Characterization of metabolic detoxifying enzymes in an insecticide resistant strain of *Aedes aegypti* harboring homozygous S989P and V1016G kdr mutations

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**Abstract:** Important insecticide resistance mechanisms in the dengue vector *Aedes aegypti* are mutations of voltage-gated sodium channel (VGSC) genes or knockdown resistance (*kdr*) and increased activity of metabolic enzymes. The objective of this study was to determine activity of mixed-function oxidases (MFO), esterases and glutathione-S-transferases (GSTs) in two strains of *Ae. aegypti*. The UPK-R strain, which harbors S989P and V1016G homozygous mutations in the VGSC, was compared with the wild-type PMD strain. Adult bioassays revealed that the UPK-R was resistant to DDT, permethrin, deltamethrin and malathion, whereas the PMD was resistant to only DDT. Enzyme activity in larvae, pupae and adults of the UPK-R strain was statistically higher than that observed in the PMD strain (mostly 1-2 fold). The current work supports previous studies which have suggested that increased MFO activity plays a partial role in pyrethroid resistance, whereas *kdr* is the major mechanism. Resistance to organophosphates and DDT is probably due to cross-resistance of MFO or increased activities of esterases and GSTs, respectively. Metabolic resistance combined with *kdr* may complicate insecticide-based control of dengue vectors in Thailand.

**Key words:** *Aedes aegypti*, insecticide resistance, *kdr*, metabolic enzymes

**Introduction**

*Aedes aegypti* is the principal vector of dengue, Chikungunya, Zika and yellow fever viruses. It is widespread in tropical and subtropical countries throughout the world. Approximately 50 million dengue infections occur every year, of which half a million lead to hemorrhagic manifestations requiring hospitalization and cause over 20,000 deaths (WHO, 2012). In Thailand, the first dengue outbreak was reported in the 1950s, and since then dengue fever has become a major public health problem. In 2016 alone, there were 50,856 dengue cases and 44 deaths reported in Thailand (Ministry of Public Health, 2016).

A vaccine against Dengue, Dengvaxia® (CYD-TDV), has been licensed since 2015, but the overall efficacy of trials has been about 60% and it has not been used on a large scale (WHO, 2017). Due to the lack of an effective vaccine or specific treatments, control of disease transmission is therefore must continue to rely on vector management, including insecticide application and the elimination of breeding sites. Several types of insecticides, including organochlorines (DDT), organophosphates (e.g., malathion, fenitrothion, and temephos), and carbamates (e.g., propoxur) were extensively used for mosquito control for over 50 years in Thailand before being replaced (except temephos) by pyrethroids in the early 1990s (Chareonviriyaphap et al., 1999). Consequently, an adverse effect of such long-term insecticide use has been the development of insecticide resistance in vector populations throughout the world. In Thailand, DDT resistance in *Ae. aegypti* was first reported in the mid-1960s (Neely, 1966). At present, *Ae. aegypti* is known to be resistant to several types of insecticides including pyrethroids (Somboon et al., 2003; Paeporn et al., 2004; Ponlawat et al., 2005; Jirakanjanakit et al., 2007; Pethuan et al., 2007).

Insecticide resistance in *A. aegypti* is generally due to two main mechanisms, metabolic enzyme-based resistance and knockdown resistance (*kdr*) (Hemingway and Ranson, 2000). Metabolic enzyme-based resistance is principally associated with three enzyme groups: mixed-function oxidases (MFO), esterases, and glutathione-S-transferases (GSTs). Knockdown resistance is the important mechanism conferring resistance to pyrethroids and DDT. It is associated with single or multiple mutations in the genes encoding voltage-gated sodium channel (VGSC) proteins. In Asia, two common *kdr* mutations...
have been reported: V1016G, a valine to glycine transversion in domain II of the VGSC (Brengues et al., 2003), and F1534C, a phenylalanine to cysteine substitution at position 1534 within domain III. (Yanola et al., 2010). V1016G mutation is associated with resistance to type I (e.g., permethrin) and type II (e.g., deltamethrin) pyrethroids, while F1534C is associated with resistance to only type I pyrethroids (Stenhouse et al., 2013). In Latin America, F1534C and V1016L, a valine to isoleucine transversion in domain II, are prevalent (Saavedra-Rodriguez et al., 2007; Harris et al., 2010). The V1016I mutation has also been reported from Vietnam, but has not been detected in Thailand (Bingham et al., 2011). Interestingly, the V1016G mutation is often found with a serine to proline mutation (S989P) in domain II (Stenhouse et al., 2013; Kawada et al., 2014). Although S989P itself does not reduce the sensitivity to pyrethroids, its combination with V1016G mutation in homozygous forms largely increases resistance to pyrethroids (Hirata et al., 2014). Recent studies in Thailand revealed three genotypes of kdr mutations in Ae. aegypti populations, C1534 homozygote, P989+G1016 homozygote and triple mutated heterozygote (S/P989+V/G1016+F/C1534), while wild type (no kdr mutations) appears to be absent (Yanola et al., 2011; Stenhouse et al., 2013; Plernsub et al., 2016a). Although kdr alleles are recessive, combination of the three kdr alleles in the triple mutated heterozygote (S/P989+V/G1016+F/C1534) confers high resistance to pyrethroids (Plernsub et al., 2016a, b).

We have previously investigated the role of metabolic detoxifying enzymes in the PMD-R strain of Ae. aegypti, which harbors the C1534 homozygous mutation, compared with the wild-type PMD strain (Somwang et al., 2011). Both strains originated from a rural area of Chiang Mai Province in Thailand. The permethrin and deltamethrin resistance levels (as determined by larval bioassays LC50s) of PMD-R were higher than those observed in the susceptible PMD strain by 25 and 13 fold, respectively (Yanola et al., 2010; Plernsub et al., 2016b). Mixed function oxidases and aldehyde hydrogenases play a partial role in pyrethroid resistance in PMD-R (Somwang et al., 2011; Lumjjuan et al., 2014).

Recently, we established another insecticide resistant strain of Ae. aegypti, UPK-R, originating from wild caught mosquitoes from Chiang Mai city. This strain harbors P989+G1016 homozygous mutations, and is resistant to DDT, permethrin and deltamethrin. This kdr genotypic form is also common in Myanmar, Singapore and Indonesia (Kawada et al., 2014; Kasai et al., 2014; Sayono et al., 2016). Although kdr mutations are generally considered to be important in conferring pyrethroid resistance, the role of metabolic enzymes in the UPK-R strain is not known. The current study reports the characterization of mixed-function oxidases, esterases and glutathione-S-transferases in this strain. Understanding these insecticide resistance mechanisms will improve our ability to detect and manage insecticide resistance.

Materials and Methods
Mosquito strains
Two laboratory strains of Ae. aegypti, PMD and UPK-R, were used in the current study. The PMD strain originated from Ban Pang Mai Daeng, a rural village of Mae Taeng District, Chiang Mai Province, Thailand (Prapanthadara et al., 2002). This strain does not have any kdr mutations. It is susceptible to pyrethroids, but resistant to DDT. The UPK-R strain was established from wild mosquitoes collected in Chiang Mai city in 2006. Several P989+G1016 homozygous isofemale lines that had survived deltamethrin (0.05%) bioassays were pooled to establish this strain. The deltamethrin resistance level of UPK-R was higher than the susceptible PMD strain by 53-fold, as determined by larval bioassays (Plernsub et al., 2016b). To establish sub-colonies, eggs were taken from the UPK-R and PMD stock colonies and allowed to hatch in distilled water. The rearing method of mosquitoes followed our routine procedures as previously described (Stenhouse et al., 2013). The sub-colony eggs were produced from females artificially blood-fed (Finlayson et al., 2015), with minor modifications. A total of 30 females and 30 males from each sub-colony were selected at random and checked for the occurrence of the V1016G and F1534C mutations by allele-specific PCR (AS-PCR) methods, and S989P mutation by DNA sequencing (Yanola et al., 2011; Stenhouse et al., 2013).

Insecticide susceptibility tests
Insecticide resistance status of the UPK-R and PMD sub-colonies was determined following WHO guidelines (WHO, 2016) in which insecticide-impregnated papers (4% DDT, 0.75% permethrin, 0.05% deltamethrin and 5% malathion) were used for adult bioassays. Twenty-five one-day old sugar-fed female mosquitoes were held for one hour in an exposure tube lined with insecticide-impregnated paper. Thereafter, they were then transferred to a holding tube and provided with a 10% (w/v) sucrose solution containing multivitamin syrup. Control mosquitoes were exposed to non-treated paper. Mortality rates were calculated 24 hr post exposure. Mosquitoes that were unable to fly or move were recorded as dead. Four replicates of each test and two control replicates were performed for each insecticide. If the mortality in the control group ranged between 5–20%, the mortality rate in the test groups was corrected by Abbott’s formula (Abbott, 1925). A test was rejected if control group mortality exceeded 20%.
Mixed-function oxidase assay (based on hemoprotein)

Mixed-function oxidase enzyme assay was performed according to the method of Brogdon et al. (1997). Heme peroxidase activity has been used to express the levels of general oxidase, which is an indirect measurement of mixed-function oxidase activity based on heme-containing enzymes (Penilla et al., 2007, Etang et al., 2007, Verhaeghen et al., 2009, Pocquet et al., 2013 and Marcombe et al., 2014). TMB dihydrochloride hydrate (TMB.2HCl.xH2O, Sigma-Aldrich) was used as substrate instead of 3,3′,5,5′-tetramethyl benzidine (TMBZ) (Sigma-Aldrich) since TMBZ tends to be less soluble than TMB.2HCl.xH2O.

Ten mosquitoes (consisting of groups of 4th instar larvae, 1- and 4-day old pupae or 1-, 7-, and 14-day old adults) were pooled and homogenized with plastic pestles in 800 µL of homogenization buffer (0.25 M sodium acetate buffer pH 5.0). Mixtures were centrifuged at 10,000×g for 20 minutes at 4°C, and supernatants were collected to be used as mosquito homogenates for the biochemical assays. A total of two hundred of mosquitoes per stage and sub-colony were examined.

One hundred microliters of larval and one day old pupal homogenates were measured directly. The four-day old pupal and adult mosquito homogenates were diluted 1:2 and 1:10, respectively, with homogenization buffer before measuring. One hundred microliters of homogenization buffer were used as a control. The reaction mixtures were prepared with 200 µL of TMB.2HCl.xH2O solution (0.01 g of TMB.2HCl.xH2O in 5 mL absolute methanol mixed with 15 mL of 0.25 M sodium acetate buffer pH 5.0), 25 µL of 3% hydrogen peroxide (H2O2) (MERCK) and 100 µL of mosquito homogenate. Reaction mixtures were then incubated for 5 minutes at room temperature and read at a wavelength of 630 nm using microplate reader (Spectra MR, DYNEX technologies). A standard curve was prepared using cytochrome c bovine heart (Sigma-Aldrich). Activity was compared with known concentrations of cytochrome c standard. Values were reported as nmol of cytochrome c equivalent unit/mg protein.

Cytochrome c from bovine heart was dissolved with phosphate buffer and used as the known concentration standard. All aliquots (100 µL of 0.1 µg/µL cytochrome c mixture) were kept in −20°C. The appropriate concentrations of standard cytochrome c were 0.4, 0.6, 0.8, 1.0, 1.2 and 1.4 ng/µL. After repeated freeze-thaw cycles of the same aliquots, there were significant decreases of cytochrome c levels, particularly after the 2nd and 3rd cycles (Fig. 1). Hence, only the first freeze-thaw cycle of aliquots was used for standard dilution.

Esterase assay

Four hundred mosquitoes per life stage were examined for esterase activity. Twenty mosquitoes were pooled and homogenized in homogenization buffer (200 µL 50 mM potassium phosphate buffer pH 6.5 with 1 µM DL-dithiothreitol 99.5% (or DTT, Bio Basic Inc.). Homogenates were centrifuged at 10,000×g for 20 minutes at 4°C, and supernatants were transferred to fresh microcentrifuge tubes. The protocol for esterase assay followed Surendran et al. (2012). p-Nitrophenyl acetate (pNPA) (Sigma-Aldrich) was used as a substrate. Each esterase assay was performed in 200 µL of pNPA working solution, containing 100 mM pNPA in acetonitrile and 50 mM sodium phosphate buffer pH 7.4 (ratio 1:100) added to 10 µL of homogenate. Ten microliters of homogenization buffer were used for the reagent blank. After hydrolysis with esterases, the p-nitrophenol product was measured at 405 nm for 2 minutes. Esterase activity was reported as µmol p-nitrophenol/min/mg protein. The extinction coefficient of p-nitrophenol at 405 nm is 6.53 mM−1 (corrected for a path length of 0.6 cm).

Glutathione-S-transferase assay

The supernatants from esterase assay were also used for Glutathione-S-transferase (GST) assay. The method was performed as described previously (Habig et al., 1974). Glutathione (GSH) and 1-Chloro-2,4-dinitrobenzene (CDNB) (Sigma-Aldrich) were used as substrates. The enzyme reaction was monitored by the conjugation of GS-DNB product which increased the absorbance at 340 nm. Two hundred microliters of 63 mM CDNB and 10 mM GSH in 0.1 M potassium phosphate buffer pH 6.5 were added to 10 µL of homogenates. Ten microliters of homogenate buffer were used for the reagent blank. Enzyme rates were measured at 340 nm for 2 minutes. The extinction coefficient for CDNB conjugate at 340 nm is 9.6 mM−1 cm−1. GST activity was reported as µmol GS-DNB conjugate/min/mg protein.
Protein determination

The protein concentration of tested mosquito samples was determined by the Bradford method using the Bio-Rad protein-assay dye reagent (Bio-Rad Laboratories Inc.) and bovine serum albumin (Thermo) as a standard (Bradford, 1976). Two hundred microliters of Bio-Rad protein reagent solution (1:4 Dye) was added into 10 µL of the mosquito homogenate. Ten microliters of homogenization buffers were used as a blank. The reaction was read at 595 nm after 5 minutes incubation at room temperature. The albumin standard concentrations were 0.1, 0.2, 0.3, 0.4 and 0.5 mg/mL. Protein values of samples were calculated from a standard curve of absorbance of albumin standard.

Statistical analysis

SPSS version 16.0 was used for statistical analysis. The independent sample t-test was used for comparing the means of enzyme activity between the two strains of mosquitoes (p<0.05). For comparing among the stages of mosquitoes, ANOVA was used (p<0.05).

Results

Insecticide susceptibility test

Testing for kdr mutations in the UPK-R sub-colony confirmed the presence of P989, G1016 and F1534 homozygous alleles in all individuals tested. No kdr mutations were found in the PDM sub-colony. Bioassays of UPK-R female mosquitoes with 4% DDT, 0.75% permethrin, 0.05% deltamethrin and 5% malathion revealed mortality rates of 0%, 0%, 7% and 84%, respectively (Table 1). The PDM strain was resistant to DDT with 1% mortality but was susceptible to the other insecticides.

Mixed-function oxidase assay (based on hemoprotein)

Mixed-function oxidase activities in various life stages of the PMD and UPK-R strains are presented in Fig. 2. The UPK-R showed significantly greater levels of activities (~1.5 fold) than the PDM in most stages, except 7-day, 14-day old female adults and 14-day old male adults. Older pupae (4-day old) of both genders had significantly higher activities than younger pupae (1-day old) and 4th instar larvae. Mixed-function oxidase activities displayed the largest increases in the adult stage. Adult males showed significantly higher activities than females of the same ages. Enzyme activities in males and females increased with age.

Esterases assay

The UPK-R strain revealed significantly higher activities of esterases than the PMD, except 14-day old female adults (Fig. 3), but such differences were also small. Within each strain, the activities of this enzyme in the larval, pupal and adult stages were similar, but there was a tendency towards decreased enzyme activity in older adults of both genders.

Glutathion-S-Transferase assay

Glutathione-S-transferase (GST) activities were significantly higher in all stages of mosquito development in the UPK-R strain compared to the PDM strain (Fig. 4). Similar to the other two enzymes, such differences were small. Larval stages had less activity than pupal and adult stages in both strains. Within each strain, enzyme activities did not differ significantly between pupal and adult stages. There was a tendency towards decreasing enzyme activity in older female adults of both strains.

Discussion

Insecticide resistance in mosquitoes is complex

![Graph showing mixed-function oxidase levels in Aedes aegypti PMD and UPK-R strains.](image)

Table 1. Adult susceptibility test of the PMD and UPK-R strains. The four replicates for each test have been pooled in this table.

| Insecticides | PMD Strain | UPK-R Strain |
|--------------|------------|--------------|
| DDT          | No. tested | No. dead | % mortality | No. tested | No. dead | % mortality |
|              | 100        | 1        | 1           | 100        | 0        | 0           |
| Permethrin   | 100        | 100      | 100         | 100        | 0        | 0           |
| Deltamethrin | 100        | 100      | 100         | 100        | 7        | 7           |
| Malathion    | 100        | 100      | 100         | 100        | 84       | 84          |
and often involves two major mechanisms, metabolic enzymes and target-site insensitivity. Normally, both mechanisms work together, but their importance may vary depending on species, genetic background, and type of insecticide. In this study, three major metabolic enzymes were quantitatively assayed to determine their role in pyrethroid resistance in the *Aedes aegypti* UPK-R strain which harbors P989+G1016 homozygous mutations. Insecticide bioassays indicated that this strain is clearly resistant to DDT, permethrin and deltamethrin (mortality <90% as recommended by WHO, 2016); however, the mosquitoes were over-exposed to the insecticides due to the high dosage of the tube bioassay. Since an insecticide susceptible strain, such as the Rockefeller strain, was not available in our laboratory, the PMD strain which is susceptible to pyrethroids but resistant to DDT was used for comparing enzyme activity. A previous study (Prapanthadara et al., 2002) revealed that the activities of these three metabolic enzymes in adult females of the pyrethroid susceptible PMD and the C1534 homozgyous mutant PMD-R strains (previously called R^S^0 and R^R^p, respectively) were similar. When compared with the Rockefeller strain, both PMD and PMD-R strains displayed higher levels of dehydrochlorinase (DDTase) (~10 fold) and cytochrome P450s (~4 fold), whereas esterase and GST activities were only slightly increased relative to the Rockefeller strain.

Since the PMD and PMD-R strains originated from the same area, they are considered to have similar genetic backgrounds. The levels of microsomal cytochrome P450s extracted from the fourth instar larvae were similar in both strains (Somwang et al., 2011). However, there are differences in another oxidative enzyme system between the two strains. In the PMD-R strain, alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) were shown to be involved in permethrin metabolism. Two ALDH genes, ALDH9948 and ALDH14080 were upregulated in the PMD-R strain and both recombinant proteins showed oxidase activity against phenoxybenzyl aldehyde, an aldehyde moiety of pyrethroid (Lumjuan et al., 2014). The addition of piperonyl butoxide (PBO), an oxidase inhibitor, to larval bioassays of both PMD-R and F/C 1534 heterozygous individuals reduced permethrin resistance by ~3 fold and ~8 fold, respectively (Yanolta et al., 2010; Somwang et al., 2011). Permethrin resistance in the PMD-R strain is conferred mainly by the F1534C mutation with oxidative enzymes playing a partial role in overall resistance. The role of ADH and ALDH in pyrethroid resistance in the UPK-R has not been studied.

In the current study, the mixed-function oxidase activity in the UPK-R strain was slightly higher (~1.5 fold) than the PMD strain in most stages. A previous study showed that deltamethrin resistance in the UPK-R strain was reduced ~1.5 fold by the addition of PBO, however this resistance was still ~38 fold higher than that observed for the PMD strain (Plernsub et al., 2016b). These results indicate that *kdr* is the major pyrethroid resistance mechanism in the UPK-R strain and oxidase enzymes play only a partial role in resistance.

In addition, mixed-function oxidase activity in the UPK-R strain varied according to stage, sex and age of mosquitoes. High activity was found in the adult stage and increased with age. Additionally, males showed greater enzyme activity than females. Such
increased activity in males and older aged mosquitoes is not considered to contribute to insecticide resistance since mortality following insecticide exposure tends to increase with age, and males are usually more susceptible than females (Hunt et al., 2005). Similarly, Amena et al. (2008) revealed an increased expression of CYP6P9 gene in older aged Anopheles funestus (from 3 to 14 days old). Later, Christian et al. (2011) demonstrated that such increased expression of CYP6P9 was not directly related to the resistance of An. funestus. This would indicate that a number of P450 genes have a primary role in metabolism of endogenous compounds rather than xenobiotics (Maibeche-Coisne et al., 2000).

The activity of esterases in the UPK-R strain was slightly higher than the PMD strain in most stages, but may not be related with pyrethroid resistance. Esterase activity in the PMD strain was shown to be slightly higher than that seen in the Rockefeller strain (Prapanthadara et al., 2002). Several studies have shown that elevated levels of non-specific esterase activity is correlated with resistance to organophosphates, such as temephos and malathion (Hemingway and Ranson, 2000). The activity of GSTs in the UPK-R strain was also slightly higher than the PMD strain (Table 1) may be partially attributed to the increased activity of esterases and/or cross-resistance mechanism of mixed-function oxidases (Hemingway and Ranson, 2000).

The activity of GSTs in the UPK-R strain was also slightly higher than the PMD strain in most stages, but this enzyme may not be related to pyrethroid resistance. These two strains and the PMD-R strain is associated with increased DDTase activity (Prapanthadara et al., 2002). Later, Prapanthadara et al. (2005) concluded that the increased DDTase activity of fractionated GST-1unBPc was involved in DDT resistance. Functional characterization of recombinant GSTE2-2 from the PMD-R strain confirmed the role of this enzyme in DDT metabolism (Lumjuan et al., 2005). However, DDTase activity conferring resistance to DDT in the UPK-R strain has not yet been investigated. In addition to increased esterase activity, increased activity of GSTs may have a secondary role in organophosphate resistance (Hemingway and Ranson, 2000).

Metabolic resistance may play an important role in insecticide resistance in some Ae. aegypti populations elsewhere. In Africa, metabolic resistance is important when kdr mutations are absent (Ngoagouni et al., 2016). A study in Vietnam reported that pyrethroid resistance in Ae. aegypti was conferred by V1016I mutations and cytochrome P450s; adding PBO enhanced the efficacy of pyrethroids (Bingham et al., 2011). In Malaysia, although kdr mutations (V1016G and F1534C) were present in Ae. aegypti populations (Ishak et al., 2015), mixed-function oxidases were the major pyrethroid resistance mechanism and PBO largely enhanced the toxicity of pyrethroids (Wan-Norafikah et al., 2010). They also found that there was no correlation between V1016G and resistance to pyrethroid and DDT. It should be noted that unlike Ae. aegypti populations in Thailand, the S989P mutation was absent from Ae. aegypti populations in Malaysia, and hence they lacked the additive resistant effect conferred by the V1016G mutation (Hirata et al., 2014; Plernsub et al., 2016b). Among Brazilian Ae. aegypti populations, increased activities of esterases and GSTs alongside the 11011M mutation might be correlated with increased pyrethroid resistance (Martins et al., 2009).

In Chiang Mai city, although the frequency of the P989+G1016 homozygous genotype was 0.15, which was lower than C1534 homozygote (0.39) and S/P989+V/G1016/F/C1534 heterozygote (0.46), this genotypic form was highly tolerant to an outdoor thermal fogging spray with deltamethrin mixed with S-bioallethrin and PBO (Plernsub et al., 2016a). Additionally, about half of the heterozygous mosquitoes (S/P989+V/G1016+F/C1534) survived the spray while about 80% of the C1534 homozygous mosquitoes died. Thus, the addition of PBO does not increase the efficacy of deltamethrin spraying against P989+G1016 mutant Ae. aegypti. Since the frequencies of P989 and G1016 alleles are very high in Myanmar (Kawada et al., 2014) and there is an increasing trend for these alleles in Thailand (Plernsub et al., 2016a) and Indonesia (Sayono et al., 2016), we emphasize the importance of monitoring the occurrence of kdr mutations in field populations of Ae. aegypti. Kdr mutations can hamper the efficacy of using pyrethroid-based control approaches, and hence there is a need for alternative methods or chemicals that can overcome kdr in the long term.

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