TEMEPHOS RESISTANCE IN TWO FORMS OF *AEDES AEGYPTI* AND ITS SIGNIFICANCE FOR THE RESISTANCE MECHANISM

Pungasem Paeporn[1,2], Narumon Komalamisra[1], Vanida Deesin[1], Yupha Rongsriyam[1], Yuki Eshita[3] and Supatra Thongrungkiat[1]

[1]Insecticide Research Unit, Department of Medical Entomology, Faculty of Tropical Medicine, Mahidol University, Bangkok; [2]Chemical Control Section, Department of Medical Sciences, Ministry of Public Health, Nonthaburi, Thailand; [3]Department of Infectious Disease Control, Oita Medical University, Japan

Abstract. *Aedes aegypti*, at the larval stage, has been subjected to the temephos selection in laboratory. The level of temephos resistance was detected in a microplate by biochemical assay using WHO bioassay technique. The major enzyme-based resistance mechanisms involved in temephos resistance include elevated nonspecific esterase, oxidase and insensitive acetylcholinesterase. After 19 generations of temephos selection, the selected group showed resistance ratios of 4.64 and 16.92, when compared with a non-selected group and the WHO susceptible strain, respectively. The two seperated forms, type form and the pale form of *Ae. aegypti* showed low levels of resistance to temephos after 19 generations of selection, with resistance ratios of 4.82 and 4.07 for the type form and the pale form, respectively; when compared with the non-selected strain, 17.58 and 14.84, when compared with the WHO susceptible strain. This showed that the type form could develop higher level resistance than the pale form. The esterase inhibitor (S,S,S-tributy1 phosphorotrithioate, DEF) or synergist implicated detoxifying esterase in all the temephos selected groups and the presence of elevated esterase were confirmed by biochemical assay. There were significant differences in elevated esterase activity between the temephos selected groups and the non-selected group. However no significant difference between the type form and the pale form was found. Besides the elevated esterase, there was no change in monooxygenase activity and no evidence of insensitive acetylcholinesterease for all temephos selected groups. These results suggest that temephos resistance could be developed in *Ae. aegypti* under selection pressure and that the main mechanism is based only on esterase detoxification.

INTRODUCTION

Dengue hemorrhagic fever (DHF) was first recognized in Thailand in 1958 (Nimmannitya, 1987). In the year 2001, a total of 139,274 cases of dengue fever (DF), DHF and dengue shock syndrome(DSS), with 239 deaths, were reported by the Epidemiology Division of the Ministry of Public Health, Thailand. Although vaccination would be an ideal method to control DHF, the development of vaccines for dengue viruses is still in progress. Thus, to date, in the absence of suitable vaccines, vector control is the only method in addition to clinical case management available to combat DHF. In Thailand, *Aedes aegypti* has been documented as the principal vector for dengue transmission. Permanent control of *Ae. aegypti* must be by the destruction of the mosquito's breeding sites. However, for immediate local control of epidemic tranmission of DHF, it is very important to carefully plan for vector control by using insecticides against larvae and adult mosquitos. At the moment, organophosphates and pyrethroids are used to control larvae and adults, respectively. Covering water containers was the most common practice to prevent mosquito breeding in drinking-water containers, whereas the addition of Abate (Temephos sand granules) or changing stored water fre-

Correspondence: Pungasem Paeporn, Chemical Control Section, Department of Medical Sciences, Ministry of Public Health, Nonthaburi 11000, Thailand.
Tel: +66 (0) 2951-0000 Ext 99252; +66 (0) 2591-5449
E-mail: pungasem@hotmail.com

quently was commonly used for non-drinking water storage (Swaddiwudhipong *et al*, 1992).

Sucharit *et al* (1997) reported that the subspecific level of *Ae. aegypti* comprised two forms, the dark form (or type form) and the pale form. The dark form is endophilic and some were peridomestic in habit, while the pale form is exophilic. These two forms showed some differences in insecticide susceptibility, as well as different susceptibility to dengue virus.

Abate (temephos) is an organophosphorus insecticide that has been used as larvicide against *Ae. aegypti* in Thailand since 1967 (Jurjevskis and Stiles, 1978). The widespread use of insecticide has led to selective insecticide resistance in mosquitos, which will be a problem for the control of the disease (Roberts and Andre, 1994). Rawlins (1998) reported high levels of resistance to temephos in some Caribbean countries. Resistance to temephos was found to be low (<5x) in *Ae. aegypti* from Venezuela (Mazzarri and Georghiou, 1995). In French Polynesia, a low, but significant, resistance to temephos (4.3x) in *Cx.p. quinquefasciatus* and 2.3x in *Ae. aegypti* (Failloux *et al*, 1994).

The two major forms of insecticide resistance mechanisms with organophosphorus insecticides are target-site resistance and detoxification enzyme-based resistance, which occurs when enhanced levels or modified activities of enzyme esterases and the cytochrome P450 oxidases (also termed monooxygenases or mixed function oxidases) prevent the insecticide from reaching its site of action (Brogdon and McAllister, 1998).

This study is designed to examine temephos resistance in *Ae. aegypti* mosquitos collected from areas where temephos sand granules had been applied continuously, so that resistant strains will be selected in the laboratory. The major forms of resistance mechanism can identified based on enzyme assay of the elevated (non-specific) esterase, oxidase and insensitive acetylcholinesterase in the subspecific levels of the type form and the pale form.

MATERIALS AND METHODS

Mosquitos

Five groups of mosquitos were used in this study. The Nonthaburi colony was derived from collecting *Ae. aegypti* larvae indoors and outdoors at the Anurajprasit Kindergarden School and the area around the school. After 1 generation in the laboratory, this colony was divided into 2 groups; the first one was subjected to temephos selection (Nonthaburi-Sel group) and the other was maintained without selection (Nonthaburi-Non-sel group). After 9 generations of temephos selection, the Nonthaburi-Sel group was characterized into another 3 subgroups: the first one was the type form (variety); the second was the pale form (variety queenslandensis) and the third were left together and identified as mixed form. *Ae. aegypti* type form and pale form were distinguished according to the method of Mattingly (1957, 1958). All 3 groups were subjected to continued selection of larvae with temephos. The last (fifth) 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

The technical grade (90% purity) of temephos (Abate), an organophosphate insecticide, was obtained from Cyanamid Co, USA.

Bioassay procedures

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

Selection procedures

The Nonthaburi groups were 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 concentration used for selection was approximately LC_{50}, which was obtained from the bioassay test of previous generations; 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 approximately LC_{80}, 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 con-

centration 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 larvae of mosquitos (late third or early fourth instar) from the Nonthaburi-Selection, Nonthaburi-Non-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 the esterase assay in preparing the larvae homogenate. Enzyme activity was determined by a microplate reader at 620 nm.

Acetylcholinesterase assay. Homogenate from 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 using 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 the individuals that might require correction factors for enzyme assay, as in the case of esterase and monooxygenase assays.

RESULTS

The percentages of adult offspring *Ae. aegypti* type form and pale form in each generation under temephos selection pressure were summarized in Table 1. In the type form group, after categorization and selection by temephos from generation 9 to 19, the percentage of the type form in the group increased. Meanwhile, in the pale form group after 11 generations of categorization and selection, the percentage of pale form mosquitos continued to fluctuate.

The LC_{50} of selected generations of the Nonthaburi-Sel group are shown in Table 2. Under temephos selection, there was increasing LC_{50} from 0.00332 mg/l to 0.010 mg/l in the S_9 generation. After each colony was categorized as the mixed form, the type form and the pale form groups were consecutively selected. In generation 19, the LC_{50} in the mixed form, type form and pale form groups increased to 0.0154, 0.016 and 0.0135 mg/l, respectively. This showed low level resistance by increasing nearly 5-fold (4.64, 4.82 and 4.07-fold) at LC_{50}, when compared with the F_1 generation (non-selected group) (Table 2).

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 increased to 17.58, 14.84 and 16.92 in the type from, pale form and mixed form, respectively (Table 3).

The addition of 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 groups; type form, pale form and mixed form groups were reduced to 4.37, 4.33 and 3.57, respectively.

Biochemical assays revealed the presence of elevated esterase activity in all Nonthaburi-Sel groups (Table 4). The Nonthaburi-Sel group mixed form, had a significantly different increase

Table 1
Percentage selection for type form and pale form under temephos selection pressure of type form and pale form groups.

Generation	Type form group	Pale form group
	%(No. type: Total)	%(No. pale: Total)
S_9	58.33 (210:360)	41.67 (150:360)
S_{10}	87.67 (64:73)	33.99 (52:153)
S_{11}	80.21 (150:187)	47.74 (74:155)
S_{12}	75.00 (126:168)	44.72 (110:246)
S_{13}	93.51 (173:185)	77.38 (585:756)
S_{14}	60.13 (95:158)	58.8 (147:250)
S_{15}	95.80 (228:238)	47.28 (87:184)
S_{16}	99.44 (179:180)	Not done
S_{17}	97.06 (165:170)	52.38 (143:416)
S_{18}	94.21 (179:190)	61.37 (143:233)
S_{19}	95.83 (69:72)	72.22 (65:90)

Table 2
LC_{50} * of temephos in selected generations of *Aedes aegypti.*

Generation	Nonthaburi-Selection			Resistance ratio		
	Mixed form	Type form	Pale form	Mixed form	Type form	Pale form
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	0.012	0.010	3.92	3.61	3.01
S_{15}	0.013	0.016	0.013	3.92	4.82	3.92
S_{19}	0.0154	0.016	0.0135	4.64	4.82	4.07

* Median lethal concentration (mg/liter)

Table 3
Effect of temephos and temephos with the esterase inhibitor, S,S,S-tributylphosphoro-trithioate (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})				
	Type form	0.016	0.031	17.58	12.5
	Pale form	0.0135	0.025	14.84	10.08
	Mixed form	0.0154	0.0388	16.52	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})				
	Type form	0.00199	0.00300	4.37	3.26
	Pale form	0.00182	0.00382	4.33	4.15
	Mixed form	0.0015	0.0031	3.57	3.37

in esterase activity from Nonthaburi-Non-sel group. The type form group and the pale form group also showed increasing enzyme activity. However, there was no significant difference between these two forms.

No change was found for monooxygenase activity (Table 5) and no evidence of insensitive acetylcholinesterase in all Nonthaburi-Sel groups (Table 6). This suggested that the resistance was not associated with monooxygenases and insensitive acetylcholinesterase.

DISCUSSION

Selection for temephos resistance showed that *Ae. aegypti*, both type form and pale form, had the potential to develop resistance to this insecticide. A low level of resistance was shown to rise nearly 5- fold in resistance ratio after 19 generations of selection (Table 2), when compared with the Non-selected group. However, in comparison with the WHO susceptible strain (Bora Bora) it showed a marked increase in resistance

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

Group	Number	Mean esterase activity[1]		
		Mean±SD[2]	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})				
Type form	25	0.264±0.064[c]	0.168	0.422
Pale form	25	0.276±0.054[c]	0.155	0.375
Mixed form	25	0.275±0.044[c]	0.210	0.376

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

Table 5
Average monooxygenase activities in the larvae of *Aedes aegypti* from Bora Bora, Nonthaburi non-selected and temephos selected groups.

Group	Number	Mean monooxygenase activity[1]		
		Mean±SD[2]	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})				
Type form	25	0.183±0.095[a]	0.086	0.465
Pale form	25	0.203±0.049[a]	0.133	0.293
Mixed form	25	0.189±0.066[a]	0.093	0.360

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

Table 6
Propoxur-inhibited acetylcholinesterase (AChE) activity expressed as percentage of uninhibited AChE activity in larvae of *Aedes aegypti* from Bora Bora, Nonthaburi non-selected and temephos-selected groups.

Group	Number	Percentage of uninhibited AChE activity		
		Mean±SD[2]	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 (S_{19})				
Type form	10	60.93±24.84[a]	33.33	95.65
Pale form	10	63.65±22.66[a]	30.00	97.22
Mixed form	10	61.49±16.20[a]	39.62	93.33

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

ratio (Table 3). It was noted that *Ae. aegypti* developed resistance to this insecticide slowly, probably due to the low selection pressure used.

Referring to categorization of the mosquitos into type form and pale form, and selection for temephos resistance, even though the adults of the type form were separated from the pale form group after each generation, the proportion of pale form mosquitos fluctuated (Table 1). This might have been due to the presence of some type form population that was more resistant to insecticide than the pale form(Sucharit *et al*, 1997). The present results showed that the resistance ratio of the type form was higher than the pale form and the resistance ratio increased as the percentage of adult type form in the colony increased (Tables 1, 2). In the mixed form, comprising the type form and the pale form, the development of resistance was intermediate, while the resistance in the pale form was lower because of greater susceptibility to insecticide (Tables 2, 3). These results were compatible with the finding of Sucharit *et al* (1997).

When the resistance to temephos had developed, the biochemical assays for enzymes revealed elevations in esterase activity in all selected groups. They showed significantly higher esterase activity than the Non-selected group (Table 4), but without statistically significant differences between the type form and pale form.

In order to confirm the association of esterase activity with temephos resistance, the esterase inhibitor (S,S,S,-tributyl phosphorotrithioate, DEF) was added to the temephos. The selected groups became more susceptible to temephos, by reducing the resistance ratio of the type form, pale form and mixed form 3-4 fold than the WHO susceptible strain and the Nonthaburi Non-selected 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 Totora, British Virgin Islands (Wirth and Georghiou, 1999) and from Trinidad (Vaughan *et al*, 1998).

The study of other enzymes showed no change in monooxygenase activity (Table 5) and no evidence of insensitive acetylcholinesterase (Table 6) in all Nonthaburi-selected groups, when compared with the Non-selected group and the WHO susceptible strain (Bora Bora). This suggested that temephos resistance was not associated with monooxygenase and insensitive acetylcholinesterase.

Following the identification of the resistance mechanism, it may useful in identifying cross resistance to the other insecticides conferred by this mechanism, since 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 resistance to temephos, which may result in a control problem. Continuous monitoring of insecticide susceptibility in *Aedes* populations is critical for decisions on insecticide use. 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.

ACKMOWLEDGEMENTS

The authors would like to thank to Dr Lee Han Lim and Ms Nazni Wasi Ahamad, Institute of Medical Research, Malaysia, Dr Mir S Mulla and Dr Margaret C Wirth, Department of Entomology, University of California Riverside, USA, and Dr William G Brogdon, Centers for Disease Control and Prevention, USA, for their kind and helpful guidance. Thank to Mr Samrerng Prommongkol for helping in separation type form and pale form of the mosquitos. Thanks to Mr Paul Adams for reviewing the manuscript.

REFERENCES

Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976: 248-54.

Brogdon WG, Beach RF, Stewart JM, Castanaza L. Microplate assay analysis of the distribution of organophosphate and carbamate resistance in Guatemalan *Anopheles albimanus*. *Bull WHO* 1988; 66: 339-46.

Brogdon WG, McAllister JC. Insecticide resistance and vector control. *Emerg Infect Dis* 1998; 4: 605-13.

Brown AWA. Insecticide resistance in mosquitoes: a

pragmatic review. *J Am Mosq Control Assoc* 1986; 2: 123-40.

Failloux AB, Ung A, Raymond M, Pasteur N. Insecticide susceptibility in mosquitoes (Diptera: Culicidae) from French Polynesia. *J Med Entomol* 1994; 31: 639-44.

Jurjevskis I, Stiles AR. Summary review of larvicides tested at stage IV/V field trials 1964-1977. Document WHO/VBC/78.688. Geneva: World Health Organization, 1978.

Lee HL. Esterases activity and temephos susceptibility in *Aedes aegypti* (L.) larvae. *Mosq-Borne Dis Bull* 1991; 8: 91-4.

Lee HL, Abimbola O, Inder Singh K. Determination of insecticide susceptibility in *Culex quinquefasciatus* Say adults by rapid enzyme microassays. *Southeast Asian J Trop Med Public Health* 1992; 23: 458-63.

Mattingly PF. Genetic aspects of the *Ae.aegypti* problem. I: Taxonomy and bionomics. *Ann Trop Med Parasitol* 1957; 51: 392-408.

Mattingly PF. Genetic aspects of the *Ae.aegypti* problem. II: Disease relationships, genetics and control. *Ann Trop Med Parasitol* 1958; 52: 5-17.

Mazzarri MB, Georghiou GP. Characterization of resistance to organophosphate, carbamate and pyrethroid insecticides in field populations of *Aedes aegypti* from Venezuela. *J Am Mosq Control Assoc* 1995; 11: 315-22.

Nimmannitya S. Dengue haemorrhagic fever in Thailand. *Southeast Asian J Trop Med Public Health* 1987; 18: 291-4.

Rawlins SC. Spatial distribution of insecticide resistance in Caribbean populations of *Aedes aegypti* and its significance. *Pan Am J Public Health* 1998; 4: 243-51.

Raymond M. Log-probit analysis basic programme of microcomputer. Cahiers ORSTOM serie. *Entomol Med Parasitol* 1985; 23: 117–21.

Robert DR, Andre RG. Insecticide resistance issues in vector–borne disease control. *Am J Trop Med Hyg* 1994; 50 (suppl): 21-34.

Sucharit S, Jirakanjanakit N, Thongrungkiat S, Komalamisra N, Surathin K. The discriminative infection of dengue virus in *Aedes aegypti* at subspecific level. *Trop Med* 1997; 39: 75-80.

Swaddiwudhipong W, Lerdlukanavonge P, Khumklam P, Koonchote S, Nguntra P, Chaovakiratipong C. A survey of knowledge, attitude and practice of the prevention of dengue haemorrhagic fever in an urban community of Thailand. *Southeast Asian J Trop Med Public Health* 1992; 23: 207-11.

Vaughan A, Chadee DD, French-Constant R. Biochemical monitoring of organophosphorus and carbamate insecticide resistance in *Aedes aegypti* mosquitoes from Trinidad. *Med Vet Entomol* 1998; 12: 318-21.

Vulule JM, Beach RF, Atieli FK, *et al*. Elevated oxidase and esterase levels associated with permethrin tolerance in *Anopheles gambiae* from Kenya villages using permethrin impregnated bednets. *Med Vet Entomol* 1999; 13: 239-44.

WHO. Insecticides resistance and vector control: Thirteenth report of the WHO Expert Committee on Insecticide. *WHO Tech Rep Ser* 1963; 265.

Wirth MC, Georghiou GP. Selection and characterization of temephos resistance in a population of *Aedes aegypti* from Tortora, British Virgin Islands. *J Am Mosq Control Assoc* 1999; 15: 315-20.