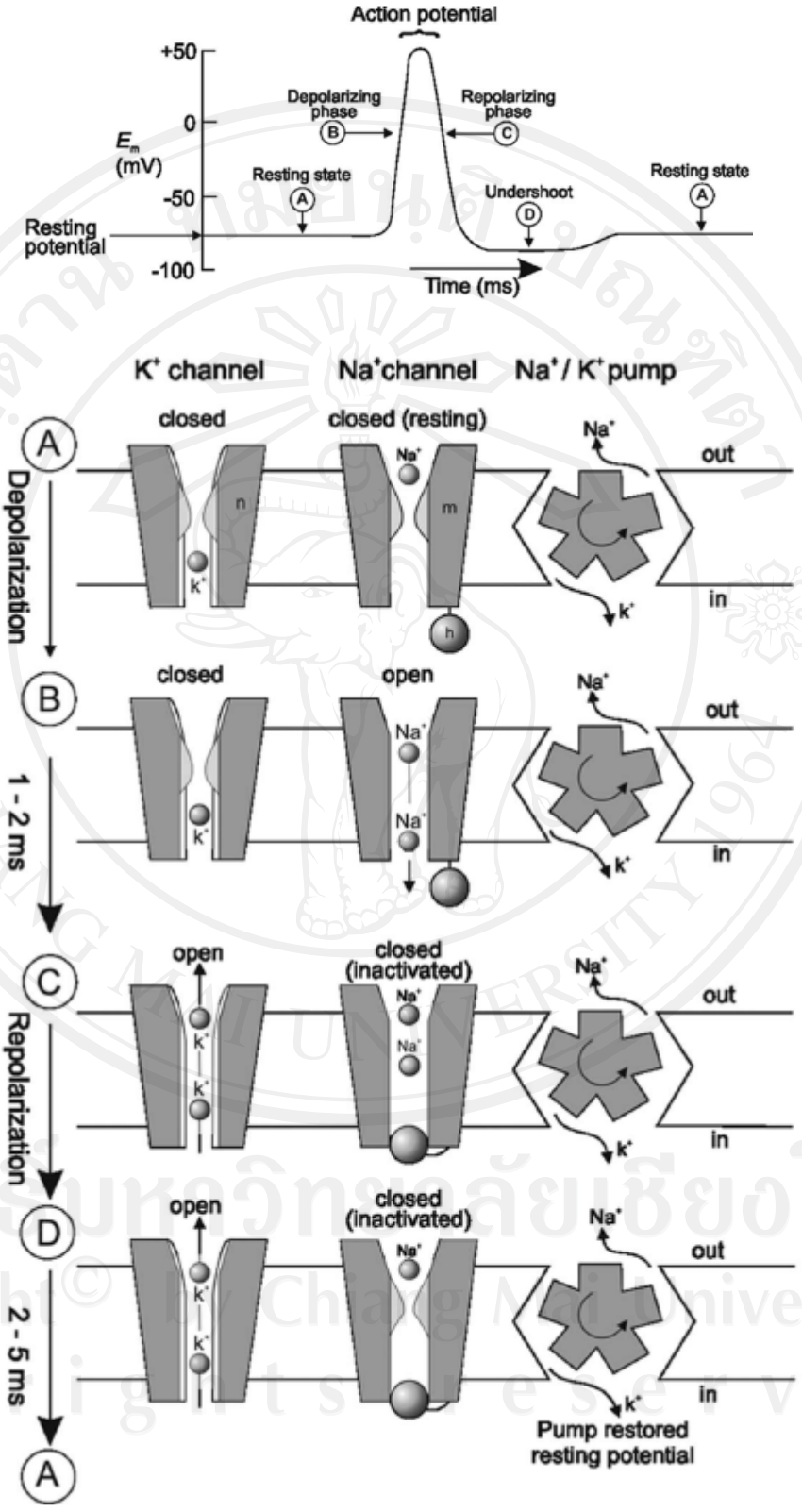


Figure 1.3 Generation of an action potential (Davies *et al.*, 2007).

Table 1.1 Neurotoxin that target the voltage-gated sodium channel and their corresponding receptor sites (Soderlund, 2005, Wang and Wang, 2003).

Receptor site	Neurotoxins	Physiological effects	Putative location
1	Tetrodoxin, saxitoxin, μ-Conotoxin	Inhibition of Na^+ permeability	p-loop at D1, D2, D3, D4
2	Batrachotoxin, veratridine, aconitine, grayanotoxin	Persistent activation, depolarization of resting potentials, repetitive firings	D1-S6, D2-S6 D3-S6, D4-S6
3	α-scorpion toxins, sea anemone II toxins, δ-atracotoxins	Prolonged sodium channel opening	D4: S3–S4, Loop D1: S5–S6, Loop D4: S5–S6, Loop
4	β-scorpion toxins	Shifts in activation gating; repetitive firings	D2: S3–S4, Loop
5	Brevetoxins, ciguatoxins	Shifts in activation gating	D1:S6, D4:S5
6	δ-conotoxins	Prolonged sodium channel opening	?
7	DDT, Pyrethroids	Prolonged sodium channel opening	D1-S6, D2-S6, D3-S6
8	Conus stratius toxin	Prolonged sodium channel opening	?
9	Gonioporatoxin	Prolong inactivation	?
10	Local anesthetics, anticonvulsants	Inhibition of Na^+ permeability	D1-S6, D3-S6, D4-S6

2.4 Insect voltage-gated sodium channels: Structure and function

In the late 1980s, two putative voltage-gated sodium channel genes, *DSC1* and *para*, were isolated from *Drosophila melanogaster* (Loughney *et al.*, 1989, Salkoff *et al.*, 1987). They were found to be structurally and functionally homologous with the α-subunit of mammalian voltage-gated sodium channel (Figure 1), consisting of four internally homologous domains (I-IV) connected by intracellular linkers with each domain consisting of six membrane-spanning segments (S1-S6) joined by intracellular or extracellular

Insect voltage-gated sodium channel gene

1. *DSC1* and Ortholog

The first putative insect sodium channel α-subunit gene was isolated from *D. melanogaster* DNA libraries using an eel (*Electrophorus electricus*) sodium channel gene probe (Salkoff *et al.*, 1987). The *DCS1* locus was localized at cytogenetic region 60E5 on chromosome 2R by genome sequencing. A *DSC1* orthologous gene was later identified from German cockroach (*Blattella germanica*) and designated *BSC1*. Like sodium channel α-subunits, *DSC1* and *BSC1* have four homologous domains, each having six transmembrane segments. Until recently, *DSC1* and *BSC1* had been predicted to encode a voltage-gated sodium channel based on their overall similarity of deduced amino acid sequence and domain organization to eel and mammalian sodium channel proteins (Littleton and Ganetzky, 2000). However, later study showed that *BSC1* and *DSC1* encode a novel family of Ca^{2+}-selective cation channels, not sodium channels (Zhou *et al.*, 2004).

2. *Para* (Paralytic-temperature-sensitive) and Orthologs

The *para* gene was isolated in *D. melanogaster* with a temperature-sensitive paralytic phenotype (Loughney *et al.*, 1989). The overall structure and the amino acid sequence of the *para* sodium channel share a high similarity with those of mammalian sodium channel α-subunits. The structural features that are critical for mammalian sodium channel function, including residues crucial for sodium selectivity, are conserved in the *para* sodium channel (Loughney *et al.*, 1989). Most importantly, subsequent functional expression and characterization in *Xenopus* oocytes conclusively demonstrated that *para* gene encodes a sodium channel (Feng *et al.*, 1995, Warmke *et al.*, 1997). Therefore, it seems that *para* is the only gene that encodes the sodium channel in *Drosophila* and presumably in other insect species as well.

The *para* orthologous genes have been isolated from several medical and agricultural important insect pest species (Soderlund and Knipple, 2003). In most cases, only partial cDNA clones were obtained. Full-length cDNA clones are available only for three *para* orthogouses gene, *Vssc1* from the house fly (Ingles *et al.*, 1996), $BgNa_v$ from the German cockroach (Dong, 1997) and $VmNa_v$ from the varroa mite (Wang *et al.*, 2003).

3. *TipE* (Temperature-induced paralysis, locus E) and Orthologs

The *TipE* gene was isolated from *D. melanogaster*. The *TipE* gene, at cytogenetic region on Chromosome 3L, is the site of a temperature-sensitive paralytic mutation having a phenotype similar to that of *para* mutants (Feng *et al.*, 1995). The *TipE* protein has two transmembrane segments connected by a large

extracellular loop and intracellular amino acid and carboxyl termini (Feng *et al.*, 1995). Coexpression of *TipE* with *para* in *Xenopus* oocytes increases the amplitude of peak current and modifies the kinetics of fast inactivation (Feng *et al.*, 1995, Warmke *et al.*, 1997). It was suggested that *TipE* functions as an auxiliary subunit like the β-subunits of mammalian sodium channels. Recently four *TipE* homologs (TEH1, 2, 3, 4) were found in *D. melanogaster* and their predicted secondary structures resemble those of *TipE* (Derst *et al.*, 2006).

The *TipE* orthologous gene was found in *Musca domestica* and designed *Vsscβ* (Lee *et al.*, 2000). The predicted *Vsscβ* protein exhibited 72% amino acid sequence identity to the *TipE* protein sequence and 97% identity within the two hydrophobic segments identified as probable transmembrane domains (Lee *et al.*, 2000). The *Vsscβ* gene of housefly appears to fulfill the same role as *TipE*. Coexpession of *Vsscβ* with the housefly sodium channel α subunit (*Vssc1*) in oocytes enhanced the level of sodium channel current expression (Tan *et al.*, 2002).

Insect sodium channel as insecticide targets

Sodium channels are targets of variety of neurotoxins including naturally occurring toxins from animals and plants, therapeutic drugs, and synthetic insecticide. Insect sodium channels are also the primary target site of synthetic insecticides, DDT, pyrethroids and oxadiazines.

1. DDT (Dichlorodiphenyltrichloroethane)

DDT was first synthesized in 1894, and its insecticidal properties were described by Paul Muller in the late 1930s. It is a persistent insecticide that was used worldwide from the mid-1940s. It binds to the voltage-gated sodium channel

and keeps the channel locked open. Prolonged influx of sodium ions causes the nerves firing repeatedly and this causes death of the insects.

2. Pyrethroids

Pyrethroids are synthetic structural derivatives of insecticidal pyrethrins present in the pyrethrum extract of *Chrysanthemum* species (Elliott, 1977). They cause prolonged opening of sodium channels primarily by inhibiting channel deactivation and stabilizing the open configuration of the sodium channel (Raymond-Delpech *et al.*, 2005). The prolonged channel opening is evidenced by a large tail current associated with repolarization under voltage-clamp conditions.

3. Oxadiazines

Indoxacarb is a new oxadiazine insecticide with high insecticidal activity and low mammalian toxicity (Wing *et al.*, 1998, Wing *et al.*, 2000, Wing *et al.*, 2005). In insects, indoxacarb is metabolically converted to *N*-decarbomethoxyllated JW062 (DCJW), which is more active than its parental compound (Wing *et al.*, 1998, Wing *et al.*, 2000). The studies showed that sodium channel is a major cellular target of indoxacarb and DCJW (Wing *et al.*, 2005).

2.5 Insect sodium channel gene and knockdown resistance

Knockdown resistance

The term “Knockdown Resistance” (*kdr*) is used to describe cases of resistance to diphenylethan (*e.g.* DDT) and pyrethroids insecticides in insects and other arthropods. The *kdr* trait confers resistance to the rapid knockdown action and lethal effects of DDT and pyrethroids (Soderlund and Knipple, 1999, Soderlund and

Knipple, 2003). *Kdr* is characterized by a reduction in the sensitivity of the insect nervous system to these compounds. *Kdr* is caused by one or more point mutations in the insect sodium channel protein that renders it less sensitive to the toxic effects of these insecticides. The *kdr* trait was first documented in houseflies in 1951 (Busvine, 1951) and isolated genetically in 1954 (Milani, 1954). A second resistance trait in the housefly (designated *super-kdr*), confering much greater resistance to DDT and pyrethroids than that found in *kdr* strain, was also isolated genetically (Soderlund and Knipple, 2003). In the early 1990s, several groups conducted genetic linkage analysis and showed that *kdr* mutations were linked to the *para*-orthologous genes in several insect species (Soderlund and Knipple, 2003). Subsequent molecular analyses led to the identification of multiple point mutations in the *para*-orthologous genes that are associated with *kdr*-type resistance to pyrethroids in these insects (Soderlund, 2005).

Sodium channel gene mutations associated with knockdown resistance

The genetic linkage of *kdr* traits and sodium channel gene sequence provided a strong impetus for the identification of resistance-associated mutations in insect sodium channel genes. Comparison of partial and complete sequences from 15 housefly strains representing multiple examples of susceptible, *kdr* and *super-kdr* phenotypes consistently identified two point mutations that were associated with resistant phenotypes: mutation of leucine to phenylalanine at amino acid residue 1014 (L1014F) in all *kdr* and *super-kdr* strains, and the additional mutation of methionine to threonine at residue 918 (M918T) only in *super-kdr* strains (Ingles *et al.*, 1996, Miyazaki *et al.*, 1996, Williamson *et al.*, 1996). Mutations in *para*-orthologous

sodium channel gene sequences corresponding to the L1014F mutation and the L1014F with M918T mutation in the housefly have been also identified to date in thirteen and three additional pest species respectively (Table 1.2) (Davies *et al.*, 2007). Further studies have also identified a number of additional amino acid substitutions in resistant insect strains that reduce sensitivity to pyrethroids and DDT (Table1.2). Several of these are clustered on the S4/S5 linker and S5 or S6 helics of domain II, or in the corresponding linker/ helices of domain I and III. These amino acids changes have been found in arrangement of importance agriculture pests and disease vectors (Table 1.2).

Table 1.2 Sodium channel amino acid sequence polymorphisms associated with knockdown resistance (Davies *et al.*, 2007).

1. Domain II S4-S6 associated mutations

Mutations identified [a]	Species	Common name
L1014F	*Anopheles arabiensis*	African malaria mosquito
	Anopheles gambiae	African malaria mosquito
	Anopheles sacharovi	-
	Anopheles stephensi	Indian malaria mosquito
	Blattella germanica	German cockroach
	Ctenocephalides felis	Cat flea
	Culex pipiens	House mosquito
	Culex quinquefasciatus	Southern house mosquito
	Cydia pomonella	Codling moth
	Leptinotarsa decemlineata	Colorado potato beetle
	Liriomyza huidobrensis	South American leafminer
	Liriomyza sativae	Vegetable leafminer
	Musca domestica	House fly
	Myzus persicae	Peach potato aphid
L1014H	*Heliothis virescens*	Tobacco budworm
	Liromyza trifolii	American serpentine leafminer
	Musca domestica	House fly
L1014S	*Anopheles arabiensis*	African malaria mosquito
	Anopheles sacharovi	-

Table 1.2 Sodium channel amino acid sequence polymorphisms associated with knockdown resistance (Davies *et al.*, 2007) . (Continued)

Mutations identified [a]	Species	Common name
L1014S	*Anopheles gambiae*	African malaria mosquito
	Culex pipiens	House mosquito
L1014F+L1014S	*Anopheles gambiae*	African malaria mosquito
I1011M+V1016G	*Aedes aegypti*	Yellow fever mosquito
S1020F	*Blattella germanica*	German cockroach
M918T+L1014F	*Haematobia irritans*	Horn fly
	Musca domestica	House fly
	Liriomyza huidobrensis	South American leafminer
	Myzus persicae	Peach potato aphid
T929I+L1014F	*Plutella xylostella*	Diamondback moth
T929C+L1014F	*Franklimiella occidentalis*	Western flower trip
T929V+L1014F	*Ctenocephalides felis*	Cat flea
F979S+L1014F	*Myzus persicae*	Peach potato aphid
M918L	*Aphis gossypii*	Cotton aphid
M918V	*Bemisia tabaci*	Tobacco whitefly
L925I	*Bemisia tabaci*	Tobacco whitefly
T929V	*Bemisia tabaci*	Tobacco whitefly
	Ctenocephalides felis	Cat flea
M827I+T929I+L932F	*Pediculus capitis*	Head louse

Table 1.2 Sodium channel amino acid sequence polymorphisms associated with knockdown resistance (Davies *et al.*, 2007).

(Continued)

2. Mutations outside of domain II

Mutations identified [a]	Location	Species	Common name
V410M	IS6	*Heliothis virescens*	Tobacco budworm
F1538I	IIIS6	*Boophilus microplus*	Southern cattle tick
L1014F+E435K+C785R	DI – II linker	*Blattella germanica*	German cockroach
L1014F+D59G+E435K+C785R+P1899L	N-terminus; DI – II linker; C-terminus	*Blattella germanica*	German cockroach
I253N; A1410V; A1494; M1524I	DI S4 – S5 linker; DIII S4 – S5 linker; DIII P-loop; IIIS6	*Drosophila melanogaster*	Common fruit fly
D1549V+E1533G	DIII-DIV linker	*Helicoverpa armigera*	Cotton bollworm
		Heliothus virescens	Tobacco budworm
L1595P+M1823I	DIII-DIV linker; IVS6	*Varroa destructor*	Honeybee mite
F1528L+L1595P+I1752V+ M1823I	IIIS6; DIII-DIV linker; IVS5; IVS6	*Varroa destructor*	Honeybee mite

[a] Position numbered according to the amino acid sequence of the most abundant splice variant of the housefly para-type sodium channel protein (Williamson *et al.*, 1996).

Knockdown resistance (*kdr*) to pyrethroids in *Aedes aegypti*

The sodium channel mutation associated with knockdown resistance (*kdr*) to pyrethroids has been observed in several insect species including the mosquitoes *An. gambiae* (Martinez-Torres *et al.*, 1998), *Cx. pipiens* (Martinez-Torres *et al.*, 1999) and *Ae. aegypti* (Brengues *et al.*, 2003). The L1014F mutation in IIS6 associated with *kdr* is identified in the sodium channel gene of *Culex* and *Anopheles* species but it has been not found in that of *Ae. aegypti*.

To date, a total of eight amino acid mutations, i.e. G923V, L982T, S989P, I1011M, I1011V, V1016I, V1016G and D1794Y, have been observed in the sodium channel gene of *Ae. aegypti* and shown to confer pyrethroids resistance (Brengues *et al.*, 2003, Chang *et al.*, 2009, Saavedra-Rodriguez *et al.*, 2007). Four of these, G923V, L982T, I1011M and V1016G, were identified in IIS6 of the sodium channel gene in a study of 11 pyrethroids resistant *Ae. aegypti* populations collected from six different countries (Brengues *et al.*, 2003). Two mutations, I1011V and V1016I, located within IIS6 were discovered in *Ae. aegypti* from Latin-America and the V1016I mutation has been positively correlated with resistance to pyrethroids (Saavedra-Rodriguez *et al.*, 2007).

The V1016G has been originally reported in a permethrin-resistant strain of *Ae. aegypti* from Thailand and Indonesia (Brengues *et al.*, 2003). The analysis of 180 individual mosquitoes collected from Thailand revealed that the V1016G was widely distributed in this country with an allele frequency of 0.23 (Rajatileka *et al.*, 2008). In addition, the V1016G mutation was found to be concurrent with the D1794Y mutation, located within domain IV, in a permethrin-resistant Per-R strain of *Ae. aegypti* (Chang *et al*., 2009). The combination of the both mutations provided highly

increasing knockdown resistance to permethrin. Recently, the co-existence of the V1016G and S989P mutations was observed in deltamethrin-resistant Khu Bua strain of *Ae. aegypti* (Srisawat *et al*., 2010).

2.5 The simplified high throughput methods for detection of the voltage-gated sodium channel mutations associated with knockdown resistance

The detection of the voltage-gated sodium channel mutations associated with *kdr* to pyrethroids is a prerequisite for resistance management strategies aimed at prolonging insecticide life while maintaining sufficient insect control. This type of monitoring requires rapid high-throughput assays.

There are currently several different methods available for detecting the amino acid substitution responsible for *kdr*, L1014F and L1014S, in *An. gambiae*. The most widely used method is based on Allele Specific PCR (AS-PCR) (Martinez-Torres *et al.*, 1998, Ranson *et al.*, 2000), but more recently a number of other assays have been described including Hot Oligonucleotide Ligation Assay (HOLA) (Lynd *et al.*, 2005), Sequence Specific Oliganucleotide Probe Enzyme-Linked Immunosorbent Assay (SSOP-ELISA) (Kulkarni *et al.*, 2006), Fluorescence Resonance Energy Transfer (FRET) / Melt Curve analysis (Verhaeghen *et al.*, 2006), PCR elongation with fluorescence (Tripet *et al.*, 2006), Real-time TaqMan assay and High Resolution Melt (HRM) analysis (Bass *et al.*, 2007). The performance and relative advantages and disadvantages of these assays were compared under comparable conditions (Bass *et al.*, 2007). The real-time TaqMan assay was both the most sensitive (with the

lowest number of failed reactions) and the most specificity (with the lowest number of incorrect scores).

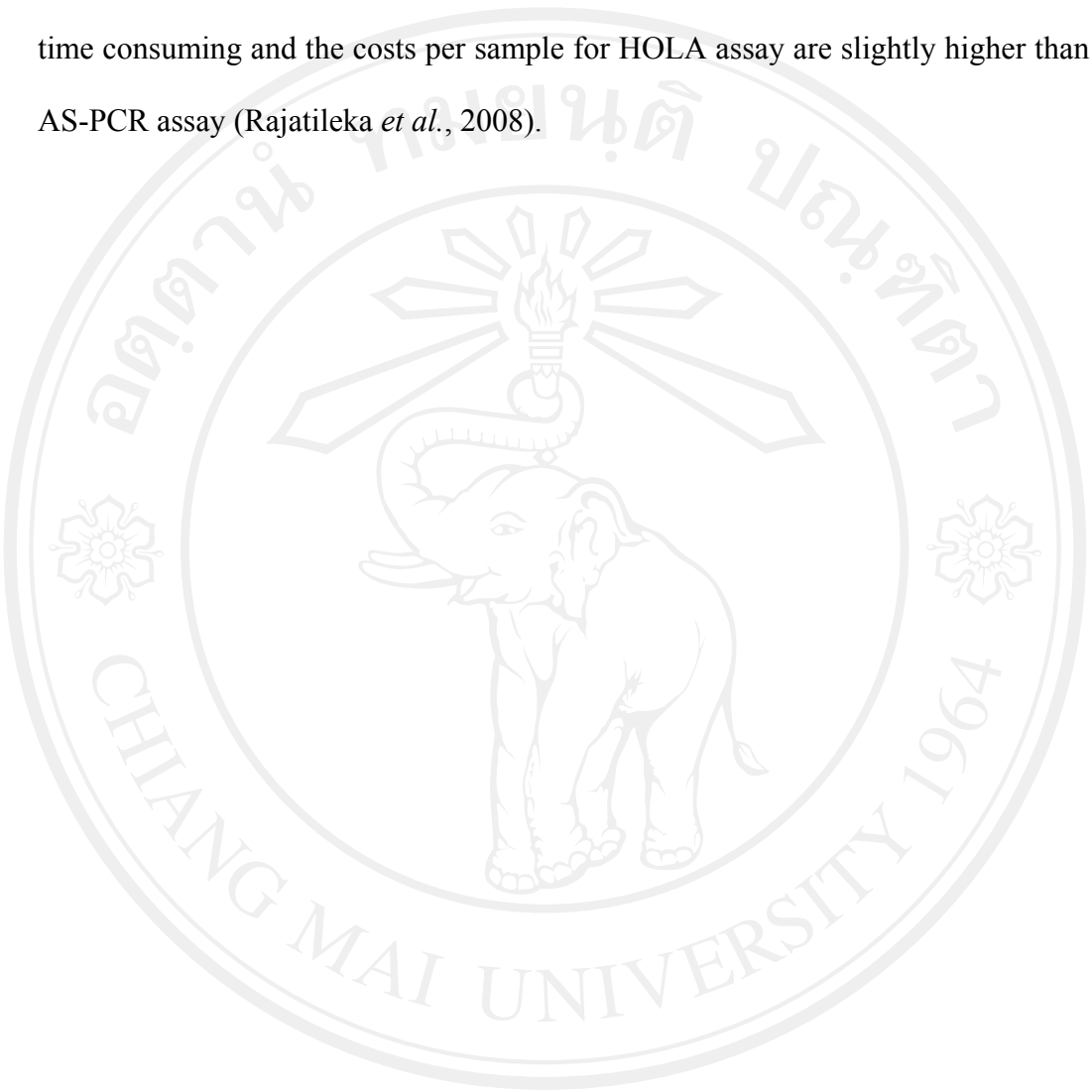
However, the running cost of this assay was slightly higher than the AS-PCR and HRM assays but comparable to other methods (Bass *et al.*, 2007). The sensitivity and specificity of AS-PCR, SSOP-ELISA and HOLA method were fairly similar with a small number of failures and incorrect scores, while HRM analysis gave the lowest of sensitivity and specificity with a higher rate of failures and incorrect scores (Bass *et al.*, 2007).

The most commonly used of method for identifying the *kdr* in malaria endemic countries is the AS-PCR method, probably due to its relatively low cost (both capital expenditure and running costs). However, a number of reports have questioned the reliability of this technique (Pinto *et al.*, 2006, Verhaeghen *et al.*, 2006).

Two methods have been used to detect the presence of the *Ae. aegypti kdr* allele and estimate its frequency from large numbers of individuals (Rajatileka *et al.*, 2008, Saavedra-Rodriguez *et al.*, 2007). The first is an AS-PCR assay for identifying all four reported substitutions within IIS6, I1011V, I1011M, V1016I and V1016G, the results of which can be read either on an agarose gel or as a melting curve on the real-time PCR machine (Saavedra-Rodriguez *et al.*, 2007). This method was a rapid and relatively inexpensive for surveying *kdr* genotypes but it had some problems with resolution of the very small amplicons by agarose gel electrophoresis (Saavedra-Rodriguez *et al.*, 2007).

The second method based on a HOLA assay was adapted from Lynd *et al* (2005) to detect these mutations (Rajatileka *et al.*, 2008). The HOLA method is more

reliable, scoring of the plates was less ambiguous and requiring no electrophoresis equipment (Rajatileka *et al.*, 2008). However this technique is more complex and time consuming and the costs per sample for HOLA assay are slightly higher than the AS-PCR assay (Rajatileka *et al.*, 2008).



3. PURPOSES OF THE STUDY

3.1. Determination of point mutations in voltage-gated sodium channel gene of the DDT/pyrethroids resistant *Ae. aegypti* strain compared to the susceptible strain.

3.2. To screen the mutations from the voltage-gated sodium channel region where putative amino acid mutations were previously reported to correlate with insecticide resistance.

3.3 Characterization of the genetic inheritance of permethrin resistance and the mutant allele based on reciprocal crosses between the susceptible and resistant mosquito strains and backcrosses.

3.4. Development of the simplified high throughput methods for detection of the voltage-gated sodium channel mutations associated with knockdown resistance in *Ae. aegypti* and compare the performance of these different methods.

3.5 The presence of this specific point mutation in resistant insects was investigated in field population of *Ae. aegypti* to confirm the correlation between the mutation and permethrin resistance.

4. SIGNIFICANT OF THE REASERCH

Pyrethroids are continuing relied to control outbreaks of dengue and those affecting human health. Understanding of voltage-gated sodium channel mutation and the association with DDT/pyrethroids resistance in *Ae. aegypti* will become a key priority in the development of strategies for resistance management and dengue control programs. Moreover, the establishment of a simplified high throughput method for identifying the mutation will be useful as a tool for monitoring resistance in field population.