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Application of non-specific esterase enzyme microassays to detect potential insecticide resistance of *Aedes aegypti* adults in Yogyakarta, Indonesia

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ABSTRAK

Sugeng Juwono Mardihusodo - ***Penerapan mikroesai enzim esterase non - spesifik untuk mendeteksi potensi resistensi insektisida pada Aedes aegypti dewasa di Yogyakarta, Indonesia.***

Dengan bioesai larvae *Aedes aegypti* yang dikumpulkan dari wilayah Yogyakarta ternyata masih rentan terhadap temefos dan malathion dan dengan uji mikroplat terbukti juga *Ae. aegypti* dari lokasi yang sama berpotensi resisten terhadap insektisida karena adanya peningkatan aktivitas enzim esterase. Karena itu, timbul dugaan bahwa nyamuk dewasa *Ae. aegypti* di Yogyakarta juga berpotensi resisten terhadap senyawa organofosfat (OP) karena mekanisme resistensi yang sama. Penelitian ini bertujuan untuk mendeteksi potensi resistensi insektisida OP pada nyamuk *Ae. aegypti* di Yogyakarta yang berkaitan dengan aktivitas enzim esterase non-spesifik. Cara penelitian meliputi penggunaan mikroesai untuk peningkatan enzim esterase dengan substrat α-naphthyl acetate pada *Ae. aegypti* stadium dewasa asal koleksi lapangan dibandingkan dengan yang koloni laboratorium. Dari rangkaian uji biokimia enzimatik itu terbukti bahwa nyamuk *Ae. aegypti* di Yogyakarta praktis masih rentan dan berpotensi menjadi resisten terhadap insektisida yang berkaitan dengan aktivitas enzim esterase non-spesifik yang mampu menghidrolisis *α-naphthyl acetate*.

ABSTRACT

Sugeng Juwono Mardihusodo - ***Application of non-specific esterase enzyme microassays to detect potential insecticide resistance of Aedes aegypti adults in Yogyakarta, Indonesia***

By bioassay *Aedes aegypti* larvae collected from Yogyakarta were found to be susceptible to temephos and malathion and by using microplate enzymatic assay *Ae. aegypti* larvae collected from the same sites were potentially resistant to the organophosphate insecticides due to elevated esterase activity in hydrolyzing α-naphthyl acetate substrate used in the enzymatic reaction from the same sites might be potentially resistant to OP insecticides due to the same mechanism. The homogenates of the mosquito adult stages from the fields were microassayed using α-naphthyl acetate substrate compared to that colonized in the laboratory. From a series of studies the results were concluded that *Ae. aegypti* adults in Yogyakarta were susceptible and potentially resistant to insecticides due to elevated α-naphthyl acetate esterase activity.

Key words*: *Aedes aegypti* - *organophosphate insecticides* - *insecticide resistance* - *non-spesific esterase* - *biochemical test.

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INTRODUCTION

Malathion and temephos, two among many organophosphate insecticides, have been commonly used for controlling *Aedes aegypti* to stop Dengue Haemorrhagic Fever (DHF) transmission in Yogyakarta since about 1974[1]. The former is applied either as a thermal fog or ultra low volume against the adult mosquitoes, while the later is applied as sand granules against the larval stages. The long practice of applying such chemicals could raise problems of insecticide re-

sistance as occurring in other countries, i.e. Malaysia[2,3]. Such crucial issues in Yogyakarta has been investigated by Mardihusodo[4] revealing the surprising evidence that *Ae. aegypti* larvae collected from different localities of Yogyakarta Municipality showed susceptible to temephos by larval bioassay and by microplate enzymatic assays for non-specific esterase. Such findings should be confirmed by susceptibility tests either by bioassay or by other method of enzymatic assay of the adult stages.

The present paper reports similar study which is aimed to determine the susceptibility status and the potential resistance of *Ae. aegypti* adult stages collected from some localities in Yogyakarta Municipality, related to the enhanced non-specific esterase activity.

Hopefully the following results would be useful to improve the control of DHF vector particularly in Yogyakarta in the future.

MATERIALS AND METHODS

Mosquitoes. *Ae. aegypti* adults of indigenous strain of Yogyakarta were subjected for the biochemical test of insecticide resistance. They were collected by ovitraps set at different localities. After hatching, the larvae were colonized in the insectary of the Department of Parasitology, Faculty of Medicine, Gadjah Mada University, until the adults emerged and the species identified and confirmed. The adult stages of 3-5 days fed only with 10% sugar water solution were subjected to all series of the biochemical tests. The same qualification were also applied to mosquitoes of the laboratory strain colony that had been free from any insecticides in the laboratory, Faculty of Medicine, Gadjah Mada University, since 1986[5].

Chemicals. Main chemicals used for enzymatic assays were (a) substrate solution: 0.5 ml α-naphthyl acetate [acetate in aceton (6 g/l] mixed with 50 ml phosphate buffer solution (0.02 M; pH = 7.0), and (b) coupling reagent: 150 mg of Fast Blue B salt in 15 ml water and 35 ml aqueous (5%; W/V) sodium dodecyl sulphate.

Equipments. Main equipments for the biochemical tests included a glass rod, a micropipette of 50 µl, microplates with flat bottomed wells, and an ELISA Reader Titertek Multiscan (MCC/340).

Biochemical test. Microplate assays as described by Lee[6,7] and also applied by Mardihusodo[4] were used for the biochemical test of insecticide resistance of the mosquitoes. The whole body of individual mosquito was used for all experiments. A single mosquito was first homogenized in 0.5 µl PBS using a glass rod. With a micropipette, 50 µl of the homogenate was transferred to a well in a microtiter plate. Using this procedure 8 replicate alliquots of the homogenate from a single specimen were available for assay. Fifty microliter of the substrate solution freshly prepared were then pipetted into each well and left for 60 seconds. The coupling reagent (50 µl) was then added. Immediately a deep purple color developed which turned to blue after standing for 10 minutes. The reaction was stopped by the addition of 50 µl 10% acetic acid into each well. The intensity of the final color, indicative of esterase activity, could be differentiated by eye and was assigned as the following score: 0 = colorless/very faint blue; 1 = faint/light blue; 2 = greenish blue; 3 = dark blue. The intensity of the final color was also scanned by an ELISA reader at γ = 450 nm to determine the color intensity quantitatively.

Data interpretation and analysis. The microplate assay for non-specific esterase hydrolizing α-naphthyl acetate substrate were interpreted in correspondence with the experimental evidence for eye score of the final color intensity of the enzymatic reactions obtained by Lee[6,7] and also applied by Mardihusodo[4]. That was as follows: 0 to 2.0 was highly susceptible (SS); 2.0 to 2.5 was moderately resistant (RS), and 2.5 to 3.0 was highly resistant (RR).

Descriptive analysis was applied to the data obtained.

RESULTS

Biochemical tests for insecticide resistance in *Ae. aegypti* adult stage due to elevated esterase activity in hydrolyzing α-naphthyl acetate substrate which reaction products of the respective replicates were eyescored in its color intensity were presented in TABLE 1. The average eyescores of the enzymatic reactions were 1.74, 2.04, 1.75, 1.95, and 2.08 respectively for mosquito specimen collected from laboratory, Mergangsan,

Ngampilan, Tegalrejo and Umbulharjo. The results of the biochemical tests verified more clearly the susceptibility/resistance status of *Ae. aegypti* adults of each district from where the mosquito were sampled. It was observed that *Ae. aegypti* of laboratory colony was highly susceptible (SS) as expected, equally to that from Ngampilan and Tegalrejo, while that of Mergangsan and Umbulharjo seemed to be developing resistant.

TABLE 1. - Non-specific esterase enzyme microassays of potential insecticide resistance in *Aedes aegypti* adults collected from Yogyakarta, Indonesia.

Sites of mosquito collection	Total No. replicates	Average score of color intensity	Susceptibility status*
Laboratory	96	1.74	SS
Mergangsan	96	20.4	RS
Ngampilan	136	1.75	SS
Tegalrejo	88	1.95	SS
Umbulharjo	96	2.08	RS

* Based on Lee[6,7]

TABLE 2. - Non-specific esterase enzyme microassays to detect potential insecticide resistance of *Aedes aegypti* adults collected from different sites of the Yogyakarta Municipality, Indonesia

Range of the Absorbance Value* ($\times 10^{-3}$)	Sites of Ae. aegypti adults collected				
	Laborat.	Mergangsan	Ngampilan	Tegalrejo	Umbulharjo
	No. Rep. (%)	No. Rep. (%)	No. Rep. (%)	No. Rep. (%)	No. Rep.** (%)
301 - 400	45 (46,88)	- -	-	1 91,14)	-
401 - 500	47 (48,96)	-	-	37 942,05)	-
501 - 600	4 (4,16)	1 (1,04)	43 (31,24)	41 (46,57)	-
601 - 700	-	22 922,92)	89 (65,63)	-	107 (83,33)
701 - 800	-	66 968,76)	4 (3,13)	3 93,42)	11 (8,33)
801 - 900	-	-	-	5 (5,69)	-
901 - 1000	-	1 (1,04)	-	-	-
1001 - 1100	-	-	-	-	10 (8,34)
1101 - 1200	-	5 (5,20)	-	-	-
> 1201	-	1 (1,04)	-	-	-
301 -> 1201	96 (100)	96 (100)	136 (100)	88 (100)	128 (100)

*Measured at = 450 nm; ** Rep. = replicate

Readings of the color intensity of the final reaction products in the microplate wells with ELISA reader at α = 450 nm revealed more clearly the pattern of distribution and frequency of the susceptibility/resistance status of *Ae. aegypti* sampled in different intervals of the absorbance value (AV) as presented in TABLE 2.

Absorbance values (AVs) of the esterase reactions in the homogenates of mosquito of laboratory colony ranged from 0.301 to 0.550 with the top frequency at AV = 0.401 to 0.500 (48.96%) (TABLE 2). AVs of esterase reactions in mosquito specimen from Mergangsan ranged from 0.501-1.201 with the top frequency at AV = 0.701-0.800 (68.76%). AVs of esterase reactions in the mosquito specimen from Ngampilan lied between 0.501-0.800 with the top frequency at AV = 0.601-0.700 (65.63%). AVs of esterase reactions in the mosquito from Tegalrejo ranged from 0.300 to 0.900 with the top frequency at AV = 0.501-0.600 (46.57%). As of esterase reactions in mosquito from Umbulharjo ranged from 0.601 to 1.100 with the top frequency at AV = 0.601-0.700 (83.33%).

Based on a number of empirical data on the present studies relating the results of esterase reaction obervations in *Ae. aegypti* of the laboratory colony and collected from the fields (Yogyakarta) either visually by color eyescoring[6,7] and colorimetrically with ELISA reader[6,7,8] it was concluded that (1) esterase reactions which was colorless/faint blue was read at AV < 0.700 corresponding to score of 2.0; (2) esterase reactions which was greenish blue was read at AV = 0.700-0.900 corresponding to score of 2.0-2.5, and (3) esterase reactions showing deep blue in color was read at AV $\geq$ 0.900 corresponding to 2.5-3.0 score. Such findings could be applied to determine the susceptibility/resistance status bio of the mosquito biochemically related to elevated α-naphthyl acetate hydrolyzing esterase activity.

Based also on the empirical data the frequency of resistance status in different levels of esterase reactions observed in sample homogenates of *Ae. aegypti* mosquito from laboratory and from the fields were presented in TABLE 3.

It was shown that 100% of mosquitoes of the laboratory colony were highly susceptible (SS), while mosquitoes collected from the fields in Yogyakarta, the frequency in decreasing order was 96.87%, 90.89%, 83.33% and 23.96% respectively collected from the Ngampilan, Tegal-

rejo, Umbulharjo and Mergangsan. *Ae. aegypti* from Mergangsan showed highest level of tolerance (68.76%), while mosquitoes from the other localities, Umbulharjo, Tegalrejo and Ngampilan, its respective frequency were 11.33%, 9.11% and 3.13%. Resistance to insecticide due to elevated non-specific esterase activity seemed to start developing in Mergangsan (6.24%) and Umbulharjo (8.34%), but this matter was not occurring in Ngampilan and Tegalrejo.

TABLE 3. – Potential resistance to insecticides in *Aedes aegypti* adults collected from some districts in the Yogyakarta Municipality, detected by non-specific esterase enzyme microassays, and read by ELISA reader at = 450 nm

Sites of mosquito collected	Frequency (%) of the susceptibility status in esterase reaction*		
	AV < 0.700 (SS)	AV = 0.700-0.900 (RS)	AV ≥ 0.900 (RR)
Laboratory	100	-	-
Mergangsan	23.96	68.76	6.24
Ngampilan	96.96	3.13	-
Tegalrejo	90.87	9.11	-
Umbulharjo	83.33	11.33	8.34

*Based on the emprical data obtained in this study

DISCUSSION

Non-specific esterase has been long recognized as an important enzyme for detoxification of related chemical insecticides and one of many insecticide resistance mechanisms known to occur in mosquitoes[7,8,9]. Yasutomi[8] reported his research work on the esterase activity in four species of mosquitoes: *Culex pipiens, Cx. pipiens fatigans, Cx. tritaeniorrhynchus*, and *Ae. aegypti* using thin layer electrophoresis. He found the presence of esterase activity hydrolyzing β-naphthyl acetate in a colony of insecticide resistant mosquito compared to other colony of susceptible mosquitoes of the same species. The enzyme is also capable of hydrolyzing other substrates, methyl n-butyrate and phenyl acetate. Lee[6] demonstrated the existing esterase enzyme activity in *Cx. quinquefasciatus* larvae hydrolyzing α-naphthyl acetate.

Thus there were at least two types of esterase enzymes: (1) esterase capable of hydrolyzing α-naphthyl acetate, and (2) esterase capable of hydrolyzing β-naphthyl acetate, coded by different genes, Est-II and Est-III[10] respectively. Besides, another esterase that is more specific in hydrolying malathion is malathion carboxylesterase[10,11]

Compared to the conventional bioassa method of insecticide susceptibility test, althoug it has been made more efficient by the introduc tion of the diagnostic doses, biochemical tests o insecticide susceptibility particularly related t the elevated esterase activity seem to be simpler quicker and more sensitive. This innovativ method is simpler due to unnecessarily need a se of complicated equipments and be observed col orimetrically (the results can be directly visual ized). The application of the biochemical metho provides immediate result of single resistance test, that is only 15 minutes due to the rapid enzymatic reaction, much quicker than that of conventional bioassay that needs 24 hrs or more for each test. The biochemical test is also highly sensitive and highly specific, shown by its capability to detect esterase enzyme actively hydrolyzing a certain substrate, i.e. γ-naphthyl acetate. Determination of the color intensity of the esterase reaction quantitatively in term of absorbance value (AV) using an ELISA reader greatly add the acuracy of resistance detection[4,6,7].

Research and development of various methods of biochemical resistance test for different resistance mechanisms of different groups of insecticides currently continuing in progress in many countries. These activities coincide with that recommended by the WHO[10]. One of the results of the development efforts in the biochemical tests is filter paper test as described by Pasteur & Georghiou[9].

Research activities in case of insecticide resistance in vectors of disease in Indonesia should proceed to research and development of biochemical method of insecticide resistance tests, aside from determination of the diagnostic doses of chemical insecticides commonly used in controlling insects of medical importance. Besides, the biochemical methods of insecticide resistance tests will be quite useful in veterinary as well as in agriculture.

CONCLUSION

Based on the microassay data of increased non-specific esterase activity in hydrolyzing α-naphthyl acetate in *Ae. aegypti* adults collected

from Mergangsan, Ngampilan, Tegalrejo and Umbulharjo districts of Yogyakarta Municipality, it was concluded that *Ae. aegypti* adult populations from the fields (Yogyakarta) were mostly sus-ceptible, and a view part of them show potential resistance to insecticides due to elevated α-naphthyl acetate esterase activity.

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