DETECTION OF NONSPECIFIC ESTERASE ACTIVITY IN ORGANOPHOSPHATE RESISTANT STRAIN OF *AEDES ALBOPICTUS* SKUSE (DIPTERA: CULICIDAE) LARVAE IN YOGYAKARTA, INDONESIA

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Abstract. Malathion and temephos, were the primary insecticides used for controlling the dengue vectors of *Aedes aegypti* and *Aedes albopictus* in Indonesia. Bioassay studies reported that *Ae. albopictus* from Yogyakarta region had been resistant to malathion. It has been proven that elevated levels of non-specific esterase are responsible for the insect resistance to organophosphate insecticides. This study aimed to determine the resistance status and mechanisms of *Ae. albopictus* larvae to organophosphates using biochemical assay. *Aedes albopictus* eggs were collected by ovitraps from four districts in Yogyakarta Special Region, where dengue is endemic (Yogyakarta City, Sleman, Bantul and Kulon Progo). The eggs were reared to larvae and biochemical assay was done by microplate assay using the substrate α-naphthyl acetate. The results showed that *Ae. albopictus* larvae in four district areas in Yogyakarta Special Region had developed resistance to organophosphate insecticide due to presence of elevated non-specific esterase activity.

Keywords: *Aedes albopictus*, organophosphate, vector control, non-specific esterase, Yogyakarta

INTRODUCTION

Dengue is emerging as a serious public health problem globally, with 2.5 billion people at risk and up to 50-100 million infections are now estimated to occur annually in over 100 endemic countries, putting almost half of the world's population at risk (Gubler, 2002). It is a mosquito-borne viral infection causing a severe flu-like illness and the incidence of dengue has increased 30-fold over the last 50 years (WHO, 2012). Dengue virus infection spread by *Aedes aegypti* (Linnaeus) and *Aedes albopictus* Skuse mosquitoes with major public health consequences for millions of people around the world, and in particular the Southeast Asia and Western Pacific Region of the World Health Organization. Of the 2.5 billion people globally at risk of dengue fever (DF) and its severe forms dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS), South-East Asia accounts for approximately 1.3 billion or 52% (WHO, 2011).

Since the first identification in Surabaya and Jakarta in 1968, dengue fever (DF) and dengue hemorrhagic fever (DHF) cases in Indonesia has been increasing

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rapidly, from 58 cases in 1968 to 158,912 cases in 2009. Unfortunately, between 2006 and 2009, Yogyakarta became high risk region for dengue infection (Ministry of Health Indonesia, 2010). Up to now, vaccine and antiviral agent to inhibit dengue virus spreading are still unavailable. Reducing dengue transmission by vector control interventions is a strategy to reduce the incidence of the infection and preventing outbreak of dengue (WHO, 2012). *Aedes albopictus* is an invasive mosquito that also has become an important vector of chikungunya. Immature stages of *Ae. albopictus* thrive in backyard household containers that require treatment with larvicides and when adult populations reach pest levels or disease transmission is ongoing, adulticiding is often required.

Vector control remains the only available intervention to prevent and control the transmission of dengue (WHO, 2009). Due to continuous exposure to insecticides, several insects have developed resistance to this chemical (Kranthi *et al*, 2001). Temephos, an organophosphate has been the main insecticide for mass larviciding in Indonesia (Ministry of Health Indonesia, 1999). Temephos is applied as 1% sand granules into household water containers and other breeding foci for larval control. However, long-term use of this chemical has contributed to the development of resistance by *Ae. aegypti*. According to French-Constant and Bonning (1989), there are two resistant mechanisms of mosquito to organophosphate insecticide (malathion and pirimiphos-methyl), *ie*, the increased activity of non-specific esterase enzyme and insensitivity of acetylcholinesterase (AchE). Successful program in controlling dengue vectors depends on mosquito resistant status to insecticide that is influenced by genetic, biological, and operational factors (WHO, 1995).

Bioassay studies reported that *Ae. albopictus* adult stage from Yogyakarta Special Region had been resistant to malathion and temephos with resistance ratio of 12.740 and 10,435, respectively (Mulyaningsih, 2004). This malathion resistance was caused by at least two resistance mechanisms, an overproduced esterase B and an insensitive acetylcholinesterase (Bisset *et al*, 1990). Biochemical assay can be used to determine resistant status of mosquito to insecticide as well as resistance mechanism (Mourya *et al*, 1993). Using biochemical methods, the increase in enzyme activities of esterase, GST and P450- mediated monooxygenase has been reported to play a role in the metabolism of pyrethroids, which has led to failures in vector-borne disease control, especially in dengue control (Strode *et al*, 2008).

The present research was designed to determine non-specific esterase enzyme activity of *Ae. albopictus* from Yogyakarta Special Region using microplate assay to detect the resistance status and its mechanism. Thus, our result can be used as a reference to control dengue vector in the future.

MATERIALS AND METHODS

Mosquito collection

Aedes albopictus mosquitoes of indigenous strain of Yogyakarta Special Region were subjected for microplate assays. They were collected from different districts through outdoor ovitraps surveys. The ovitraps located around houses in shaded areas in one collection site in every district (Yogyakarta City, Sleman, Bantul and Kulon Progo).

Colonization

The collected eggs were colonized in

the laboratory to get adult stage. The adult mosquitoes were identified to confirm the presence of *Ae. albopictus*. The mosquitoes were maintained at 25 ± 2°C, 80% relative humidity, and a 12 h light:12 h dark photoperiod with a 10% sucrose solution as mosquito feed. Colonization of the mosquitoes was continued to obtain the F1 generation of larval stages. The larvae were placed in dechlorinated water in a pan 30 cm by 20 cm kept at a temperature of 25 ± 2ºC and relative humidity of 80% with dried beef liver as larval feed. The larvae were reared until third instar larvae which were used for the assay. The same methods of colonization were also applied to mosquitoes of Parasitology Laboratory, Medical Faculty, Universitas Gadjah Mada, Yogyakarta-Indonesia as positive and negative control. The positive control were F 83, highly resistant (RR) to malathion and the mortality were 67%. The negative control were F 1056, highly susceptible (SS) to malathion and the mortality were 100%. The susceptibility and resistance status was done by CDC method (CDC, 2010).

Chemicals

For microplate assays, the substrate solution contained 0.5 ml α-naphthyl acetate (Sigma, N 8505; St Louis, MO) in acetone (6 g/l) mixed with 50 ml phosphate buffer solution (PBS) of 0.02 M and pH=7.0, while coupling reagent contained 150 mg of Fast Blue B salt (Sigma D 3502) in 15 ml H_2O and 35 ml aqueous sodium dodecyl sulfate (Sigma L 3771) (5% w/v).

Equipment

The main equipment for microplate assays were paper cups of 250 ml, microplates with 96 flat bottomed wells, micropipettes of 50 ml, glass rod and Bio-Rad Benchmark Microplate Reader (Bio-Rad, Hercules, CA).

Biochemical assay using microplate assays

Whole body of individual larva was homogenized in 0.5 ml PBS using a glass rod. With a micropipette, 50 µl of the clear homogenate was taken up and transferred into a well of microplate. Fifty µl freshly prepared substrate solution were added into each well and incubated for 60 seconds. Then, 50 µl coupling reagent was added to each well and incubated for 10 minutes. When red color developed which turned to blue, reaction was stopped immediately by adding 50 µl 10% acetic acid into each well. In each population, 16 larvae were examined and replicated three times. The intensity of the final color, indicative of esterase activity could be differentiated by eye score: 0 = colorless; 1 = light blue; 2 = greenish blue and 3 = dark blue. The intensity of the final color was also scanned by a microplate reader at λ 450 nm (Lee, 1991; Mardihusodo, 1995).

Data interpretation and analysis

Data of microplate assays of α-naphthyl acetate hydrolyzing esterase (α-NA Est) in *Ae. albopictus* larvae showing the resistance status to organophosphate insecticides were interpreted in accordance with experimental evidence for eye score of final color intensity of the enzymatic reaction by Lee (1991), Lee *et al* (1992) and Mardihusodo (1995) as follows: 0 to 2.0 was highly susceptible (SS); 2.0 to 2.5 was moderately resistant (RS) and 2.6 to 3.0 was highly resistant (RR). From the other study it is known that α-NA Est reaction in the well of microplate showing colorless to light blue and read at absorbance values (AVs) of 0.700, greenish blue to blue at AVs of 0.7001 - 0.900, and dark blue color at AVs ≥ 0.900 (Mardihusodo, 1995). Average of AVs were also analyzed based on cut-off positive value, calculated from average AV of negative control ± 2

Table 1
Determination of α-naphthyl acetate hydrolyzing esterase (α-NA Est) activities by microplate assays in *Aedes albopictus* larvae collected from 4 districts in Yogyakarta Special Region based on eye score.

Population locality (District)	Esterase reaction and susceptibility status			
	Total larvae	Total replicate[a]	Average eye score[b]	Susceptibility status[c]
Yogyakarta city	48	144	2.22 (blue)	RS
Sleman regency	48	144	2.15 (blue)	RS
Bantul regency	48	144	2.66 (dark blue)	RR
Kulon Progo regency	48	144	2.07 (blue)	RS
Lab (negative control)	48	144	1.38 (faint blue)	SS
Lab (positive control)	48	144	2.71 (dark blue)	RR

[a]Each replicate was 50 µl homogenate from single larva.
[b]Eye score: 0 = colorless; 1= faint blue; 2 = greenish blue; 3 = dark blue.
[c]Susceptibility status for eye score (Lee, 1991): ≤2.0 = highly susceptible (SS); 2.0-2.5 = moderately resistant (RS) and 2.6-3.0 = highly resistant (RR).

SD (Mulyaningsih, 2004).

RESULTS

Aedes albopictus larvae of wild field strain from four districts (Yogyakarta City, Sleman, Bantul and Kulon Progo) in Yogyakarta Special Region together with mosquito larvae of laboratory strain for negative and positive control were assayed for determination of α-NA Est activities that were considered to be related to organophosphate insecticides (Table 1).

All samples of mosquito larvae for negative control were susceptible (less than 2.0) and for positive control were resistant (more than 2.5) to organophosphate insecticide based on the average eye score. The score in negative control indicated very low non-specific esterase activity to hydrolyze insecticide (organophosphate), and allowed the insecticides through a mechanism to kill the mosquitoes. In positive control the eye score indicated high specific esterase activity to hydrolyze insecticide (organophosphate), and the capability to hydrolysis carboxyl ester from organophosphate was high.

Esterase activity in hydrolyzing substrate (α-NA) could be determined quantitatively by measuring the color intensity with a microplate reader at certain wavelength *ie*, λ 450 nm (Mardihusodo, 1995). Distribution and frequency absorbance value (AV) of non-specific esterase activity of *Ae. albopictus* larvae from four districts in Yogyakarta Province is shown in Fig 1. These presentations add further verifications on the potentials for organophosphate resistance related to the elevation of α-NA Est activities in *Ae. albopictus* larvae collected from four districts in Yogyakarta Special Region.

The level of the esterase activities of all larvae of the positive control were read at the AV range 0.801 to 1.400 and all larvae of the negative control were 0.801 to 1.100, while all mosquito larvae collected from the field were 0.801 to 1.400. Accord-

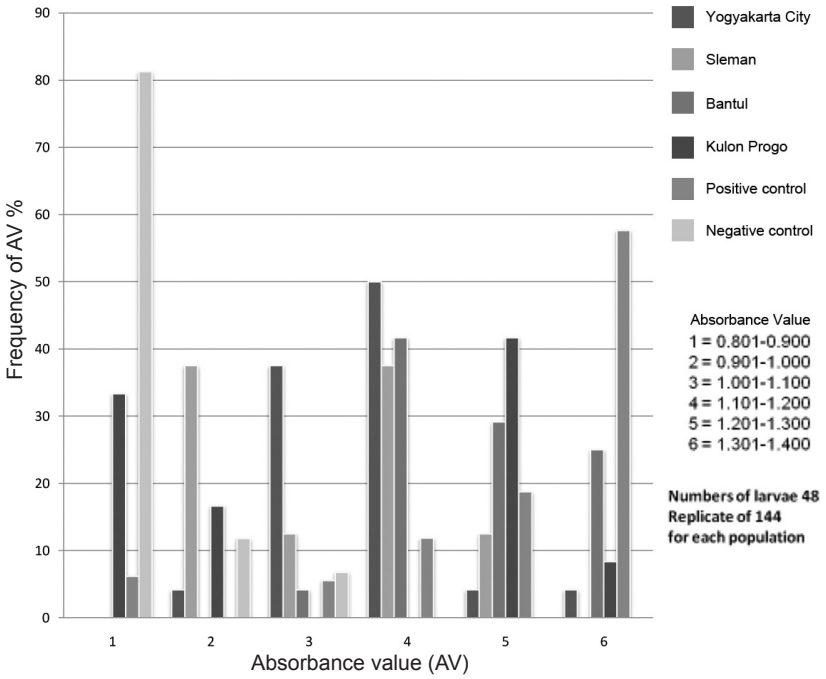

Fig 1–Distribution and frequency of Absorbance Value (AV) of α-naphtyl acetate hydrolyzing esterase activities of *Ae. albopictus* larvae from four districts in Yogyakarta Special Region-Indonesia.

ing to Mardihusodo (1995), any α-NA Est reaction in the wells of microplates showing colorless to light blue and reads at AVs 0.700 correspond to eye score of < 2.0 for susceptible (SS).

The reaction showing greenish blue to blue and with AV values of 0.701-0.900 corresponded to eye score of 2.0 to 2.5 for moderately resistant (RS) mosquito, and for reaction showing dark blue and with AVs ≥ 0.901 corresponded to eye score of 2.6 to 3.0 for highly resistant (RR) strain. The *Ae. albopictus* larvae susceptibility status based on empirical correlation between the AVs range and eye score classification was presented in Table 2.

Mosquito resistance to organophosphate insecticide due to elevated Est activities was found in *Ae. albopictus* larvae from the field and laboratory. However, mosquito larvae from Yogyakarta, Sleman and Bantul showed much higher level of resistance compared to positive control, while the resistance in *Ae. albopictus* larvae from Kulon Progo was lower compared to positive control. Hence *Ae. albopictus* larvae from Yogyakarta, Sleman, Bantul, and Kulon Progo showed various level of resistance to organophosphate insecticide.

The mean AVs were also analyzed based on cut-off positive value, calculated from mean AVs of negative control + 2 SD (Table 3). According to cut-off positive calculation from average AV of negative control + 2 SD (0.742 + 2 x 0.234 = 1.210), thus AV from several populations can be grouped into categories of highly susceptible (SS), if average AV < 1.210; moderately resistant (RS) if average AV were 1.210 - 1.543 and highly resistant (RR) if average were AV ≥ 1.543. *Aedes albopictus* larvae from Yogyakarta, Sleman and Kulon Progo can be determined as moderately resistant, since the average AV was 1.431; 1.352; and 1.362, respectively, while average AV *Ae. albopictus* larvae from Bantul was 1.671 and determined as highly resistant.

DISCUSSION

The widespread use of chemical insecticides has led to development of in-

Table 2
Determination of α -naphtyl acetate hydrolyzing esterase activities related to susceptibility status of *Ae. albopictus* larvae from four districts in Yogyakarta Special Region-Indonesia.

Population locality (District)	Total larvae (Replicates)	Frequency (%) and susceptibility status in esterase reaction		
		AV≤ 0.700 SS	AV= 0.701-0.900 RS	AV ≥ 0.901 RR
Laboratory				
Negative control	48 (144)	0	81.25	18.75
Positive control	48 (144)	0	6.21	93.79
Yogyakarta	48 (144)	0	0	100
Sleman	48 (144)	0	0	100
Bantul	48 (144)	0	0	100
Kulon Progo	48 (144)	0	33.34	66.66

Table 3
Average absorbance value of α-naphtyl acetate hydrolyzing esterase activities of *Ae. albopictus* larvae from four districts in Yogyakarta Special Region-Indonesia.

No.	Population locality (District)	Average AV[a]	Standard deviation
1	Yogyakarta	1,431	+ 0,145
2	Sleman	1,352	+ 0,209
3	Bantul	1,671	+ 0,149
4	Kulonprogo	1,362	+ 0,391
5	Laboratory (positive control)	1,543	+ 0,356
6	Laboratory (negative control)	0,742	+ 0,234

[a]Absorbance value.

secticide resistance in several insect species including mosquitoes (Cui *et al*, 2006). Currently insecticide resistance management is considered as a serious public health challenge throughout the world. For the control of mosquito population, continuous monitoring of insecticide resistance is important. Mechanisms of insecticide resistance in mosquito vectors are being studied worldwide, as they elucidate the pathways of resistance development and help in newer strategies of preventing and delaying insecticide resistance.

Biochemical techniques are essentially based on the detection and quantification of enzymes known to be responsible for resistance (Lee *et al*, 1992). Biochemical estimations have been the method of choice for understanding the mechanism of insecticide resistance among insects. With sophisticated and sensitive biochemical assays, it is now possible to analyze the mechanisms of insecticide resistance with a fair degree of accuracy (Muthusamy *et al*, 2014).

Microplate assay was used to deter-

mine α-NA Est activities in *Ae. albopictus* from four districts in Yogyakarta Special Region, and eye score determination indicated that larvae from Yogyakarta, Sleman and Kulon Progo Districts were moderately resistant to organophosphate insecticide group. Further quantitative determination of the α-NA Est activities by measuring the absorbance values (AVs) of the color intensities of the resultant enzymatic reaction in microplate wells with microplate reader at λ 450 nm, indicated variations in the range of the AVs (Fig 1). The result indicated that *Ae. albopictus* in the field were heterogenic and reflected the presence of genotypic polymorphism in the population of mosquitoes, particularly in relation to the detoxification activity of esterase genes (Mardihusodo, 1995).

In this study, we performed analyses of esterase enzyme activity, which revealed the resistance status of *Ae. albopictus* larvae in Yogyakarta Special Region. Data analysis based on average eye score can be observed easily because special equipment are not needed and the result is reliable. Data analysis using empirical criteria based on average AV has also been proven to be valid (Lee, 1991; Mardihusodo, 1995). Because of microplate reader variability in each laboratory, were also did analysis according to cut-off positive value using calculation of average AV from negative control + 2 SD (Mulyaningsih, 2004). The analysis was aimed at achieving high sensitivity detection of resistant larvae. Resistance status of mosquito vector will determine the effectiveness of the applied insecticide.

This present study indicated that *Ae. albopictus* larvae from Yogyakarta City, Sleman, Bantul, and Kulon Progo Districts were resistant to organophosphate insecticide. Previous studies reported that *Ae. aegypti* mosquito from Yogyakarta had been resistant to temephos and malathion insecticide (Mardihusodo, 1995; Mulyaningsih, 2004; Boewono and Widiarti, 2007). These results were expected because of the long term use of temephos and malathion since 1974 in DHF control program at Yogyakarta Special Region.

As most of the insecticides are neurotoxic in nature, and target insect nervous system, acetylcholinesterase plays an important role in detoxification of organophosphate and carbamate insecticides by overproduction of acetylcholinesterase in resistant insects (Byrne and Toscano, 2001). Mosquitoes have specific mechanism to survive from organophosphate insecticide by increasing esterase enzyme activity (Mardihusodo, 1995). Esterase is an important enzyme for insects, because its function is to detoxify insecticide poison. Detoxification mechanism mediated through non-specific esterases is another major mechanism of resistance in insects. These esterases detoxify organophosphate, carbamates and synthetic pyrethroid pesticides by two main ways, hydrolysis of the ester bond and binding of the pesticide to the active site of esterase (Crow *et al*, 2007). Most of the insecticide groups contain ester linkages which are susceptible to hydrolysis by esterase. Resistant insects usually show a very high activity of esterases (Wu *et al,* 2004; Yang *et al*, 2004).

In susceptible mosquitoes, non-specific esterase enzyme activity is low, so capability to hydrolyse carboxylester from organophosphate is low, and hence is toxic to the mosquito.. In resistant mosquitoes, non-specific esterase enzyme activity is high, and so the capability to hydrolyse carboxylester in organophosphate is high, rendering the insecticide ineffective.

Elevation of non-specific esterase enzyme activity also reduces insecticide

dose, from lethal dose to sub-lethal dose, so mosquito target remain alive (Ferrari, 1996; WHO, 2006). Esterase plays an important role in conferring or contributing to insecticide resistance and changes in esterase sensitivity to inhibition by organophosphate and carbamate insecticides, can confer high levels of resistance in insects (Georghiou and Pasteur, 1978). Development of resistance to organophosphate and pyrethroids in *Ae. aegypti* larvae is reportedly due to increased activity and metabolism of both insecticides by AChE, GST and esterase metabolic enzymes (Muthusamy *et al*, 2014).

In conclusion, based on microplate biochemical assay using the substrate of α-naphthyl acetate, *Ae. albopictus* larvae from Yogyakarta, Sleman, Kulon Progo were moderately resistant, while those from Bantul was highly resistant to organophosphate insecticide due to elevated α-naphthyl acetate esterase activity. Based on these findings, the use of organophosphate insecticides for dengue vector control program in Yogyakarta Province should be reviewed and re-evaluated to ensure effectiveness is not compromised.

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