POTENTIAL DEVELOPMENT OF TEMEPHOS RESISTANCE IN *AEDES AEGYPTI* RELATED TO ITS MECHANISM AND SUSCEPTIBILITY TO DENGUE VIRUS

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Abstract. The addition of temephos to water containers as a larvicide against *Aedes aegypti* was commonly used as a part of DHF control programs. The widespread, or long-term, application of insecticides can lead to the development of mosquito resistance to the insecticides through selection pressure. This presents a problem for disease control. Therefore, this study was conducted in the laboratory to observe the potential development of resistance to temephos and the mechanism involved in *Ae. aegypti,* and to study the significance for dengue infection. The larvae were selected in consecutive generations. The level of resistance to temephos was detected by WHO assay technique. After 19 generations of selection, a low level of resistance was found. The resistance ratio at LC_{50} was 4.64 when compared with the non-selected group. The assay for major enzyme-based resistance mechanisms was done in a microtiter plate to detect elevated non-specific esterases, monooxygenase, and insensitive acetylcholinesterase in the temephos-selected and non-selected groups. It revealed a significant increase in esterase activity when compared with the non-selected group. There was no elevation of monooxygenase or insensitive acetylcholinesterase activities. However, when an esterase inhibitor (S, S, S-tributyl phosphorotrithioate, or DEF) was added to temephos and the susceptibility in the selected group was studied, the resistance ratio was reduced from 16.92 to 3.57 when compared with a standard susceptible strain (Bora Bora). This indicates that the esterases play an important role in temephos resistance.

Dengue-2 virus susceptibility was studied by oral feeding to females of the temephos-selected (S19) and the non-selected groups. The dissemination rates, when the titer of virus in the blood meal was 7.30 MID_{50}/ml, were 11.11% and 9.38% for the selected and non-selected groups, respectively. When the titer of virus in the blood meal was 8.15 MID_{50}/ml, the dissemination rates increased to 24.24% and 33.33%, respectively. A statistical difference in viral susceptibility was not found between the two groups. This suggested that the low level of temephos resistance might not affect oral susceptibility. However, this needs further study.

INTRODUCTION

Dengue hemorrhagic fever first appeared as an epidemic in Bangkok in 1958. The epidemic pattern has changed from one of alternate years to an irregular pattern (Kantachuvessiri, 2002), with more than 20,000 deaths per year. The mosquito *Aedes (Stegomyia) aegypti (L.)*, the main vector of dengue virus, was presumably introduced to Thailand and other parts of Southeast Asia by vessels sailing across the Indian Ocean from Africa, where this species originated (Mattingly, 1957; Laird, 1994). *Ae. aegypti* was primarily urban, with the highest densities in cities and towns.

Until an effective, safe and affordable vaccine becomes available, no adequate prevention or control measures, other than control of the vector, *Ae. aegypti*, are available to deal with epidemics of disease (Gratz, 1993). The only effective approach to *Ae. aegypti* control has been the elimination of larval habitats from the domestic environment, or source reduction (Soper *et al*, 1943; Schliessman and Calheiros, 1974). However, difficulty with control has been encountered due to the emergence of insecticide-resistant mosquitos. Data on insecticide susceptibility, resistance detection and characterization of the mechanisms of *Ae. aegypti* will provide base-line data for planning control programs and making decisions about insecticide usage. In this study, the differences in insecticide susceptibility, and susceptibility to dengue-2 virus in a temephos-selected group were examined.

MATERIALS AND METHODS

Mosquitos

Three groups of mosquitos were used in this study. The Nonthaburi colony was derived from the

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collection of *Ae. aegypti* larvae indoors and outdoors at Anurajprasit Kindergarten School and the area around the school. After one generation in the laboratory, this colony was divided into 2 groups; the first was subjected to temephos selection (Nonthaburi-Sel group) and the other was maintained without selection (Nonthaburi-Non-sel group). The third group, *Ae. aegypti* Bora Bora strain (WHO susceptible strain), which was obtained from Prof Yap Han Heng, Universiti Sains Malaysia, Penang, was used as the reference susceptible group.

Insecticides

Technical grade (90% purity) temephos (Abate), an organophosphate insecticide, was obtained from Cyanamid Co.

Bioassay procedures

The late third or early fourth instar larvae of all groups were used for bioassay test. The procedures were as recommended by WHO (1963). The results were analyzed for median lethal concentration (LC_{50}) and LC_{95} by probit analysis using a Basic program (Raymond, 1985).

Selection procedures

The Nonthaburi group was used for selection. The groups of 25 late third or early fourth instar larvae were exposed to temephos in 250 ml of dechlorinated tap water for 24 hours. The concentrations used for selection were 0.0025 mg/l for S_1 to S_4 generations, 0.005 mg/l for S_5 to S_7, 0.01 mg/l for S_7 to S_{14} and 0.02 mg/l for S_{15} to S_{19} generations. The surviving larvae from each exposure were reared for further selection.

Synergism test for confirmation of the defence mechanism

This test was similar to the bioassay tests, except that 0.5 ml of the maximum sublethal concentration of an esterase inhibitor, S,S,S-tributyl phosphorotrithioate, (0.5 μg/ml) was added to each cup with 0.5 ml of insecticide.

Microtiter plate assay

Esterase assay. Total esterase activity in individual, frozen mosquito larvae (late third or early fourth instar) from Nonthaburi-Non-sel, Nonthaburi-Sel and Bora Bora strains were dertermined according to the method of Lee (1991). Enzyme activity was determined as an OD value by microplate reader at 450 nm.

Monooxygenase assay. To measure the activity of monooxygenases from individual larvae, the procedure described by Vulule *et al* (1999) was adopted with a single modification, by using the same buffer (potassium phosphate buffer) as esterase assay in preparing the larval homogenate. Enzyme activity was determined by a microplate reader at 620 nm.

Acetylcholinesterase assay. Homogenates of the mosquito larvae were tested for insensitive AChE using the method of Lee *et al* (1992), which was modified from the Ellman test (Brogdon *et al*, 1988). Enzyme activity was determined by a microplate reader at 410 nm.

Protein concentration determination

The protein in each larva was determined by the method of Bradford (1976), in order to detect the differences in size among individuals that might require correction factors for the enzyme assays, as in the case of esterase and monooxygenase assays.

Virus susceptibility

After 19 generations of selection with temephos, the susceptibilities to dengue 2 virus by oral feeding (Sucharit *et al,* 1997) were compared among the temephos selected-group, the non-selected group and the standard susceptible strain (Bora Bora). The method for determining susceptibility to dengue virus is as follows:

Preparation of dengue virus antigen. Den-2 infected mosquitos, *Toxorhynchites splendens*, were ground with phosphate-buffered saline (PBS) pH 7.5 containing 30% inactivated fetal calf serum (FCS) and clarified by centrifugation at 7,000 rpm at 4°C for 1 hour.

Oral feeding. Infectious blood meals were prepared by mixing 2 parts virus suspension, 1 part washed red cells, and 2 parts 10% sugar solution. Three to five days old, fasted female mosquitos were forced to feed on infectious blood meals using an artificial membrane feeder for 30 minutes. Fully engorged mosquitos were kept, provided with 10% sugar solution and maintained at 32°C, 70-80% RH for 14 days.

Detection of dengue antigen. After 14 days' incubation, squashed heads of *Aedes aegypti* were checked by direct immunofluorescence (DFAT), using fluoro-isothiocyanate (FITC) conjugated to determine the infection.

Virus assay. Virus titration was done by intrathoracic inoculation of 10-fold serial dilution of virus suspension into *Tx. splendens*. The mosquito infectious dose 50 (MID_{50}) was calculated from the

positive mosquito head squash after inoculation for 14 days, by the method of Reed and Meunch (1938).

RESULTS

In generation 19 of temephos selection, the LC_{50} in the Nonthaburi-Sel group increased to 0.0154. It showed low level resistance by an increase of nearly 5-fold at the LC_{50}, when compared with the Nonthaburi-Non-selected strain (Table 1).

In the absence of selection pressure, the temephos resistance ratio of the Nonthaburi-Non-selected group, compared with the Bora Bora strain was 3.65, at LC_{50}. After 19 generations of selection, the temephos resistance ratio at LC_{50} increased to 16.92 (Table 3).

Table 1
LC^{a}_{50} of temephos in selected generations of *Aedes aegypti.*

Generation	Nonthaburi-Sel	Resistance ratio
S_0 (F_1)	0.00332	1.00
S_2	0.003	0.90
S_4	0.006	1.81
S_6	0.005	1.51
S_8	0.010	3.01
S_9	0.010	3.01
S_{10}	0.013	3.92
S_{15}	0.013	3.92
S_{19}	0.0154	4.64

[a] Median lethal concentration (mg/liter).

The addition of the esterase inhibitor, DEF, to the temephos resulted in a reduction in the resistance ratio, as shown in Table 3. The resistance ratio in the Nonthaburi-Sel group was reduced to 3.57 at LC_{50}.

Biochemical testing revealed the presence of elevated esterase activity in the Nonthaburi-Sel group (Table 2). No changes were found for monooxygenase activity (Table 4) and no evidence of insensitive acetylcholinesterase in the Nonthaburi-Sel group (Table 5). This suggested that the resistance was not associated with monooxygenases or insensitive acetylcholinesterase.

The susceptibility of mosquitos to dengue-2 virus showed the dissemination rates, when the titer of virus in the blood meal was 7.30 MID_{50}/ml, were 11.11%, 9.38% and 3.13% for the selected, non-selected groups and Bora Bora, respectively. When the titer of virus in the blood meal was 8.15 MID_{50} /ml, the dissemination rates increased to 24.24%, 33.33% and 8.57%, respectively (Table 6). However, there was no significant difference in virus susceptibility between each group.

DISCUSSION

Selection for temephos resistance showed that *Ae. aegypti* had the potential to develop resistance to this insecticide. A low level of resistance was shown as an almost 5-fold increase in resistance ratio after 19 generations of selection (Table 1) when compared with the non-selected group. However, in comparison with the WHO susceptible strain (Bora Bora) it showed a marked increase in resistance ratio (Table 3). It was noted that *Ae. aegypti* developed resistance to this insecticide slowly, probably due to the low selection pressure used.

Table 2
Average esterase activities in the larvae of *Aedes aegypti* from Bora Bora, Nonthaburi-Non-sel and temephos-selected groups.

Group	Number	Mean esterase activity[a]		
		Mean ± SD[b]	Minimum value	Maximum value
Ae. aegypti Bora Bora	25	0.132 ± 0.031[a]	0.069	0.181
Nonthaburi-Non-sel	25	0.189 ± 0.042[b]	0.124	0.294
Nonthaburi-Sel (S_{19})	25	0.275 ± 0.044[c]	0.210	0.376

[a] Esterase activity expressed as absorbance / minute / mg protein.
[b] Means followed by the same letter are not significantly different (p = 0.05, LSD test).

Table 3
Effect of temephos and temephos with esterase inhibitor, S, S, S-tributyl phosphorotrithioate (DEF), on resistance levels to temephos of *Aedes aegypti* groups in comparison with the susceptible strain (Bora Bora).

Insecticide	Group	LC_{50}(mg/l)	LC_{95}(mg/l)	Resistance ratio	
				LC_{50}	LC_{95}
Temephos	Bora Bora	0.00091	0.00248	1	1
	Nonthaburi-Non-sel	0.00332	0.00985	3.65	3.97
	Nonthaburi-Sel (S_{19})	0.01540	0.03880	16.92	15.65
Temephos + DEF	Bora Bora	0.00042	0.00092	1	1
	Nonthaburi-Non-sel	0.00044	0.00097	1.05	1.05
	Nonthaburi-Sel (S_{19})	0.00150	0.00310	3.57	3.37

Table 4
Average monooxygenase activities in the larvae of *Aedes aegypti* from Bora Bora, Nonthaburi Non-sel and temephos-selected groups.

Group	Number	Mean monooxygenase activity[a]		
		Mean ± SD[b]	Minimum value	Maximum value
Ae. aegypti Bora Bora	25	0.188 ± 0.052[a]	0.111	0.277
Nonthaburi-Non-sel	25	0.187 ± 0.091[a]	0.107	0.420
Nonthaburi-Sel (S_{19})	25	0.189 ± 0.066[a]	0.093	0.360

[a] Monooxygenase activity expressed as absorbance / minute / mg protein.
[b] Means followed by the same letter are not significantly different (p = 0.05, LSD test).

Table 5
Propoxur-inhibited acetylcholinesterase (AChE) activity expressed as a percentage of uninhibited AChE activity in larvae of *Aedes aegypti* from Bora Bora, Nonthaburi Non-sel and temephos-selected groups.

Group	Number	Percentage of uninhibited AChE activity		
		Mean ± SD[a]	Minimum value	Maximum value
Ae. aegypti Bora Bora	10	64.16 ± 8.42[a]	49.02	75.86
Nonthaburi-Non-sel	10	67.54 ± 16.53[a]	33.00	90.90
Nonthaburi-Sel (S19)	10	61.49 ± 16.20[a]	39.62	93.33

[a] Means followed by the same letter are not significantly different (p=0.05, LSD test).

When the resistance to temephos had developed, the biochemical assay for enzymes revealed elevations in esterase activity in the selected group. It showed significantly higher esterase activity than the non-selected group (Table 2). In order to confirm the association of esterase activity with temephos resistance, the esterase inhibitor (S, S, S,-tributyl phosphorotrithioate, or DEF) was added to the temephos. The selected group became more susceptible to temephos by reducing the resistance ratio

Table 6
Dissemination rates in *Aedes aegypti* after oral infection with Den-2 viruses.

Titer of blood meal Log10 MID50/ml[a]	No. infected/No. tested (%)[b]		
	Bora Bora	Nonthaburi-Non-sel	Nonthaburi-Sel
7.30	1/32 (3.13)	3/32 (9.38)	4/36 (11.11)
8.15	3/35 (8.57)	8/24 (33.33)	8/33 (24.24)

[a] MID50 = 50% mosquito infectious dose.
[b] Determined by direct fluorescent antibody test (DFAT) on head squashes.

nearly 4-fold, than the WHO susceptible strain and the Nonthaburi-Non-sel group (Table 3). This indicated that esterases play a significant role in temephos resistance. Elevated esterase activity associated with temephos resistance was also reported in *Ae.aegypti* from Tortola, British Virgin Islands (Wirth and Georghiou, 1999) and from Trinidad (Vaughan *et al*, 1998).

Study of other enzymes showed no change in monooxygenase activity (Table 4) and no evidence of insensitive acetylcholinesterase (Table 5) in the selected group. This suggested that the temephos resistance was not associated with monooxygenase and insensitive acetylcholinesterase.

Following the identification of the resistance mechanism, it may be useful to be aware of possible cross-resistance to other insecticides conferred by this mechanism. Brown (1986) also reported cross-resistance between temephos and chlorpyrifos in a strain of *Ae. nigromaculis* (Ludlow) from California.

It is evident that this important vector species, *Ae aegypti*, has the potential to develop temephos resistance, which may result in control problems. Continuous monitoring of insecticide susceptibility in *Aedes* populations is critical for decisions on insecticide usage. Source reduction, environmental manipulation and self-protection must be emphasized in order to reduce insecticide use, and to delay the further development of organophosphate resistance.

Regarding the susceptibility of the resistant mosquito to dengue-2 virus, a statistical difference in dissemination rate was not found between each group. This is supported by the work of Gokhale *et al* (2000), where their selection of mosquito strain with malathion showed increased esterase activity but no increase in dengue virus susceptibility. However, this needs further study for higher levels of temephos resistance.

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