

Characterization of deltamethrin resistance in field populations of *Aedes aegypti* in Thailand

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ABSTRACT: Five field collections of adult *Aedes aegypti* mosquitoes from different areas in Bangkok and Pathum Thani provinces were subjected to susceptibility tests against deltamethrin. Low levels of resistance were detected among all populations tested (RR_{50} = 8-17.2) compared to the susceptible strain, Bora (French Polynesia). Among the five populations tested, the BKH (Bang Khen, Bangkok) and PSC (Phasicharoen, Bangkok) populations showed a higher level of deltamethrin resistance than the other three populations (RR_{50} of BKH= 17.2, and of PSC= 13.6) and cross-resistance to DDT was observed in these strains. Biochemical analysis showed a significant elevation of mixed function oxidases enzyme activity in all populations. There was an elevation of non-specific esterases in all populations except BKL, and there was no consistent association of glutathione S-transferases with deltamethrin and DDT resistance, although not all populations were bioassayed for DDT. The partial cDNA sequence of the *para*-type voltage-dependent sodium channel (IIS4-IIS6) was determined for BKH and PSC populations. Common amino acid substitution, leucine to phenylalanine in the IIS6 region, found for insects including *Anopheles gambiae* was not found in either the BKH or the PSC populations. However, two other amino acid substitutions (proline substituted with serine at position 64 in the PSC population and leucine with phenylalanine at position 69 in the BKH population) were found in the IIS5-IIS6 inter-segment region sequenced. The role these substitutions play in target site resistance is uncertain at this time. *Journal of Vector Ecology* 30 (1): 144-150. 2005.

Keyword Index: *Aedes aegypti*, deltamethrin, resistance mechanism, biochemical assays, Thailand.

INTRODUCTION

Dengue haemorrhagic fever (DHF) remains a serious disease in Thailand, and the mosquito *Aedes aegypti* has been incriminated as the main vector. Over 140,000 cases and 250 deaths were reported annually in Thailand (Annual Epidemiological Surveillance Report 2001). Control of *Ae. aegypti* vectors in Thailand has relied on the organophosphate and carbamate insecticides, including temephos, fenitrothion, malathion, and propoxur since 1950 (Chareonviriyaphap et al. 1999a). At the same time, DDT was used for insect control in Thailand particularly in agricultural areas (Chareonviriyaphap et al. 1999a). Since 1992, synthetic pyrethroids have been the primary insecticides used in agriculture and public health. During endemic seasons, deltamethrin, cypermethrin, and permethrin are the main synthetic pyrethroids used to control adult *Aedes* mosquitoes through mass spraying (Vector Borne Disease Annual report 2002-2003). Additionally, household insecticide products (aerosols, mosquito coils, mats, and liquid forms) containing synthetic pyrethroids such as permethrin, d-tetramethrin, and esbiothrin have been commonly used, especially in cities such as Bangkok (Paeporn et al. 1996). To date, deltamethrin is used for space spraying in adult *Aedes sp.* mosquito control, either alone or in combination with other synthetic pyrethroids (Vector Borne Disease Annual report 2002-2003).

Common insecticide resistance mechanisms include alteration of target sites and increased enzyme activities of non-specific esterases, glutathione S-transferases (GSTs), and P450-mediated monooxygenases or mixed function oxidases (MFOs) (Oppenoorth 1985, Price 1991, Hemingway and Ranson 2000). GST has been shown to be responsible for DDT resistance in mosquitoes (Brogdon and Barber 1990, Grant et al. 1991), while non-specific esterases have been shown to be involved in resistance to organophosphates, carbamates and, to a lesser extent, pyrethroids. The monooxygenases are reported to play a role in the metabolism of pyrethroids, activation and/or detoxification of organophosphorus insecticides and, to a lesser extent, carbamate resistance (Hemingway et al. 2004). Target site resistance in pyrethroids and DDT is due to reduced affinity of sodium channels resulting in delayed knockdown time (knockdown resistance; *kdr*). The *kdr* is associated with point mutations in the *para*-type voltage-dependent sodium channel gene (Sorderlund and Knipple 2003). A single mutation (leucine to phenylalanine) at position 1014 in the domain II segment 6 (IIS6) of the sodium channel gene is the molecular basis of *kdr* in several insects such as *Musca domestica* (Williamson et al. 1996), *Anopheles gambiae*, and *Culex pipiens* (Martinez-Torres et al. 1998, 1999).

In Thailand, resistance to DDT has been recognized for decades in *Ae. aegypti* (Neely 1966). Recently, several cases

of field-associated resistance have been reported in *Ae. aegypti* against pyrethroid aerosol products (Wattanachai and Tintanon 1999). Resistance to DDT and pyrethroids including permethrin and deltamethrin in *Ae. aegypti* and *Aedes albopictus* was documented in Northern Thailand (Somboon et al. 2003). This study was undertaken to obtain information on susceptibility to deltamethrin in adult *Ae. aegypti* field-collected mosquitoes from Bangkok and Pathum Thani provinces where high numbers of dengue hemorrhagic fever cases have been reported. These populations had a prior history of exposure to pyrethroid insecticides. Possible mechanisms responsible for resistance were investigated by biochemical analysis and by determination of partial sodium channel cDNA sequence.

MATERIALS AND METHODS

Mosquito test populations

Larvae of five *Ae. aegypti* mosquito populations were collected from individual houses in Bangkok and Pathum Thani provinces during October 2002 to January 2003. Mosquito populations were collected from individual houses in the districts of Bang Khen (BKH), Bang Kholaem (BKL), Phasicharoen (PSC), and Pravet (PRV) in Bangkok province and the district of Ladlumkaew (LLK) in Pathum Thani province, north of Bangkok. In each house five collections of mosquito larvae were made and pooled.

Mosquito larvae were reared to adults in the laboratory (Department of Entomology, Faculty of Agriculture, Kasetsart University, Bangkok, Thailand). A batch of approximately 250 larvae was reared with guinea pig food in a finely ground mixture in clean water. Adult mosquitoes were provided with 5% sugar solution. Females were fed on guinea pig blood before laying eggs. Second generation (F_2) adult female mosquitoes were used for bioassay, biochemical analysis, and molecular study. The Bora (French Polynesia) *Ae. aegypti* strain was used as a susceptible control.

The history of insecticide exposure of each population was obtained. The LLK population was exposed to temephos (OP), deltamethrin (PY), and cypermethrin (PY). BKH population was continuously exposed to aerosol sprays containing various synthetic pyrethroids more than 10 years, whereas PSC was continuously exposed for the last few years to synthetic pyrethroids, mostly allethrin from mosquito coils. The BKL and PRV populations were not intensively exposed to insecticides, although various pyrethroids in the form of aerosols were used in the house.

Bioassays

The susceptibility of adult *Ae. aegypti* mosquitoes to deltamethrin was conducted by using WHO test kits with some modification (WHO 1981). Twenty to 25 adult females, 5-6 days old, non-blood-fed were exposed to 0.05% deltamethrin impregnated paper (World Health Organization, Penang, Malaysia). Control mosquitoes were exposed to paper without insecticide. In each mosquito population, triplicate bioassays were performed on different batches of mosquitoes. Each population was divided into four sets and each set (consisting

of two tubes) was exposed to deltamethrin-impregnated paper at different time intervals, including 30 min, 1 h, 2 h, and 3 h. Right after the defined time exposure, mosquitoes were transferred to clean tubes where sugar was provided before survivors were counted after 24 h. Scores of mortality at different exposure time periods were used to further calculate lethal time (LT_{50}). Bioassay data were pooled and LT_{50} was obtained by probit analysis basic program as described by Finney (1971). Resistance ratio (RR_{50}) was calculated by comparing LT_{50} of each population with LT_{50} of susceptible strain. Cross-resistance in adult mosquitoes to DDT was tested against BKH and PSC populations that had shown higher resistance than the remaining three populations. Mosquitoes were exposed to 4% DDT impregnated paper following the WHO standard method as described (WHO 1981).

Biochemical assays

Adult female mosquitoes that survived from the deltamethrin susceptibility test were kept at -80°C prior to subsection to biochemical analysis. A total of 46 to 52 mosquitoes per population surviving from the susceptibility test was subjected to biochemical assays in duplicate per assay. Total protein content of each mosquito was determined using a commercial protein assay kit (Bio-Rad, California, U.S.A.). Protein concentration was determined by converting the OD values into concentrations using bovine serum albumin as standard. Prior to enzyme assays, individual female mosquitoes were homogenised in 100 μl of 0.02M potassium phosphate buffer at pH 7.2 and the volume was made up to 1 ml with the same buffer. After centrifugation at 10,000 rpm, 3 min at 4°C , homogenates were used for assay of enzyme activities in a microtiter plate and OD values were measured by the microtiter plate reader, Multiskan EX (Thermo Labsystems, Finland).

The assay for mixed function oxidases (MFOs) activities was performed according to Brogdon et al (1997). The OD values were measured at 620 nm after 5 min incubation and the activity was measured using cytochrome C as a standard. Total oxidase activity was expressed as nmol equivalent cytochrome-P450. min^{-1} . mg protein^{-1} . Assay for activity of non-specific esterases was carried out as previously described (Brogdon and Dickinson 1983). The concentration of naphthol product was measured at 540 nm after 10 min as an end point. The OD values were converted to nmol of naphthol product. min^{-1} . mg protein^{-1} using either α or β naphthol as the standard. Glutathione S-transferase activity was measured according to the method described by Brogdon and Barber (1990). The OD values were measured at 414 nm at 0 and 20 min and the activity of GSTs was calculated as nmol CDNB. min^{-1} . mg protein^{-1} . Means of enzyme activities in each mosquito population were compared by analysis of variance (ANOVA) using the SPSS statistical program (SPSS Inc., 2001). Fisher's least significant difference (LSD) test was used to separate means at $\alpha = 0.05$.

RT-PCR and sequencing of partial cDNA of sodium channel gene

The same group of individual female mosquitoes

surviving from the susceptibility test subjected to biochemical assays was used for detection of mutation in the S4 -S6 of domain II of the sodium channel gene by RT-PCR. Eleven individuals from the PSC population and seven from the BKH population that were survivors from the bioassay against deltamethrin were homogenized and the total RNA was prepared using the NucleoSpin RNA II kit (Macherey-Nagel, Guitenberg, France) following the manufacturer's instructions. RT-PCR was carried out as previously described (Martinez-Torres et al. 1998). RNA was reverse transcribed to single stranded cDNA using a Superscript RNaseH⁻ reverse transcriptase kit (Gibco/BRL, New York, U.S.A.) and D1 primer (5' AA (G/A) (C/T) T (G/A/C/T) GC (G/A/C/T) AA (G/A) TCTTGGCC 3'). The resulting cDNAs were subjected to primary PCR using D1 primer and Dg2 (5' GC (T/G/A) AT (C/T) TT (A/G) TT (G/A/T/C) GT (G/A) TC (G/A) TT (G/A) TC 3') primer. Nested PCR was performed with primers D1 and Dip2 (5' TTG GAC AAA AGCAA (G/A) GCTAAG 3'). The PCR products from the BKH and PSC populations were subjected to DNA sequencing (BSU, Thailand).

RESULTS

Bioassays

In this study, bioassay baselines were established by time/mortality against deltamethrin in each population collected from the districts of Bang Khen (BKH), Bang Kholaem (BKL), Phasicharoen (PSC), and Pravet (PRV) in Bangkok province, and the district of Ladlumkaew (LLK) in Pathum Thani province, north of Bangkok. These susceptibility tests indicated low levels of deltamethrin resistance in all field populations compared to the susceptible Bora strain as shown in Table 1. Resistance to deltamethrin was consistent with the history of pyrethroid exposure in all five populations tested. Among the five populations tested, higher level resistance observed in BKH ($RR_{50}=17.2$) and PSC ($RR_{50}=13.6$) populations was correlated with the history of continuous

exposure to aerosol sprays containing various synthetic pyrethroids in BKH and exposure to mosquito coil containing allethrin in PSC. However, the LLK, BKL, and PRV populations that were not intensively exposed to pyrethroid group showed a lower resistance level against deltamethrin than the BKH and PSC populations.

To investigate whether deltamethrin resistance in BKH and PSC populations was cross-resistant to DDT that was used for decades, DDT susceptibility was tested in these populations. The LT_{50} values against DDT were 6 to 8 times higher than the susceptible Bora strain indicating that both BKH and PSC populations were resistant to DDT ($RR_{50}=8$ and 6.39 respectively) (Table 2). However, high LT_{50} (2.25 h) was observed in the susceptible strain, Bora, which may indicate tolerance to DDT in this strain. The remaining BKL, PRV, and LLK populations that showed resistance to deltamethrin were also observed to be resistant to DDT. Mortality lower than 35% after 7 h exposure was observed for the remaining three populations. However, the resistance ratio compared with Bora was not recorded in these three populations (unpublished data).

Biochemical assays

There were significant differences in MFOs activities between the Bora strain and field populations (Table 3). The increase in MFOs activities could be correlated with deltamethrin resistance observed among test populations and their history of pyrethroid exposure. In all populations, except BKL, there were significant differences in non-specific esterases between the Bora and field strains in association with deltamethrin resistance. There was no correlation of GSTs activities and bioassay results in populations tested against DDT. There was elevation of GST activity in BKH, BKL and LLK populations, while in PSC and PRV the GST activity was not elevated, although all populations showed resistance to DDT.

Table 1. Level of resistance to 0.05% deltamethrin in adult *Ae. aegypti* indicated by LT_{50} values, slope and resistance ratios among populations from Bangkok (BKH, BKL, PSC, PRV) and Pathum Thani (LLK) provinces.

Populations	Collection area	N ¹	LT_{50} ²	Slope (±SD)	RR_{50} ³
BKH	Bang Khen, Bangkok	684	86	2.136 ± 0.323	17.2
PSC	Phasicharoen, Bangkok	501	68	1.517 ± 0.262	13.6
BKL	Bang Kholaem, Bangkok	495	42	2.163 ± 1.100	8.4
PRV	Pravet, Bangkok	611	40	2.091 ± 0.560	8
LLK	Ladlumkaew Pathum Thani	809	52	3.12 ± 0.254	10.4
Bora	Bora, French, Polynesia	703	5	1.61 ± 0.164	1

¹ Total number of adult mosquitoes tested (excluding control).

² Expressed as minutes within 95% confidence limits.

³ Resistance ratio calculated at the LT_{50} level with reference to the susceptible Bora strain.

Table 2. Level of resistance to 4% DDT in *Ae. aegypti* indicated by LT_{50} values, slope and resistance ratios among populations from Bang Khen (BKH) and Phasicharoen (PSC), Bangkok.

Populations	Collecting area	N ¹	LT_{50} ²	Slope (SD)	RR_{50} ³
BKH	Bang Khen, Bangkok	417	18.30	4.605 ± 1.16	8
PSC	Phasicharoen, Bangkok	317	14.38	4.906 ± 1.12	6.39
Bora	Bora, French, Polynesia	528	2.25	2.61 ± 0.345	1

¹ Total number of adult mosquitoes tested (excluding control).

² Expressed as hours within 95% confidence limits.

³ Resistance ratio calculated at the LT_{50} level with reference to the susceptible Bora strain.

Sequencing of cDNA fragment of the sodium channel gene

To investigate whether an alteration of the sodium channel gene is involved in resistance mechanisms, a 348 bp cDNA sequence comprising the S4 to S6 hydrophobic segment of domain II of this gene was PCR amplified, sequenced, and its deduced amino acid sequence was obtained. Figure 1 shows alignment of the deduced amino acid sequences of BKH and PSC populations along with those of *An. gambiae*, *Cx. pipiens*, and previously sequenced *Ae. aegypti* (Martinez-Torres et al. 1998, 1999, Brengues et al. 2003). The amino acid sequence for these two field sites showed 97% identity to *An. gambiae* and 99% identity to *Cx. pipiens* and *Ae. aegypti*. Two amino acids previously identified as polymorphic alleles, valine (V, GTC) and histidine (H, CAT) at positions 42 and 51 (Brengues et al. 2003) noted in the sequence, shown in this study, were substituted with isoleucine (I, ATC) and lysine (K, AAG), respectively (Figure 1). The amino acid substitution, leucine (L, CTC) to phenylalanine (F, TTC) at position 104 found in pyrethroid *kdr* strains of *An. gambiae*, and *Cx. pipiens* (Martinez-Torres et al. 1998, 1999) was not found in sequences reported in this study. However, two amino acid

substitutions, namely substitution of serine (S, TCA) with proline (P, CCA) at position 64 in PSC and phenylalanine (F, TTC) with leucine (L, CTC) at position 69 in BKH were found. But these two substitutions were not observed in all cDNA sequences of the mosquitoes.

DISCUSSION

The present studies reported low-level deltamethrin resistance in *Ae. aegypti* populations in central Thailand. Similar levels of resistance were previously reported in Northern Thailand, in Chiang Mai and Nan provinces (Somboon et al. 2003). In the districts of Mae Kud and Mae Kasa in Chiang Mai province, northern Thailand, *Ae. aegypti* mosquitoes showed low-level resistance to permethrin ($RR_{95} = 5.4$ and 7 respectively) (Brengues et al. 2003). The results imply that various populations of *Ae. aegypti* in Thailand have developed resistance to pyrethroids. Low levels of pyrethroid resistance observed from *Ae. aegypti* mosquitoes in Thailand agreed with the level of resistance from previous reports (Mazzarri and Georghiou 1995), except for a few strains from

Figure 1. Deduced amino acid sequences of the domain II of the voltage gated sodium channel gene from *Ae. aegypti* populations of Bang Khen (BKH) and Phasicharoen (PSC) compared to *An. gambiae* (Genbank Accession no. Y13592), *Cx. pipiens* (Genbank Accession no. AJ012476), and *Ae. aegypti* (Genbank Accession no. AF534112). The IIS5 and IIS6 regions are boxed. Dots indicate identical amino acids among the populations. Arrows define intron/exon boundaries corresponding to *An. gambiae* sequence (Martinez-Torres et al. 1998). Shaded letters indicate amino acid positions where common substitution occurred (L104F). The substitution of two amino acids (V42I and H51K) previously identified as polymorphic alleles are shown. Substitutions of the two amino acids (Proline at position 64 and Leucine at position 69) referred to in the text are presented.

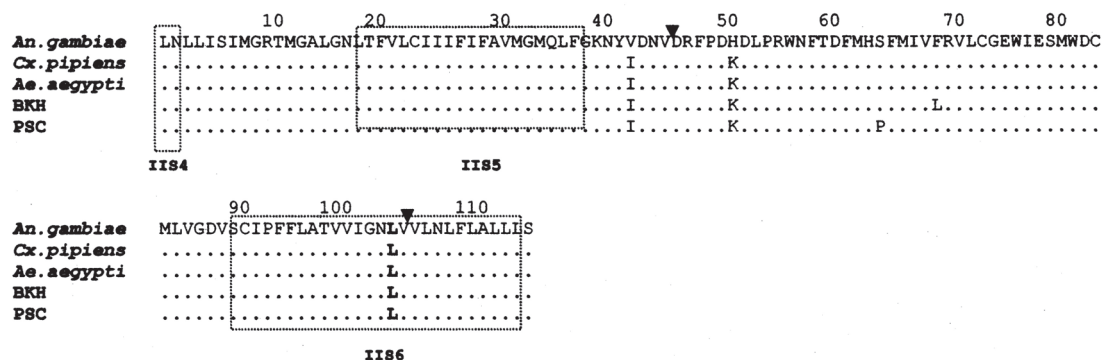


Table 3. Mean values and standard deviation of activities of mixed function oxidases (MFOs), non-specific esterases (α and β), and glutathione S-transferases (GSTs) in *Ae. aegypti* populations collected from different areas in Bangkok (BKH, BKL, PSC, PRV) and Pathum Thani (LLK) provinces compared to the Bora susceptible strain.

Population	Collecting Area (number of mosquito)	Total protein (mg protein/ml)	MFOs (nmole product/ min/mg protein)	α Esterase (nmole α naphthol/ min/mg protein)	β esterase (nmole β naphthol/ min/mg protein)	GSTs (nmole CDNB/ min/mg protein)
BKH	Bang Khen, Bangkok (49)	0.088 \pm 0.17	47.088 \pm 8.528 *	358.339 \pm 60.808 *	86.888 \pm 79.566 *	31.144 \pm 10.320 *
PSC	Phasicharoen, Bangkok (52)	0.078 \pm 0.026	33.112 \pm 9.398 *	329.311 \pm 44.243 *	87.035 \pm 17.480 *	7.039 \pm 3.666
BKL	Bang Kholaem, Bangkok (46)	0.042 \pm 0.015	36.764 \pm 8.331 *	215.191 \pm 80.250 *	48.859 \pm 20.333	29.415 \pm 10.312 *
PRV	Pravet, Bangkok (46)	0.060 \pm 0.020	28.089 \pm 6.274 *	220.572 \pm 77.413 *	56.563 \pm 22.342 *	8.928 \pm 6.667
LLK	Ladlumkaew, Pathum Thani (48)	0.078 \pm 0.020	39.206 \pm 10.525 *	271.727 \pm 72.183 *	61.657 \pm 12.280 *	13.86 \pm 7.956 *
Bora	Bora, French Polynesia (43)	0.145 \pm 0.031	20.303 \pm 4.551	163.875 \pm 29.931	43.737 \pm 11.250	8.655 \pm 4.471

*Significant increase in mean differences compared to the Bora susceptible strain ($p < 0.005$, Fisher's least significant difference test).

Indonesia, Vietnam, and Martinique where resistance levels between 35-296 folds were found (Bregues et al. 2003). Deltamethrin resistance in *Ae. aegypti* in Thailand could result from pre-selection with other pyrethroids constantly used, and /or cross-resistance to DDT. This can be demonstrated by the history of insecticide exposure in BKH, PSC, and LLK populations that had a history of prior exposure to pyrethroids. Despite DDT not being used in control programs for *Aedes sp.*, it was commonly used inside households, resulting in contaminated mosquito habitats. An effect on pyrethroid susceptibility by prior selective effects of DDT used in other insect control measures was reported by Brogdon et al. (1999).

Detoxification of pyrethroids by MFOs either alone or in combination with esterases and GSTs is suggested to play a role in pyrethroid resistance (Hemingway et al. 2004). Previous studies demonstrated that MFOs were the predominant enzyme responsible for high levels of permethrin and deltamethrin cross-resistance in *Cx. quinquefasciatus* in Saudi Arabia (Amin 1989). In another study, the elevation of monooxygenases levels has been reported in field populations of several insects including mosquitoes after exposure to pyrethroids and is an indicator of resistance to pyrethroids (Vulule et al. 1999). Our study found similar results, namely increases in MFOs were observed in all populations. Biochemical analysis in most populations except BKL suggested that esterases could be involved in deltamethrin resistance as well. Esterase metabolism alone or in combination with oxidases was reported to be associated with resistance to pyrethroids in *An. albimanus* and *An. gambiae* (Beach et al. 1989, Chareonviriyaphap et al. 1999b, Vulule et al. 1999). Alternatively, the elevated non-specific esterases activities could reflect historical exposure to organophosphates of *Ae. aegypti* populations, resulting in the existent amounts of these enzymes detected in this study. The use of synergists will help elucidate whether MFOs or esterases actually play a role in resistance.

Previous reports suggest that increased GST activity confer resistance to DDT in *Ae. aegypti* and *An. gambiae* (Grant et al. 1991, Prapanthadara et al. 1993). But there was no consistent increase in GSTs activity in association with DDT resistance in this study. The results agree with previous studies in *An. maculatus*, *Cx. quinquefasciatus*, and *Ae. aegypti* resistance to DDT in Malaysia (Lee and Chong 1995) and *An. albimanus* in Guatemala (Brogdon et al. 1999) where other enzyme systems, such as MFOs, may have been involved in DDT-pyrethroid cross-resistance. The variability of enzyme metabolism observed in the populations reported here could be related to the history of insecticide pressure against each population, through intensive exposure to household insecticides or insecticides used for agricultural purposes. A similar result has been observed in a *Blatella germanica* pyrethroid resistance strain in Alabama, U.S.A. (Pridgeon et al. 2002).

We have sequenced the IIS4-IIS6 region to detect any possible amino acid substitution compared to the *Ae. aegypti* sequences previously reported. Our results detected serine to proline (S64P) and phenylalanine to leucine (F69L) amino acid substitutions in PSC and BKH populations, respectively.

Such substitutions could represent either allelic polymorphisms or be associated with sodium channel insensitivity as previously described for isoleucine to methionine (I101M) and valine to glycine (V106G) mutations reported by Bregues et al. (2003). Further investigation on more mosquito field samples from various collections could help reveal whether the amino acid changes are related to resistance.

The deltamethrin resistance reported here indicates the limitation of new pyrethroid candidates for use in mosquito control programs. Further susceptibility assays on populations from various locations and associated enzyme activities will help determine the prediction susceptibility status among populations and lead to the selection of effective insecticides for *Aedes sp.* mosquito control in Thailand.

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