Biochemical detection of pyrethroid resistance mechanism in *Aedes aegypti* in Ratchaburi province, Thailand.

Pungasem Paeporn[1], Kasin Supaphathom[1], Raweewan Srisawat[2], Narumon Komalamisra[2], Vanida Deesin[2], Phubeth Ya-umphan[1], Somjai Leeming sawat[2]

[1]Chemical Control Section, Department of Medical Science, Mintstry of Public Health, Nonthaburi 11000, Thailand; [2]Departmentment of Medical Entomology, Faculty of Tropical Medicine, Mahidol University, Bangkok 10400, Thailand.

Abstract. The emergence of insecticide resistance in mosquito vectors was an important issue to be considered as one of factors influencing the success of vector control. The early detection of resistance could help the health personnel to plan and select appropriate alternative control measures or insecticide for effective control. Therefore biochemical assay of enzymes in mosquito was conducted to detect the emergence of insecticide resistance and to define the machanisms involved in pyrethroid resistance. Adults of *Aedes aegypti* from two localtities in Ratchaburi province were subjected to permethrin and deltamethrin selection in laboratory. After three generations of selection, permethrin-selected and deltamethrin-selected strains were established. Their LT_{50} increased to 7.46 and 1.18 folds in the F_3 strains that were selected with permethrin and deltamethrin respectively. The enzymes of these mosquitoes were assayed biochemically to study the mechanisms of resistance. The results revealed significant increase of esterase activity and monooxygenase levels in both strains when compared with labolatory susceptible strain. Glutathione-S-transferase activity was found to increase in permethrin-selected strain but not in deltamethrin-selected strain. This suggested that not only esterase and monooxygenase but also glutathione-S-transferase were associated with permethrin resistance in *Ae. aegypti*. The exposing of permethrin-selected and deltamethrin-selected mosquitoes to diagnostic concentration of permethrin (0.75%) and deltamethrin (0.05%) indicated no cross resistance for permethrin to deltamethrin while slight cross resistance from deltamethrin to permethrin was evident. It seemed that glutathione S-tranferase was not associated with cross resistance since its activity in deltamethrin-selected strain remained unchanged as compared with that of laboratory susceptible strain.

INTRODUCTION

Dengue haemorrhagic fever is still one of the major mosquito borne diseases in Thailand with *Aedes aegypti* as the principal vector. As long as an effective, safe and effordable vaccine is not available, no adequate prevention or control measure other than control of vector are available. Especially during epidemics of the disease, the use of insecticides are needed. For example, application of temephos for larval control, thermal fogging or ULV spray of certain organophosphates and pyrethroids for adult control. Insecticide resistance could develope and would thus be a major problem in controlling the vectors and other pest insects.

The major metabolic enzymes involved in resistance against pyrethroids in insects include P450 mediated monooxygenases, elevated non-specific esterases, and reduced sensitivity of sodium ion channels along nerve axons (Oppenoorth, 1985; Georghiou,1986; Nelson *et al.*, 1996; Roberts & Andre, 1994; Scott *et al.*, 1998; Feyerisen, 1999). Moreover, increased levels of glutathione-S-tranferase (GSTs) have been associated in *Ae. aegypti* (Grant & Matsumura, 1988).

In this study, we conducted biochemical assays of enzymes integrated with dose-mortality bioassays for detection of resistance and to define the underlined mechanisms involved in pyrethroid resistance in *Ae. aegypti* which were selected for insectcide resistance under laboratory conditions.

0 ATERIALS AND METHODS

Test populations

A susceptible colony of *Ae. aegypti* from the Department of Medical Sciences, Ministry of Public Health, Thailand has been used as a laboratory susceptible strain.

Aedes aegypti from Tambon Pongsawai and Tambon Wat KhuBua were established as permethrin-selected strain and deltamethrin-selected strain through a series of laboratory selection with permethrin and deltamethrin.

Selection procedure

Aedes aegypti from Tambon Pongsawai, Ratchaburi province, was subjected to permethrin selection and *Ae. aegypti* from Wat KhuBua; Tumbon KhuBua, Ratchaburi province was as subjected to deltamethrin selection respectively. The procedure was as in WHO(1981), as previously described by Paeporn *et al.*, (2004). These two strains were continually selected for 3 generations by exposing non-blood

fed females aged 2-3 days old for a certain time that produced 60-70 % mortality to these insecticides.

Diagnostic susceptibility assay

Non-blood fed females aged 2-3 days old of the Tambon Pongsawai and Wat KhuBua; Tambon KhuBua strains were exposed to the diagnostic concentrations of permethrin(0.75%) and deltamethrin(0.05%) impregnated paper respectively for 1 hour and the mortality was recorded at the end of 24 hours holding period. The cross resistance between these two insecticides was studied.

Biochemical test

Individual mosquitoes were homogenized in 200 µl distilled water. Each of 10 µl of the homogenate was used for monooxygenase, glutathion S-transferase and protein assay. The other twenty µl of homogenate was used for esterases assay. The protocol for each assay followed WHO(1998); technique to detect insecticide resistance mechanisms (field and laboratory manual). The details of each assay is as followed;

Protein assay

The total protein content of individual mosquitoes was determined using the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories) in order to detect the differences in size among individuals that might require correction factors for the enzyme assays.

Esterase assay

Twenty µl of homogenated were placed in separate wells of microtitre plate. Two hundred µl of 0.3 mM α - napthyl acetate were added to each well. Leave the plate at room temperature for 1 min and then added 50 µl of fast blue strain solution. After 5 minutes, enzyme activity was determined as an OD value by microplate reader at 450 nm. Esterase activity was expressed as : OD/min/mg protein

Monooxygenase (Cytochrome p450) assay

Ten µl of homogenate were placed in separate of microtitre plate followed by addition of 80 µl 0.625 M potassium phosphate buffer (pH 7.2). Ten mg of 3,3,5'5',-Tetramethyl Benzidine(TMBZ) in 5 ml methanol was prepared and a 15 ml of 0.25 M sodium acetate buffer(pH 5.0) was prepared. Two hundred µl of the above TMBZ solution was added in to each well followed by 25 µl of 3% hydrogen peroxide. The plate was read after 2 hours at 630 nm.

This assay measures the haem content of insect based on that of Brogdon *et al.,* (1997). It is therefore a rough means of monooxygenase content. It is not a

measure of monooxygenase activity in the insect. Monooxygenase level was expressed as: OD / mg protein

Glutathione -S-transferase (GST) assay

Ten µl of each homogenate was transferred to a microplate well followed by 200 µl of the GSH/CDNB working solution which was prepared by adding 125 µl of 63 mM 1-chloro-2,4-dinitrobenzene (CDNB) to 2.5 ml 10 mM glutathione solution(GSH). The plates were read after 5 mins with the ELISA plate reader at a wavelength of 340 nM. GST activity was expressed as: mMoles/ min / mg protein

Data analysis

Abbott's formula was used to correct the observed mortality in adult susceptibility test (WHO 1981) the LT_{50} value was analyzed by probit analysis using a Basic program (Raymond,1985). A one way analysis of variance (ANOVA) was used to compare the enzyme expression levels between population by using SPSS 10.0 program. All levels of statistical significant were determined at $P < 0.05$.

RESULTS

Aedes aegypti were selected for permethrin and deltamethrin resistance. Resistance ratios after 3 generations of permethrin selection of the *Ae. aegypti* from Tambon Pongsawai were increased to 513.93 and 7.46-fold when compared with laboratory susceptible strain and with non selected strain (Table1).

The resistance ratios after 3 generations of delta methrin selection of *Ae. aegypti* from Wat KhuBua; Tambon KhuBua increased to 62.29 and 1.18-fold when compared with laboratory susceptible strain and with non selected strain (Table 2).

Diagnostic dose test

The adults of laboratory susceptible strain showed complete mortality to the diagnostic concentration of permethrin (0.75%) and deltamethrin (0.05%) impregnated papers. While the mosquitoes from Tambon Pongsawai non-selected strain and Tambon Pongsawai F_3 permethrin-selected strain were exposed to 075% permethrin, the mortalities of 22 % and 7.27% were observed. And these two strains showed the same mortality (31%) when exposed to 0.05% deltamethrin.

On the other hand, the adults from Wat KhuBua non-selected and F_3 deltamethrin selected strains showed 17% and 16% mortality respectively when exposed to diagnostic concentration of deltamethrin. The test also showed 46% and

30% mortality respectively when exposed to permethrin at diagnostic concentration(Table 3).

Biochemical test

The results of the biochemical assay showed significant elevation of esterase activity and monooxygenase content in permethrin and deltamethrin selected as compared with laboratory susceptible strains (Table 4 and 5).

The Pongsawai strain after 3 generations of permethrin selection revealed an increase of glutathione-S-transferase activity when compared with Wat KhuBua deltamethrin selected strain and laboratory strain (Table 6). While no evidence of elevated glutathione-S-transferase activity was found in Wat KhuBua deltamethrin selected strain.

DISCUSSION

The selection of two strains of *Ae.aegypti* with permethrin and deltamethrin for 3 consecutive generations revealed increase in resistance ratio to 7.46 and 1.18 in permethrin-selected and deltamethrin-selected strains respectively. When the permethrin – selected strain was exposed to diagnostic concentration of permethrin the mortality was 7.27% as compared with 22% of the non-selected strain (Table 3) which was compatible with the increase of resistance ratio to permethrin. The development of permethrin resistance was suggested. When exposed to deltamethrin diagnostic concentration the cross resistance to deltamethrin was not observed as shown by the same percentage mortality (31%) as observed in non – selected strain. While the resistance ratio of deltamethrin-selected strain was almost unchanged from the non-selected (1.18 : 1) strain . This indicated probably resistance to deltamethrin has not developed yet. Cross resistance to permethrin was slightly observed since its mortality decreased from 46% to 30% when exposed to diagnostic concentration of permethrin.

Biochemical analysis showed significant increase of esterases, glutathione-S-tranferase activities and monooxygenase content in permethrin-selected strain. It revealed the association of these enzymes to the development of resistance to permethrin. While the glutathione S tranferase activity seemed to be not associated with cross resistance to deltamethrin. In contrast a low level of cross resistance to permethrin was observed in deltamethrin-selected strain eventhough the level of glutathione-S-tranferase was not elevated.

In deltamethrin-selected strain only esterases activity and monooxygenase content were found to increase. It might be due to glutathione-S-transferase activity was not associated with resistance to deltamethrin or the level of resistance ratio to deltamethrin was still very low. Hence, further selection pressure to obtain deltamethrin resistant strain is needed for verification this phenomenon.

As the elevated glutathione-S-transferase activity was related to DDT resistance (Mourya *et al.*,1993,1994,1994) and continuous use of permethrin could induce higher activity of this enzyme. The cross resistance between these insecticides would probably become a problem in the area where DDT was used for malaria control.

Acknowledgement. Our work was supported by the Department of Medical Sciences for research fund. We would like to thank to the staff of the Provincial Health Office in Ratchaburi Province, the staff of the Vector-borne Disease Control unit No.4, Ratchaburi Province for providing data of Dengue cases and chemical control usage, the staff of Chemical Control Section, Department of Medical Sciences for collecting larvae, Ms. Mariam Santithipong for taking care of mosquito colonies and conducting the experiments.

REFERENCES

Brogdon, W.G., McAllister, J.C. & Vulule , J. (1997). Heme peroxidase activity measured in single mosquitoes identifies individuals expressing an elevated oxidase for insecticide resistance. *Journal of American Mosquito Control Associate* **13(3)**: 233-237.

Feyereisen, R. (1999). Insect P450 enzymes. *Annual Review of entomology* **44**: 507-533.

Georghiou, G.P. (1986). The magnitude of Resistance Problem. Pesticide Resistance: Strategies and Tactics for Management. National Acadamic Press: Washington D.C. pp.14-43.

Grant, D.F.& Matsumura, F.(1988). Glutathione S-transferase in *Aedes aegypti* larvae.Purification and properties. *Insect Biochemistry* **18**:615-622.

Mourya, D.T., Gokhale, M.D., Chakraborti, S., Mahadev, P.V. & Banerjee, K. (1993). Insecticide susceptibility status of certain populations of *Aedes aegypti* mosquito from rural areas of Maharashtra state. *Indian Journal of Medical Research* **97**: 87-91.

Mourya, D.T., Gokhale, M.D.& Banerjee, K. (1994). Effect of sublethal dosages of

insecticides on chikungunya virus susceptible and refractory strains of *Aedes aegypti*. *Southeast Asian Journal of Tropical Medicine and Public Health* **25(3)**: 536-542

Mourya, D.T., Gokhale, M.D., & Mishra, A.C. (1994). Biochemical basis of DDT-resistance in *Aedes aegypti* population from dengue affected area in Shahjahanpur city. *Indian Journal of Medical Research* **99**:212-215.

Nelson, D.R., Koymans,L., Kamataki, T., Stegeman, J.J., Feyereisen, R., Waxman, D.J., Waterman, M.R., Gotoh, O., Coon, M.J., Eastbrook, R.W., Gunsalus I.C.& Nebert, D.W.(1996). P450 superfamily: Update on new sequences, gene mapping, accession numbers and nomenclature. *Phamacogenetics* **6**:1-42.

Oppenoorth, F.J.(1985). Biochemical and genetic in insecticide resistance. In: *Comprehensive Insect Physiology Biochemistry and Pharmacology* (eds. Kerkut, G.A. and Gilbert L.I.) Pergamon Press. pp 731-773.

Paeporn, P., Supaphathom, K., Ya-umphan, P., Savanpanyalert, P., Wattanachai, P. & Patimaprakorn, R.(2004). Insecticide susceptibility and selection for resistance in a population of *Aedes aegypti* from Ratchaburi Province, Thailand. *Tropical Biomedicine* (in press).

Raymond, M.(1985). Log-probit analysis basic programme of microcomputer-Cohiers ORSTOM Serie. *Entomology Medicale et Parasitologie* **23**;117-121.

Roberts, D.R.& Andre,R.G.(1994). Insecticide resistant issues in vector. *American Journal of Tropical Medicine and Hygine* **50(suppl)**:21-34.

Scott, J.G., Liu, N. & Wen, Z.(1998). Insect cytochromes P450: Diversity, insecticide resistance and tolerance in plant toxins. *Comparative Biochemistry and Physiology* **121C**: 147-155

World Health Organization (1981). Instruction for determining the susceptibility or resistance of adult mosquito to organochlorine, organophosphate and carbamate insecticides establishment of base line. *WHO/VBC/81.805*, 7 pages.

World Health Organization (1998). The technique to detect insecticide resistance machanisms (field and laboratory manual). *WHO/CDS/CPC/98.6*, 35 pages.