

## RESEARCH NOTE

# FIELD-COLLECTED PERMETHRIN-RESISTANT *Aedes Aegypti* FROM CENTRAL THAILAND CONTAIN POINT MUTATIONS IN THE DOMAIN IIS6 OF THE SODIUM CHANNEL GENE (*KDR*)

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**Abstract.** One of the mechanisms responsible for pyrethroid resistance in mosquitoes is mutations in domain IIS6 of voltage-gated sodium channel gene (*kdr*). *Aedes aegypti* larvae were collected from the central provinces of Thailand (Bangkok, Prachin Buri and Ratchaburi) and colonized until they became adults. Partial fragment of *kdr* of permethrin-resistant mosquitoes were amplified by RT-PCR and sequenced. Among the four nucleotide mutations detected, two mutations resulted in two amino acid substitutions, S(TCC) 989 P(CCC) and V(GTA)1016 G(GGA). Among 94 permethrin-resistant mosquitoes, the SS genotype (SS/VV) was found to predominate ( $n = 74$ ), followed by SR (SP/VG) ( $n = 15$ ) and RR (PP/GG) genotypes ( $n = 5$ ), with the resistant allele frequency ranging from 0.03 to 0.17. As pyrethroid insecticides are currently being advocated for use in Thailand, investigations of pyrethroid resistance in other regions of the country are needed to prevent potential cross-resistance among different types of insecticides.

**Keywords:** *Aedes aegypti*, permethrin resistant, voltage-gated sodium channel, *kdr* resistance

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## INTRODUCTION

Insecticidal pyrethroids are widely used to control vectors involved in transmission of viral diseases which are of public health concern, such as dengue and chikungunya fever, due to their beneficial

characteristics *viz* low toxicity to humans and mammals and limited persistence in soil. Hence, they are used in indoor residual sprays, for impregnating bed nets, curtains, and screens, as well as in coils, mats and aerosols (Chareonviriyaphap *et al*, 2003).

When a case of dengue fever is reported, the Ministry of Public Health of Thailand initiates spraying of the area surrounding the patient's house with pyrethroid or organophosphate insecticides within 24 hours in order to kill any adult mosquitoes in the vicinity (Limpawitthayakul and Sorngeng, 2006). However, the long-term and frequent usage of these insecticides has led mosquitoes in Thailand to become resistant to these compounds. In Thailand, permethrin and DDT resistance are now widely distributed in *Aedes aegypti* (Prapanthadara *et al*, 2002; Thanispong *et al*, 2008; Paeporn *et al*, 2010), and *Ae. aegypti* is also resistant to deltamethrin in Central Thailand including Bangkok (Yaicharoen *et al*, 2005; Komalamisra *et al*, 2011; Srisawat *et al*, 2011).

The mechanism of pyrethroid and DDT resistance in insects generally involves changes to the sensitivity of the target voltage-gated sodium channels to these insecticides (Brenques *et al*, 2003). The voltage-gated sodium channel contains four homologous repeated domains (I, II, III, and IV), each domain consisting of six putative transmembrane regions arranged to form an ion pore. Mutations to this gene (*kdr*), known as "knockdown resistance", have been linked to resistance of this target in a range of insects (Brenques *et al*, 2003). Single nucleotide substitutions in domain II of the voltage-gated sodium channel gene induce resistance to pyrethroids in *Ae. aegypti*, including Val → Gly or Val → Ile at position 1016, Ile → Met

or Ile → Val at 1011, Leu → Trp at 982, Gly → Val at 923 and Ser → Pro at 989 (Brenques *et al*, 2003; Saavedra-Rodriguez *et al*, 2007; Srisawat *et al*, 2010). The S989P mutation was first reported in a laboratory colony selected for deltamethrin resistance.

In this study, we identified a mutation in domain IIS4-6 of *kdr* that is associated with permethrin resistance in field collected *Ae. aegypti*.

## MATERIALS AND METHODS

### Field collected permethrin-resistant *Ae. aegypti*

*Ae. aegypti* larvae were collected from areas of Central Thailand in which permethrin resistance had been reported (Bang Khae District, Bangkok; Mueang District, Prachin Buri Province; and Pong Sawai District, Ratchaburi Province) (Paeporn *et al*, 2004; Srisawat *et al*, 2011). The global positioning system (GPS) coordinates of the study sites were recorded. Larvae were colonized in the laboratory of the Department of Medical Entomology, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand until adults emerged. *Ae. aegypti* that survived 1 hour exposure to 0.75% permethrin (WHO diagnostic dose) were considered to be permethrin-resistant. The susceptible strain originating 10 years ago from Buri Ram Province was colonized at the Department of Medical Entomology, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand.

### Insecticide susceptibility test

Twenty-five 2-5-day-old, non-blood fed female mosquitoes were tested for insecticide susceptibility according to standard WHO procedure (WHO, 1998). In brief, mosquitoes were kept in a holding tube for 1 hour and then transferred

to an exposure tube lined with 0.75% permethrin paper for 1 hour before being transferred back to the holding tube and provided with 10% sugar solution. Mortality was examined after 24 hours. Susceptibility tests were carried out using 100 female mosquitoes from each strain. The control mosquitoes were exposed to paper containing silicone oil. When the mortality of control mosquitoes was > 20%, the experiment was terminated. When the mortality of the control group ranged from 5% to 20%, the mortality rate was adjusted using Abbott's formula:

$$\frac{(\% \text{ test mortality} - \% \text{ control mortality})}{(100 - \% \text{ control mortality})} \times 100$$

#### **Amplification and sequencing of *kdr* from permethrin-resistant *Ae. aegypti***

Total RNA was extracted from individual mosquitoes of permethrin-resistant strain using TRIzol® reagent (Invitrogen, Carlsbad, CA). Subsequently, RT-PCR was performed using primers NaF (forward primer 5' CGT GGC GCT GTC GTT GCT C 3') and NaR (reverse primer 5' CTT GTT CGT TTC GTT GTC GGC 3'), which cover *kdr* regions S4 and S6 of domain II (Sri-sawat *et al*, 2010). The PCR mixture 12.5

l total volume contained 10 picomole of each primer, 1X SuperScript™ One Step RT-PCR PLATINUM Taq reaction mixture (Invitrogen, Carlsbad, CA), and 200 ng of RNA template from each mosquito. Thermal cycling consisted of 50°C for 30 minutes, 94°C for 2 minutes, followed by 35 cycles of 94°C for 30 seconds, 65°C for 30 seconds and 68°C for 1 minute, and final heating step at 68°C for 2 minutes. PCR amplicons were analyzed by electrophoresis at 100 V in 1.5% agarose gel for 30 minutes, stained with ethidium bromide, and visualized under UV light. Amplicons then were purified and sequenced

by Macrogen (Seoul, Korea), using ABI BigDye Terminator Cycle Sequencing kit and a 3730xl Automated DNA sequencer (PE Applied Biosystems, Foster City, CA).

#### **Effect of the two mutations (989, 1016) of *kdr* in relation to permethrin exposure time**

Permethrin-resistant Pongsawai strain of *Ae. aegypti*, collected in Ratchaburi Province, Thailand in 2003 (Paeporn *et al*, 2004), was subjected to an examination of the relationship between the frequency of the two resistance alleles and permethrin exposure time according to standard WHO protocol (WHO, 1998). Mosquitoes were exposed to 0.75 % permethrin for 60, 270, or 360 minutes, and only the surviving *Ae. aegypti* were examined for their resistant allele frequency.

## RESULTS

All *Ae. aegypti* were highly resistant to 0.75% permethrin [mortality rate of <80%, the criterion used by World Health Organization (1998), to designate resistant mosquitoes]. Mortality rate of *Ae. aegypti* in Bangkok ranged from 0% (BK-2) to highest of 63% (BK-5) (Table1). *Ae. aegypti* from Ratchaburi and Prachin Buri Provinces also showed high levels of permethrin resistance.

Four nucleotide substitutions were detected in domain IIS4-S6 of the sodium channel gene in the surviving permethrin-resistant *Ae. aegypti* mosquitoes (data not shown). One nucleotide substitution at 982 position and one at 1011 were synonymous. However the other two resulted in S(TCC) 989 P(CCC) and V(GTA) 1016 G(GGA), both occurring together in the same permethrin-resistant mosquito. The mutant *kdr* allele is designated R and wild type allele S.

An examination of *kdr* genotypes in

Table 1  
Percentage mortality of *Ae. aegypti* exposed to 0.75% permethrin for 1 hour.

Collection site	Code	GPS coordinates	Mortality rate (%)
Bang Khae District, Bangkok	BK-1	13° 70' 91.5" N, 100° 42' 59.5" E	9
	BK-2	13° 74' 88.9" N, 100° 35' 42.7" E	0
	BK-3	13° 41' 47.8" N, 100° 25' 44.6" E	52
	BK-4	13° 41' 98.6" N, 100° 23' 32.3" E	35
	BK-5	13° 42' 13.4" N, 100° 24' 51.2" E	63
Pong Sawai District, Ratchaburi Province	RB-1	ND	35
Mueang District, Prachin Buri Province	PR-1	14° 06' 49.1" N, 101° 19' 52.1" E	51
Laboratory susceptible strain (Buri Ram Province)	SBU	ND	100

ND, no data

Table 2  
Genotypic frequency of mutations in domain IIS6 of *kdr* in field collected permethrin-resistant and laboratory susceptible strain of *Ae. aegypti*.

Insecticide status	Locality	No.	Genotype frequency at 989/1016			Frequency of R allele
			SS	SR	RR	
Permethrin- resistant strain	Bangkok	73	0.75 (55) <sup>a</sup>	0.09 (13)	0.07 (5)	0.17
	Prachin Buri	15	0.93 (14)	0.03 (1)	0 (0)	0.03
	Ratchaburi	6	0.83 (5)	0.08 (1)	0 (0)	0.08
Permethrin- susceptible strain	Buri Ram	6	1 (6)	0 (0)	0 (0)	0

<sup>a</sup> Number of mosquitoes

94 samples, SS genotype was found to predominate (74 mosquitoes), followed by SR (15) and RR (5) (Table 2). The resistant allele frequency was highest in *Ae. aegypti* from Bangkok (0.17), followed by those from Ratchaburi (0.08) and Prachin Buri (0.03), while the susceptible strain possessed homozygous susceptibility allele. The permethrin-resistant allele increased in frequency in *Ae. aegypti* from Ratcha-

buri Province surviving longer exposure time and SS genotype was no longer found in these mosquitoes (Table 3).

## DISCUSSION

At present, most *Ae. aegypti* in Thailand are resistant to permethrin including those in Bangkok, Ratchaburi and Prachin Buri (Jirakanjanakit *et al*, 2007;

Table 3

Genotypic frequency of two mutations sites in *Ae. aegypti* collected from Ratchaburi Province, Thailand after exposure to 0.75% permethrin for various time.

Exposure time (min)	N	Frequency of mutation site (989/1016)			Frequency of R allele
		SS	SR	RR	
		SS/VV	SP/VG	PP/GG	
60	6	0.83 (5) <sup>a</sup>	0.08 (1)	0 (0)	0.08
270	5	0 (0)	0.3 (3)	0.4 (2)	0.7
360	6	0 (0)	0.33 (4)	0.33 (2)	0.7

<sup>a</sup> Number of mosquitoes

Thanispong *et al*, 2008; Paeporn *et al*, 2010; Komalamisra *et al*, 2011; Srisawat *et al*, 2011; Yanola *et al*, 2011). The permethrin-resistant allele (R) frequency in *Ae. aegypti* in Bangkok (an urban area) was higher than those in Prachin Buri and Ratchaburi (rural areas). Houses in urban areas are small and enclosed, and once an insecticide (*viz*, permethrin) has been sprayed inside a house, mosquitoes are likely to become exposed to it because of the limited space within the house. On the other hand, in rural areas, houses are large, open and distant from each other, thereby mosquitoes have less chance to come into contact with insecticides. In addition, mosquito coils, which repel mosquitoes, are the preferred anti-mosquito measure in rural areas.

Permethrin-resistant *Ae. aegypti* possesses two point mutations within domain IIS6 of *kdr*, with a number of mutations being reported *viz* G923V, L982W, S989P, I1011M/V, and V1016G (Bregues *et al*, 2003; Rajatileka *et al*, 2008; Srisawat *et al*, 2010). Rajatileka *et al* (2008) reported I1011V mutation in a mosquito sample collected from the southern part of Thailand, but a synonymous change was found in our samples, which were collected from

the central part of the country. The two non-synonymous nucleotide substitutions found in this study, resulting in S989P and V1016G mutations, were the same as those found in deltamethrin-resistant *Ae. aegypti* (Srisawat *et al*, 2010). In contrast, Yaicharoen *et al* (2005) could not find either S989P or V1016G in field *Ae. aegypti* from Bangkok.

The frequency of resistant allele in permethrin-resistant *Ae. aegypti* in this study is low compared with those reported from Vietnam, Brazil, and Mexico (Saavedra-Rodriguez *et al*, 2007; Kawada *et al*, 2009; Martins *et al*, 2009). It might be due to the fact that only 20% of permethrin-resistant mosquitoes were tested. The mosquitoes in those countries have demonstrated an increasing *kdr* resistant genotype frequency. However, there are signs of increasing *kdr* resistant allele frequency in Thai populations, especially in Bangkok population when compared to the result of Yaicharoen *et al* (2005).

A possible reason for the discrepancy between low *kdr* resistant allele frequency and high level of permethrin resistance detected in this study is the presence of other resistance mechanisms, increases in the expression of detoxification enzymes

might also be involved in insecticide resistance mechanism (Hemingway and Ranson, 2000; Brooke, 2008). Mixed function oxidase and esterase activities are elevated in pyrethroid resistant *Ae. aegypti* (Paeporn *et al.*, 2004; Yaicharoen *et al.*, 2005). In addition, mutations in domain I and domain III associated with permethrin resistance should be considered (Yanola *et al.*, 2010). Recently, Yanola *et al.* (2011) reported that *Ae. aegypti* with F/F1534 domain III susceptibility genotype was able to survive after being exposed to permethrin because of the presence of homozygous mutations in domain II, S989P and V1016G. Thus enzyme detoxification mechanism and/or mutations in other domains may contribute permethrin resistance, which needs further studies.

This study clearly demonstrated that loss of SS genotype and appearance of R allele are associated with high resistance to permethrin in field collected *Ae. aegypti*, as was reported in a deltamethrin-resistant *Ae. aegypti* laboratory strain (Srisawat *et al.*, 2010). However, the distribution of resistant alleles remains unknown in many parts of Thailand. It is therefore necessary to investigate insecticide resistance status and to prevent potential cross-resistance among different type of insecticides.

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