Tropical Biomedicine 32(1): 140–150 (2015)

Low efficacy of delthamethrin-treated net against Singapore *Aedes aegypti* is associated with *kdr*-type resistance

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Received 7 January 2014; received in revised form 18 August 2014; accepted 19 August 2014

Abstract. There has been a worldwide surge in the number and severity of dengue in the past decades. In Singapore, relentless vector control efforts have been put in to control the disease since the 1960's. Space spraying, fogging, chemical treatment and source reduction are some commonly used methodologies for controlling its vectors, particularly *Aedes aegypti*. Here, as we explored the use of a commercially available delthamethrin-treated net as an alternative strategy and the efficacy of the treated net was found to be limited. Through bioassays and molecular studies, the failure of the treated net to render high mortality rate was found to be associated with the knockdown resistance (*kdr)* mutation. This is the first report of *kdr*- mutations in Singapore's *Ae. aegypti*. At least one point mutation, either homozygous or heterozygous, at amino acid residue V1016G of DIIS6 or F1269C of DIIIS6 was detected in 93% of field strains of *Ae. aegypti*. Various permutations of wild type and mutant amino acids of the four alleles were found to result in varying degree of survival rate among local field *Ae. aegypti* when exposed to the deltamethrin treated net. Together with the association of higher survival rate with the presence of both V1016G and F1269C, the data suggest the role of these mutations in the resistance to the deltamethrin. The high prevalence of these mutations were confirmed in a country wide survey where 70% and 72% of the 201 *Ae. aegypti* analysed possessed the mutations at residues 1016 and 1269 respectively. The highest mutated frequency combination was found to be heterozygous alleles (VG/FC) at both residues 1016 and 1269 (37.8%), followed by homozygous mutation at allele 1269 (24.4%) and homozygous mutation at allele 1016 (22.9%). The *kdr*- type of resistance among the vector is likely to undermine the effectiveness of pyrethroids treated materials against these mosquitoes.

INTRODUCTION

Dengue Fever, a mosquito-borne febrile disease, has become a major international public health challenge especially in countries situated in the tropical and sub-tropical regions of the world (WHO 2009), including Singapore. Singapore's first reported case of dengue fever occurred in 1901, with all 4 serotypes circulating all year round. The implementation of law enforcement in 1969 suppressed the flare of dengue epidemics however, it further led to successive episodes of dengue outbreaks in 1986, 1989, 1992, 1998 and 2004 with increasing dengue incidence from 16.7 per 100,000 to 223.1 per 100,000 from 1987 to 2004 respectively (FW 1904; WHO 1990; Committee of Epidemic Diseases 1993; Committee of Epidemic Diseases 1999; Kita, 2005; Koh *et al.*, 2008). Dengue cases increased in 2005 with 14209 reported cases.

In later years, dengue cases fluctuated between 8826 and 3127 cases from year 2006 to 2012 (MOH).

Aedes aegypti is the primary vector responsible for major outbreaks in many Southeast Asian countries. In the absence of specific treatment and effective vaccines against dengue, prevention and control of the disease rely mainly on the control of mosquito vectors (Bangs *et al.*, 2001; Kroeger *et al.*, 2006). Effective vector control requires an integrated vector management programme which includes a consortium of tools such as environmental management, source reduction of breeding areas, routine larviciding in breeding areas that cannot be eliminated and adulticidal chemical space spray (Tan 1997; Ng & Vythilingum 2011).

In recent years, bed nets have been used as a barrier to protect man from being bitten by mosquitoes and other haematophagus insects. The incorporation of insecticides into these bed nets and curtains, collectively known as ITMs, has been widely used in malaria control and prevention as significant reduction of morbidity and mortality associated with vector-borne diseases especially malaria (Nahlen *et al.*, 2003; Lengeler 2004). Several studies conducted in Africa, Asia and Latin America have shown the impact of ITMs in reducing malaria morbidity and mortality, especially among children and expecting mothers (Zimmerman & Voorham 1997; Modiano *et al.*, 1998; Lengeler 2004; Gamble, *et al.*, 2007).

In dengue control, the use of ITMs as curtains and/or water storage jar covers, have shown to effectively reduce the population of *Ae. aegypti* in Mexico, Venezuela, Thailand, Cambodia and Haiti (Kroeger, *et al.*, 2006; Lenhart, *et al.*, 2008; Seng, *et al.*, 2008; Vanlerberghe, *et al.*, 2010). These studies have demonstrated the potential of ITMs in residential settings for dengue control. It was widely known that *Ae. aegypti* is a diurnal urbanized mosquito, which is commonly found in residential areas especially in a developed country like Singapore. They are frequently found seeking hosts indoors and majority of this mosquito are found resting on non-sprayable surfaces such as furniture, hanging objects such as clothes and curtains, and especially prefer dark, humid and secluded places within the house (Perich, *et al.*, 2000; WHO 2011). Thus, this study aims to improvise current dengue control by placing ITMs at *Aedes* mosquitoes' favourite hideouts so as to expose both resting and host seeking mosquitoes to insecticides that were impregnated onto the substrates.

Majority of ITMs, including long lasting insecticidal nets (LLIN), are impregnated with active ingredients from pyrethroids class. Resistance to this class of insecticides involved two mechanisms: enhanced rate of metabolic detoxification of the insecticide by enzymes; and insensitivity of target sites, which is also known as "knockdown resistance" or *kdr* (Brengues, *et al.*, 2003; Kawada, *et al.*, 2009; Martins *et al.*, 2009). The voltage-gated sodium channel (Na_v) is the common target site for pyrethroids which bind to the channel and provoke the phenomenon known as "knockdown" – a rapid, uncoordinated and involuntary movements followed by paralysis and death (Martins, *et al.*, 2009). Mutations in the Na_v genes, which reduces pyrethroids affinity to the Na_v, have been shown to be associated with *kdr* to pyrethroids (Brengues, *et al.*, 2003; Saavedra-Rodriguez, Chang *et al.*, 2009). *Kdr*-type of resistance has been reported in several species of mosquitoes such as *Anopheles gambiae*, *Anopheles stephensi*, *Culex quinquefasciatus* and *Ae. aegypti*. Singapore first detected *Ae. aegypti* resistance to permethrin in 1991 (Ping, *et al.*, 2001), and has confirmed cross-resistance within the class of insecticides (Lee & Lam-Phua, unpublished data). Therefore, in this study, we investigated the usefulness of ITMs, as part of our effort to enhance Singapore's integrated vector management program.

MATERIALS AND METHODS

Long lasting insecticidal net

The efficacy of PermaNet® 2.0, a long lasting insecticidal net made of multifilament polyester fabric treated with deltamethrin at a target dose of 1.8g/kg (equivalent to 55 mg/m^2), against local *Ae. aegypti* was

determined. The net was supplied by Vestergaard Frandsen SA (Denmark). A locally available untreated 100% polyester mosquito net was used as control.

Test mosquitoes

Aedes aegypti (field strain) collected by field surveillance officers during routine larval checks in houses from all five regions of Singapore were pooled and colonized. Approximately two hundred larvae were collected within a week and kept in trays until emergence. All female wild caught *Ae. aegypti* were subjected to blood feeding for the propagation of the next progeny (F_1). Five hundred randomly selected F_1 *Ae. aegypti* eggs were left in water with yeast to hatch and reared to adults stage. All F_1 female mosquitoes were subjected to blood feeding for propagation of F_2 progeny. For exposure to insecticide treated nets and subsequent molecular analysis – F_2 population of field strain and F_{111} *Ae. aegypti* Bora-bora strain (WHO susceptible strain) were used. Three to five days old, sugar-fed females were used throughout the study. To determine the prevalence of *kdr*-mutation in the Na_v genes in local *Ae. aegypti*, larvae collected from five different regions of Singapore were analyzed. All mosquitoes were reared/held in the insectary at 25 ± 2°C and 75 ± 5% Relative Humidity, with a photoperiod of 12:12 (Light:Dark) hours.

Laboratory bioassay

Bioassays were carried out using contact bioassay cones, according to the WHO guidelines for laboratory and field-testing of long-lasting insecticidal mosquito nets (WHO 2005). Briefly, 5 mosquitoes were introduced into each cone, which was fixed on PermaNet® 2.0 or control net (15 cm x 15 cm). Each net was supported by a black-colored paper to encourage the mosquitoes to rest on the net surface, so as to compensate for the excito-repellency property of the deltamethrin. After 3 minutes, mosquitoes were then aspirated and transferred into holding cups; cotton soaked with 10% sugar solution were provided as food source. The number of dead mosquitoes at 24-hrs post-exposure was recorded. The control was carried out in a similar manner using untreated polyester net.

The first assays were performed on Day 0, when PermaNet® 2.0 was first removed from its packaging. To study the residual efficacy of the net, the bioassay was repeated on the same nets one, two and three weeks after Day 0. Five 15cm x 15cm nets were cut randomly from each net, serving as replicates for each of the above experiments. A total of 4 nets were used.

Sequencing of *Ae. aegypti* voltage-gated sodium channel gene (Na_v)

Mosquitoes that survived the 24 hours post-exposure to PermaNet® 2.0 were separated from those that died, and killed by placing them inside –20°C freezer for a few minutes. Dead and sacrificed mosquitoes were individually transferred into a 1.7ml microcentrifuge tube (Axygen, USA) and labeled accordingly.

Each mosquito was individually homogenized with a pestle and DNA was extracted using the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. PCR was performed on each DNA sample to amplify part of the DIIS6, DIIIS6 and DIVS5 of the Na_v fragments. The sequences of the primers and targeted domains of the gene are shown in Table 1 and Figure 1, respectively. The 50 µl PCR reaction mixture contained the reaction buffer (Promega, USA), 1.5mM (for primers AaSCF1 and AaSCR4; AaSCF7 and AaSCR7) and 3.0mM (for primers DL1787F, DL1787R, KDRF20 and KDRR21) $MgCl_2$, 0.2mM of each dNTP, 0.3 µM of each primer, 1.5U *Taq* DNA polymerase (Promega, USA) and 3 µl of the genomic DNA template. PCR amplification parameters were: 95°C for 4 min, 30 cycles at 95°C for 30 sec, 55°C (for primers AaSCF1 and AaSCR4; AaSCF7 and AaSCR7) or 57°C (for primers KDRF20 and KDRR21) for 30 sec, and 72°C for 30 sec, followed by 72°C for 3 minutes. The PCR products, confirmed on a 2% agarose gel, were purified using Purelink PCR purification kit (Invitrogen Corp., USA) according to the manufacturer's recommendations. Sequencing of the purified

Table 1. Sequencing and PCR primers used for amplification of DIIS6, DIIIS6 and DIVS5

Primer	DNA fragment	Primers' Sequences	Use	Product Size (bp)	Reference
KDR20F	DIIS6	5' atg tgg gat tgt atg ctt g 3'	PCR	357-377	Saavedra-Rodriguez K *et al.*, 2007
KDR21R	DIIS6	5' gat gaa ccg aaa ttg gac 3'			Saavedra-Rodriguez K *et al.*, 2007
AaSCF7	DIIIS6	5' gag aac tcg ccg atg aac tt 3'		~ 800	Hitoshi Kawada *et al.*, 2009
AaSCR7	DIIIS6	5' gacgac gaa atc gaa cag gt 3'			Hitoshi Kawada *et al.*, 2009
AaSCF1	DIIS6	5' aga caa tgt gga tcg ctt cc 3'		~ 700	Hitoshi Kawada *et al.*, 2009
AaSCR4	DIIS6	5' gga cgc aat ctg gct tgt ta 3'			Hitoshi Kawada *et al.*, 2009
DL1787F	DIVS5	5' ttg gtc atg ttc atc ttc gcc 3'		~ 100	EHI designed primer
DL1787R	DIVS5	5' gct ctg gcc gaa cgt ctt gaa a 3'			EHI designed primer
AaSCF3	DIIS6	5' gtg gaa ctt cac cga ctt ca 3'	Seq	NA	Hitoshi Kawada *et al.*, 2009
AaSCR6	DIIS6	5' cga ctt gat cca gtt gga ga 3'			Hitoshi Kawada *et al.*, 2009
AaSCR8	DIIIS6	5' tag ctt tca gcg gct tct tc 3'			Hitoshi Kawada *et al.*, 2009
KDR22F	DIIS6	5' gac tga aag taa att gga gc 3'			EHI designed primers
KDR23R	DIIS6	5' ggt tag cac gat aga atc g 3'			EHI designed primers

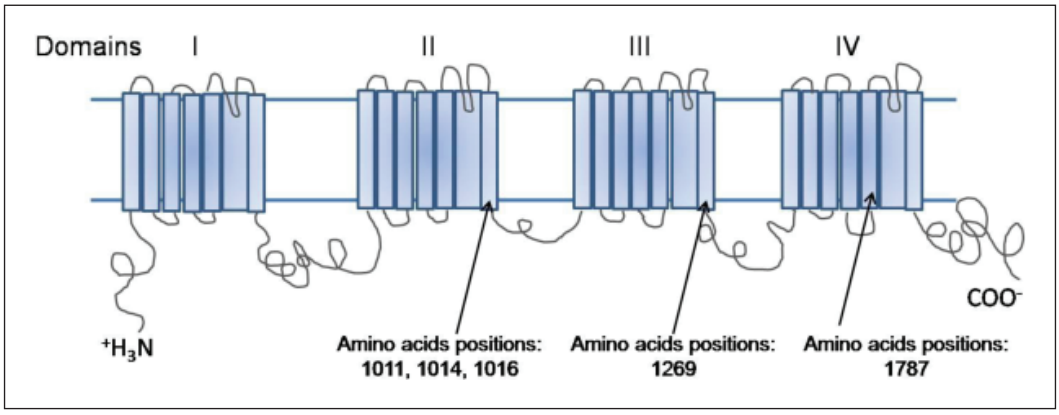

Figure 1. Illustration of targeted residues on domain II, III and IV.

PCR products were performed by a commercial laboratory according to the BigDye Terminator Cycle Sequencing kit (Applied Biosystems, USA) protocol. Primers used for sequencing of the DIIS6, DIIIS6 and DIVS5 of the Na_v fragments are also shown in Table 1. Consensus sequence was obtained by assembling a contiguous sequence from raw sequencing data using Seqman software and the predicted amino acid peptide fragments were generated using MegAlign software (Lasergene, DNASTAR, USA). Sequences obtained from field strain *Ae. aegypti* were compared with those obtained from the Bora-Bora strain. Heterozygous positions were identified by the presence of two overlapping peaks of similar height from the electropherograms while homozygous allele would only show one peak at the same position.

Statistical analysis

Chi-square test of association, paired-sample T-test and one-way analysis of variance (ANOVA) were performed using SPSS version 19.0 to determine the association of survival rates and frequencies of mutations, the significant differences in the mortalities between both strains, and the effect of extending exposure times of PermaNet® 2.0 on the mosquitoes, respectively.

RESULTS

Efficacy of PermaNet® 2.0 against *Ae. aegypti*

Initial exposure of Bora-Bora strain to PermaNet® 2.0 for three minutes resulted in 100% mortality rate in all replicates. However, the same exposure treatment to field strain resulted in less than 80% mortality. The differences in the means of % mortality of field strain and the Bora-Bora strain at each time point, was significant when analysed by paired-sample T-Test ($T = 4.37$; $P < 0.05$). By week 3, less than 30% mortality was observed.

Distribution of kdr –type resistance alleles in *Ae. aegypti*

Point mutations at amino acid residues I1011M, I1011V, L1014F, L1014S, V1016G, V1016I, F1269C and D1787Y have been shown to confer *kdr*-type of resistance to mosquitoes against pyrethroids (Martinez-Torres *et al.*, 1998, 1999; Brengues *et al.*, 2003; Soderlund & Knipple 2003; Saavedra-Rodriguez, *et al.*, 2007; Chang *et al.*, 2009; Kawada *et al.*, 2009). In order to determine if these mutations could be correlated with the low kill rate of PermaNet® 2.0 against Singapore *Ae. aegypti*, PCR and direct sequencing of the DIIS6, DIIIS6 and DIVS5 fragments of *Ae. aegypti*'s voltage-gated sodium channel gene were performed. A total of 60 field and 25 Bora-Bora strain *Ae. aegypti* used in the PermaNet® 2.0 bioassays were processed and analyzed for *kdr*-associated mutations. Of these, seven of the field mosquitoes and one of the Bora-bora strains did not yield readable sequences. All of the 24 Bora-Bora strain analyzed and 15 (28.3%) out of the 53 analyzed field mosquitoes did not survive the exposure to PermaNet® 2.0.

All Bora-Bora strain mosquitoes showed the wild type amino acid at the respective residues analyzed. Out of the known amino acid substitutions that confer resistance, only V1016G and F1269C were detected (Table 3) among field mosquitoes. None of the field mosquitoes had wild type genotype at both amino acid residues.

The number and frequencies of genotypes at amino acid residues 1016 and 1269 are shown in Table 3. Thirty-seven (69.8%) out of 53 field mosquitoes examined possessed the V1016G mutation. Of these, 29 (54.7%) occurred as heterozygous (V/G) while 8 (15.1%) were homozygous (G/G) (Table 3). On the other hand, 46 (86.8%) of the field strains possess the F1269C mutation. Twenty-nine (54.7%) of those having the F1269C mutations were heterozygous (F/C) and 17 (32.1%) were homozygous (C/C). Seven out of 15 and 13 out of 15 of the *Ae. aegypti* having the V1016G and F1269C mutations, respectively, did not survive PermaNet® 2.0 exposure.

Despite the lower frequency of mutation, it was observed that the survival rate of mosquitoes is higher with the presence of mutation at amino acid residue 1016 (81.1%) when compared to 1269 (71.7%). However, there is no significant association ($\chi^2 = 0.98$, $P = 0.32$) between the frequencies of mutation at amino acid residues 1016 and 1269, and their respective survival rates (Table 3).

When these mutations were analyzed together, it was observed that all field mosquitoes expressed resistant alleles, either as homozygous or heterozygous mutants, at amino acids residues 1016 or 1269 (Table 4). Furthermore, it was also observed that whenever a heterozygous allele was observed at amino acids residue 1016 (V/G), amino acids residue 1269 was also always heterozygous (F/C). However, a homozygous resistant allele at amino acids residue 1016 may not necessarily give rise to a homozygous resistant allele at amino acids residue 1269 and vice versa (Table 4).

Fifty percent of mosquitoes with a homozygous mutation at F1269C coupled with wild type V1016 survived PermaNet® 2.0 exposure, while mosquitoes having the homozygous mutation at V1016G but wild type F1269 showed a higher survival rate (71.4%). However, the highest survival rate against PermaNet® 2.0 exposure was achieved in mosquitoes having the heterozygous resistant alleles at both amino acids residues (82.8%). This combination resulted in significantly higher survival rate than those with homozygous mutation at F1269C only, but not those with homozygous mutation at V1016G. When comparison was made, the relationship of survival rates

Table 2. Total number and percentage mortality of *Ae. aegypti* (field and Bora-bora strains) after exposing to PermaNet® 2.0 at different days and exposure time

Days	Exposure time	*Aedes aegypti* strains			
		Field strain		Bora-Bora strain	
		Mortality		Mortality	
		Total no. of dead mosquitoes/ total number of mosquitoes exposed	% (±SE) [#]	Total no. of dead mosquitoes/ total number of mosquitoes exposed	% (±SE) [#]
Day 0	3 min	76/100	76 (±11.66)	100/100	[#]100 (±0.00)
Day 7	3 min	32/100	32 (±13.57)	100/100	[#]100 (±0.00)
Day 14	3 min	60/100	60 (±10.95)	100/100	[#]100 (±0.00)
Day 21	3 min	26/100	26 (±14.14)	100/100	[#]100 (±0.00)

[#] means of % mortality of field strain is significantly different from Bora-Bora strain by Paired-sample T-Test ($P<0.05$)
SE = Standard error of proportion, where *STDEV* is standard deviation and *N* is sample size.

$= SE = STDEV/\sqrt{N}$

Table 3. Heterozygous and homozygous allele mutation frequencies of amino acids residue 1016 and 1269 and their respective survival rates after PermaNet® 2.0 exposure

	Amino acids residues					
	[#]V1016	V1016G		[#]F1269	F1269C	
		[#]V/G	[#]G/G		[#]F/C	[#]C/C
Dead	8	5	2	2	5	8
Alive	8	24	6	5	24	9
Total (%)	16(30.2)	29 (54.7)	8 (15.1)	7 (13.2)	29 (54.7)	17 (32.1)

[#]No significant association between survival rates and mutation frequencies of amino acid residues 1016 and 1269 by Chi-square test of association (c^2= 0.98, P= 0.32)

Table 4. The relationship of the combined allele of amino acids residue1016 and 1269 and field strain *Ae. aegypti* surviving PermaNet® 2.0 exposure

Strains	Residues V1016G/F1269C	N	Dead		Alive	
			n	%	n	%
Field strain	VV/CC	16	8	50	8	50[1,2]
	GG/FF	7	2	28.6	5	71.4[1,3]
	GG/CC	1	0	0	1	100[*]
	VG/FC	29	5	17.2	24	82.8[2,3]

[1,3]No significant difference between the residues combination (VV/CC and GG/FF, GG/FF and VG/FC) and their survival rates (c^2= 0.91, P= 0.34; c^2= 0.46, P= 0.50) by Chi-square test of association.
[2]Significant difference between the residue combination (VV/CC and VG/FC) and the survival rate (c^2= 5.39, P= 0.02) by Chi-square test of association.
[*]data for GG/CC was omitted for SE due to small sample size.

Table 5. *Kdr* genotype frequencies in Na_v gene (residues V1016G and F1269C) of 201 *Ae. aegypti* larvae in five regions of Singapore

Residues V1016G/F1269C	No. of larvae of various genotypes from different regions of Singapore					Total no. of larvae of various genotypes
	Central	South Western	South Eastern	North Western	North Eastern	
VV/CC	12	15	3	15	4	49^
GG/FF	12	6	9	8	11	46*
VG/FC	14	18	12	14	18	76*^
VV/FF	2	0	4	0	0	6
GG/FC	1	1	5	2	0	9*^
VG/CC	0	0	2	0	2	4*^
VG/FF	1	0	2	0	2	5*
VV/FC	0	0	2	2	1	5^
GG/CC	1	0	0	0	0	1*^
Total	43	40	39	41	38	201

*^ Average of the sums of % mutation frequencies at residue *1016 and ^1269 of the 5 regions are 70% and 72% respectively.

with the mutation combination (VV/CC and VG/FC) showed a significant difference (χ^2=5.39, P=0.02) by Chi-square test of association. Due to the small number of mosquito (n=1) having both homozygous resistant alleles, it remains unclear whether this will confer a higher level of *kdr*-type of resistance to SP. Thus, this data was omitted for statistical analysis. Here, we provide evidence that the ineffectiveness of the deltamethrin-treated net could be associated with mutations in the voltage-gated sodium channel (Na_v) genes that confer *kdr*-type of resistance.

Prevalence of kdr mutation in Singapore

To determine the prevalence of these mutations in the local population, genetic analysis was performed directly on 201 larvae collected from the field. Only 6 out of the 201 mosquitoes surveyed had wild type genotype at both amino acid residues. The high prevalence of mutations were confirmed in the countrywide survey where 70% and 72% of the 201 *Ae. aegypti* analyzed possessed V1016G and F1269C alleles respectively (Table 5). The most frequent genotype was V1016G/F1269C at 37.8% (n=76) prevalence among the mosquitoes surveyed – the heterozygous genotype in both amino acids residues that seems to confer a high resistance, with only 17.6% of *Ae. aegypti* exposed to the deltamethrin net succumbing to the insecticide. The next higher frequencies were VV/CC and GG/FF at 24.4% and 22.9% respectively. The rest of the alleles were less than 4.5% (n=9).

DISCUSSION

This study demonstrated a low efficacy and poor residual effect of PermaNet® 2.0 against local field *Ae, aegypti* (Table 2) but excellent results against Bora Bora strain. This might be due to resistance present in our local field *Ae. aegypti*. The active ingredient (deltamethrin) of PermaNet® 2.0 falls under pyrethroids class of insecticides, which is the main insecticide being used to treat LLIN to combat the scourge of malaria (WHO 2009; Tungu, *et al.*, 2010). However, the rapid development and spread of pyrethroids resistance among disease vectors may undermine the effectiveness of LLIN against these mosquitoes. Despite the concern, reports from Western and Central Africa and Vietnam have shown the effectiveness of pyrethriod-treated LLIN against pyrethroids resistant malaria vectors (Van Bortel, *et al.*, 2009; Corbel, *et al.*, 2010), particularly where mosquito populations displayed only low frequency of *kdr* mutation, or metabolic associated resistance.

In Singapore, the evidence of *Ae. aegypti* resistance to pyrethroids such as permethrin has been reported (Ping, *et al.*, 2001). Local studies on fogging and insecticide resistance test, using WHO test methods and guidelines, also reported no efficacy and high resistance ratio (RR_{50}) against Singapore's wild caught *Ae. aegypti* when using deltamethrin (EHI unplublished data). However, the mechanisms that confer resistance towards these insecticides among local dengue vectors are poorly understood.

Molecular investigation in Na_v showed high prevalence of two point mutations (69% for V1016G in DIIS6 and 77% F1269C in DIIIS6) among the field *Ae. aegypti*. The Na_v sequence is highly conserved among insects (Martins, *et al.*, 2009). Most studies on *kdr* mutations have focused on the segment 4 to 6 (S4-S6) region of domain II of the Na_v gene (DIIS4- DIIS6). Several mutations in DIIS6 of the Na_v that confers *kdr*-resistance to pyrethroids in *Ae. aegypti* have been identified (Martinez-Torres, *et al.*, 1998; 1999; Brengues, *et al.*, 2003; Soderlund & Knipple 2003; Saavedra-Rodriguez, *et al.*, 2007; Chang, *et al.*, 2009; Raweewan, 2010). These point-mutations, and those associated with *Culex* species and *Anopheles* species, were investigated in this study to provide insights on the possible resistance mechanism of *Ae. aegypti* towards deltamethrin.

The two point mutations detected amongst Singapore's *Ae. aegypti* at amino acids residues 1016 and 1269 were previously found in the resistant strain of *Ae. aegypti* from Thailand and Vietnam. High prevalence of these two mutations (70% for V1016G and 72% F1269C) was confirmed in a countrywide survey. Only 3% of *Ae. aegypti* surveyed had wild type genotype in both residues. All possible pairing of mutation at amino acids residues 1016 and 1269 were detected. However, the highest mutated frequency combination was found to have heterozygous genotype (VG/FC) at both residues 1016 and 1269 (37.8%), followed by homozygous mutation at amino acids residue 1269 (24.4%) and homozygous mutation at amino acids residue 1016 (22.9%). Only one out of 201 larvae was found to possess homozygous resistant alleles at both amino acids residue. This might indicate the budding of homozygous mutations at both residues among Singapore's *Ae. aegypti* populations. Interestingly, in the current study, the various permutations of wild type and the four mutant alleles at both amino acids residues were found to result in varying degree of survival rate of *Ae. aegypti* when exposed to deltamethrin. More study is needed to understand these interesting genotypes. This is the first local study to provide evidence on the *kdr*-type of resistance mechanism found in field strain *Ae. aegypti*.

Although *kdr*-type of resistance is widely known to contribute to resistance to pyrethroids class of insecticide in *Ae. aegypti*, multiple studies also revealed that pyrethroids metabolic resistance might potentially confer pyrethroids resistance in *Ae. aegypti* (Ahmad, *et al.*, 2007; Strode, *et al.*, 2008; Harris, *et al.*, 2010; Stevenson, *et al.*, 2012). Some comprehensive studies determined that both metabolic and *kdr*-type of resistance were found in *Ae. aegypti* (Flores, *et al.*, 2006; Marcombe, *et al.*, 2009). Thus, we do not rule out the possibility of metabolic pyrethroids resistance or both pyrethroids resistance mechanisms (i.e. metabolic and *kdr*-type) in local *Ae. aegypti* that might be involved in conferring pyrethroids resistance. More comprehensive researches are necessary to address this field of study.

In Singapore, fogging with pyrethroids used to be performed as part of a long standing dengue control program (Ping, *et al.*, 2001). Furthermore, an assortment of pyrethroids in aerosol cans is readily available and commonly used by general households where *Ae. aegypti* is likely to be found. These had probably imposed a significant pressure for the selection of the resistant alleles among Singapore's *Ae. aegypti*. The presence of *kdr*-type of resistance in local *Ae. aegypti* population may undermine the effectiveness of pyrethroids in controlling the major dengue vector in Singapore. In view of the high frequency of *kdr* mutations among local mosquitoes, the synergistic effect of PBO will most likely render limited improvement in the effectiveness of pyrethroids class of insecticide. The findings demonstrated the

need to understand the insecticide resistance mechanisms amongst resistant mosquito population that will be crucial for the development of strategies for resistance management and the need to evaluate new control methods before being incorporated into existing vector control program.

Acknowledgements. We would like to acknowledge Png Ah Bah and Foo Siew Yoong for the collection of larvae for the study. We would also like to extend our special thanks to Lien Tran and Helen Pates Jamet from Vestergaard Frandsen SA (Switzerland) for providing PermaNet® 2.0 for this study.

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