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Detection of insecticide resistance in the larvae of some *Aedes aegypti (Diptera: Culicidae)* strains from Java, Indonesia to Temephos, Malathion and Permethrin

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Abstract
Larval insecticide resistance status of the *Ae. aegypti* in Indonesia is hardly known. Therefore, assay to determine resistance status of *Ae. aegypti* larvae collected from Bogor, Tasikmalaya, Sumedang, Garut and Semarang to temephos, malathion and permethrin was conducted using the WHO standard test. The results showed that, there was absent to moderate resistance to temephos, malathion, and permethrin showed by Resistance Ratios (RR) ranged from 0.78 to 7.40 as compared to the susceptible VCRU strain. In addition, this is the first report that larvae of *Ae. aegypti* in Indonesia (Sumedang, Garut and Tasik) have developed resistance to malathion. Biochemical analysis showed that in general, detoxifying enzymes, i.e., Esterase B and Mixed-Function Oxidase (MFO) were involved in the mechanism of resistance to insecticide in the majority of strains, although the results also suggest that other possible mechanism(s) might also be involved.

Keywords: *Aedes aegypti*, Indonesia, insecticide, resistance

1. Introduction
Aedes aegypti is considered as one of the most dangerous insects in public health due to their role as vector of Dengue Fever (DF) and Dengue Hemorrhagic Fever (DHF) in tropical, subtropical and temperate regions of the world [11, 24]. Annually, millions of people are infected by DF and DHF [13], and Indonesia has been considered as one of the major infection area after Brazil. Since its first occurance in 1968, several dengue outbreaks have occured. For example, in 2010 150,000 cases with 1317 deaths were reported by the Indonesia's Ministry of Health. Furthermore, until the end of 2012 the number of cases were stable at around 150,000 cases.
In an effort to reduce or prevent the dengue transmission, Indonesian Government together with the Pest Control Operators (PCOs) extensively used insecticides to control *Ae. aegypti* [2]. Since the early 1970's, organophosphates (temephos and malathion) had been used, and in the 1980's, pyrethroids (permethrin and deltamethrin) were introduced and had gradually replaced malathion to control adult population of *Ae. aegypti*. Whilst for larvacide, temephos has been the insecticide of choice. However, the frequent use of insecticides, especially those containing pyrethroids, either by mosquito eradication program initiated by the government and PCOs, or by community at large may lead to the development of mosquito's resistance to insecticides, thus hampering control and increasing the rate of transmission of the disease in question. Reports on insecticide resistance in *Ae. aegypti* showed increasing cases in Asia, Carribean, Central, and South America [3, 19, 29]. However, even though considered as one of major area of occurrence and transmission of DF, report on the effectiveness of certain insecticides to control *Ae. aegypti* population in Indonesia is limited (see [3, 4, 5, 7]). Moreover, given the very limited number of reports, the reports unfortunately only focused on adult mosquitoes, and not on larval resistance to insecticides, which might give different results [18, 28]. Therefore, the availablility of adequate data about resistance status of both larvae and adult mosquitoes in the area in question is imperative. Since this data could be used to develop a good mosquito control strategy, that is effective and does not encourage further resistance. To complictate the matters, it is the fact that only two of the four classes of insecticide are available for use to control *Ae. aegypti* in Indonesia.

Therefore it is imperative to have an updated data about the Effectiveness insecticides being used to control the mosquito.
Besides, eventhough the resistance of *Ae. aegypti* to several insecticides has been reported in Indonesia, the possible mechanism of the resistance in question is hardly reported. It is known that one of the common mechanisms of insect resistance to insecticides has been attributed to the increasing activity of detoxification enzymes such as esterases, mixed function oxidases and glutahion S-transferases (GSTs) [6]. A given example, Ahmad *et al*. [4] reported that some strains of adult *Ae. aegypti* from Indonesia had elevated detoxifying enzimes, i.e. oxidase and esterase associated with permethrin and deltamethrin resistance.
This report describes the current resistance status of *Ae. aegypti* larvae collected from geographically distant areas in Java Island, Indonesia to the three commonly used insecticides. In addition, we also documented the possible resistance mechanism responsible for insecticide resistance strains through observation on activity of detoxiciation enzymes. This study complements the existing data about resistance status of *Ae. aegypti* adults to several insecticides that could be used to develop a better mosquito control program in Indonesia.

2. Materials and methods

2.1 Mosquitoes

Five strains of *Ae. aegypti* were tested in this study. They were collected from five cities in Java, i.e. Bogor, Tasikmalaya, Sumedang, Garut, and Semarang (Figure 1). A pure insecticide-susceptible strain obtained from Vector Control Research Unit (VCRU) University Sains Malaysia was used as reference strain. All strains were reared in the laboratory of Entomology, School of Life Sciences and Technology, where they were held at 25±2 ºC and 75% RH before used.

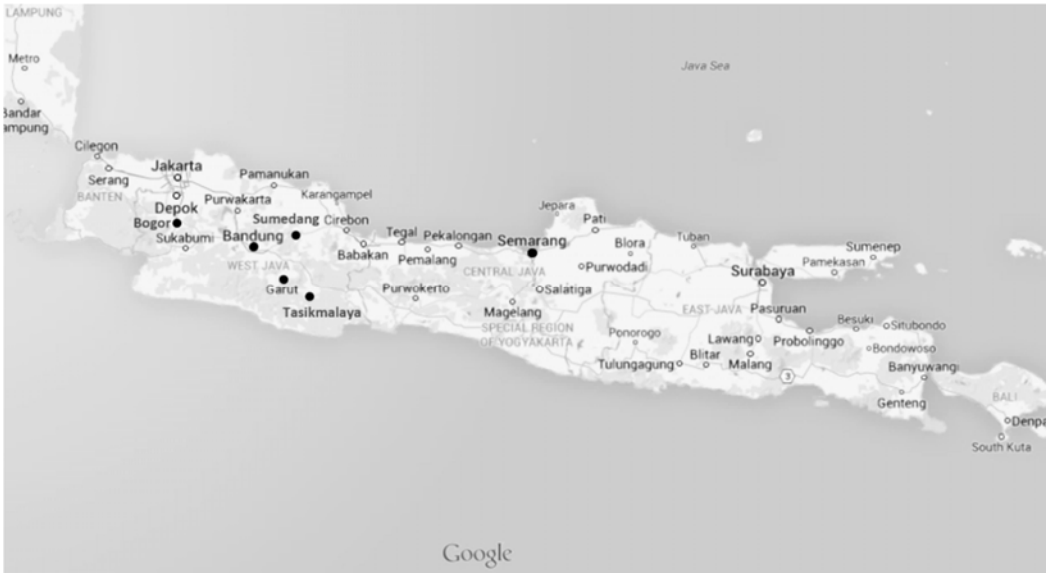

Fig 1: Sampling area (closed circles) of *Aedes aegypti*.

2.2 Insecticides

Insecticides used in this study were Temephos (ABATE 1%) from BASF, Malathion 1%, and Permethrin 1%. Malathion and Permethrin were obtained in the form of stock solution from Vector Control Research Unit University Sains Malaysia.

2.3 Resistance Assay

Assays to determine resistance status were conducted to all using WHO standard method [29]. Five replicates of twenty 4th instar larvae were added to 200 ml of insecticides solution (5 serial concentrations) in 300 ml water container. Mortality was measured after 24 hours of exposure.

2.4 Biochemical Assay

Biochemical assay was conducted using two groups of mosquitoes for each strain; Control mosquito (not exposed to insecticides) and treatment mosquito (survived after exposure to insecticides with concentration above LC_{50}). Individual mosquito larvae was homogenized using homogenizer in 500 µl of Potassium Phosphate Buffer (PPB) 0.1M pH 7.2. The homogenate was centrifuged at 13.000 RPM 4 ^{0}C for 10 minutes. The supernatant was collected and stored in -80 ^{0}C freezer before being used.

2.5 Total Protein Quantification

Total protein content was measured using Biorad Bradford micro assay method. 150 µl of homogenate were pipetted to each well of 96 well microplate. 150 µl of bradford reagent was added to each well and shook gently. Absorbance was measured at 595 nm using microplate reader. Results were compared to a standard curve using Bovine Serum Albumin (BSA) as protein standard.

2.6 Esterase Activity

100 µl of homogenate was pipetted into well of microplate. 50 µl of substrate solution was then added to each well. Mixtures were incubated in room temperature for 15 minutes and O-dianisidine and SDS 1% were added as coloring agent and stop solution, respectively. Absorbance was measured at 570 nm using microplate reader. β-naphtyl acetate were used as substrate for esterase B. Results were compared to a standard curve using β-naphtol as enzyme product standard. Activity of esterase was expressed in µmol product/minute/mg protein.

2.7 Oxidase Activity

100 µl of homogenate was pipetted into well of microplate. 100 µl of TMBZ solution was added to each well and 25 µl of

H_2O_2 3% was also added to each well as substrate solution. Mixtures were incubated in room temperature for 2 hours before 25 µl of H_2SO_4 2M were added as stop solution. Absorbance was measured at 450 nm using microplate reader. Results were compared to a standard curve using Cytochrome C P450 as standard. Activity of oxidase was expressed in nmol Cytochrome C Equivalent Unit/mg protein.

2.8 Data Analysis

Bioassay data were analyzed using POLO PC [17] to determine 50% lethal concentration (LC_{50}) values. Control mortality was corrected by Abbott's formula [1]. Resistance ratios (RR_{50}) were calculated by comparing the LC_{50} and of each field strain to the LC_{50} of susceptible strain (VCRU). Mixed function oxidase activities were log transformed to fit normal distribution and the difference between treatments was analyzed using student's *t* test. However, esterase B enzyme activity was analyzed using non-parametric Mann Whitney U-test since the data were not normally distributed even after transformation. All statistical tests were performed using JMP version 5.

3. Results and Discussion

3.1 Resistance assay

Using the classification resistance ratio developed by Lee and Lee [16], we found that the majority of field strain mosquito larvae showed low resistance (RR_{50}< 5) to all insecticides tested except for the Garut strain which had a moderate resistance to permethrin with RR_{50} 7.40 (Table, 1, 2, 3). Interestingly, Bogor and Semarang strains were more susceptible to malathion (RR_{50}< 1) as compared to the VCRU reference strain. Unfortunately, due to the unforeseen laboratory rearing problems in the laboratory, we lost Tasikmalaya and Semarang strains. Consequently, we were not able to test Tasikmalaya and Semarang strains to permethrin.

Table 1: Temephos resistance level of four field and VCRU strains of *Ae. aegypti* larvae

Strain	n	Slope (±SE)	LC_{50} (90%)	LC_{95} (90%)	RR_{50}	RR_{95}
Vcru	361	4.5(0.50)	0.24(0.22-0.27)	0.57(0.48-0.75)	1	1
Sumedang	121	2.44(0.73)	0.58(0.44-1.34)	2.75(1.24-7.40)	2.41	4.82
Garut	118	3.36(0.65)	0.31(0.24-0.38)	0.98(0.68-2.36)	1.29	1.72
Bogor	119	3.77(0.81)	0.48(0.39-0.76)	1.32(0.81-7.54)	2	2.32
Tasik	80	4.97(1.09)	0.28(0.24-0.31)	0.60(0.49-0.89)	1.16	1.05
Semarang	60	4.33(1.1)	0.30(0.24-0.35)	0.73(0.56-1.33)	1.25	1.28

Table 2: Malathion resistance level of four field and VCRU strains of *Ae. aegypti* larvae

Strain	N	Slope (±SE)	LC_{50} (90%)	LC_{95} (90%)	RR_{50}	RR_{95}
Vcru	202	2.60(0.66)	0.09(0.07-0.11)	0.40(0.28-0.93)	1	1
Sumedang	40	2.76(1.02)	0.14(0.07-0.19)	0.56(0.35-3.18)	1.56	1.40
Garut	149	4.93(0.65)	0.20(0.16-0.25)	0.43(0.32-0.82)	2.22	1.08
Bogor	120	1.89(0.64)	0.07(0.008-0.11)	0.52(0.32-4.49)	0.78	1.30
Tasik	80	4.57(1.40)	0.12(0.08-0.14)	0.27(0.21-0.54)	1.33	0.68
Semarang	120	2.15(0.64)	0.08(0.03-0.12)	0.50(0.33-1.91)	0.89	1.25

Table 3: Permethrin resistance level of three field and VCRU strains of *Ae. aegypti* larvae

Strain	N	Slope (±SE)	LC_{50} (90%)	LC_{95} (90%)	RR_{50}	RR_{95}
Vcru	82	2.00(0.52)	0.010(0.03-0.017)	0.067(0.044-0.13)	1	1
Sumedang	90	3.31(0.71)	0.028(0.02-0.03)	0.089(0.06-0.16)	2.80	1.33
Garut	120	3.62(0.67)	0.07(0.039-0.10)	0.212(0.13-1.67)	7.40	3.16
Bogor	164	2.33(0.33)	0.021(0.016-0.027)	0.108(0.07-0.22)	2.10	1.61
Tasik	DNA	DNA	DNA	DNA	DNA	DNA
Semarang	DNA	DNA	DNA	DNA	DNA	DNA

DNA: Data not available

The findings that all strains had low resistance (RR_{50}>1 to ≤ 5) to temephos are similar to those obtained by previous studies conducted in Indonesia with adult mosquitoes [2, 10]. Whilst Mulyatno *et al*. [22] reported that field strains of *Ae. aegypti* larvae in Surabaya were more resistant to temephos with RR ranging from 2.8-8.5 (low to moderate resistance). Actually, their findings regarding the RR values might have been higher had they used the standard susceptible strain such as the Rockefeller reference strain or VCRU strain line which were used in this study. Knowing the fact that temephos, as the principle larvicide, has been widely used in Indonesia since the early 1980's, this finding is not surprising. Similarly, as reported by Ponlawat *et al*. [25], resistance of *Ae. aegypti* larvae to temephos was detected in Thailand with one strain which had high levels of resistance (RR_{50}= 82).

Furthermore, our findings provide evidence that in general, *Ae. aegypti* strains collected from Sumedang, Garut, and Tasikmalaya have developed low resistance to malathion (RR_{50}>1 to ≤ 5), as compared to a susceptible laboratory strain (VCRU). Larvae from Bogor and Semarang were more susceptible as compared to the VCRU strain with RR_{50}=0.78 and 0.89, respectively. Besides, this is the first report that larvae of *Ae. aegypti* in Indonesia (Sumedang, Garut and Tasik) have developed resistance to malathion, the common insecticide used in fogging to control adult mosquitoes. We compare the finding reported here with the previous study conducted in our laboratory [3] with adults of *Ae. aegypti* which showed that in general, adults were still susceptible to malathion, despite that fact that malathion has been used in Indonesia for more than 36 years. The finding that *Ae. aegypti* larvae demonstrated low resistance to malathion is similar with the previous study conducted by Ponlawat *et al*. [25] which shows that one strain of *Ae. aegypti* from southern Thailand had low level of resistance (RR_{50}=2.8). Ahmad *et al*. [2]

reported that adults *Ae. aegypti* from Palembang, Surabaya and Bandung were resistance to permethrin with RR_{50} ranging from 4.9 - 45. This study also found that *Ae. aegypti* from Bogor, Garut and Sumedang were also resistance to permethrin with RR_{50} ranging from 2.10-7.40 (Table. 3). Although the strains that we compared were not the same between adults and larvae, the comparison suggests that higher resistance was shown in adults with RR_{50} 45.7-fold occurring in Bandung strain. We expected that larval resistance to permethrin would be similar with those of the adults, because permethrin as adulticide has been widely used since 1980 in the government and PCOs dengue-control programs, as well as household insecticide. But, this study found that resistance at larval stages is much lower compared with adult stages.

3.2 Biochemical assay

The results of the biochemical assays, in this study showed that larval exposure to temephos, malathion and permethrin caused a changed in the activity of the enzymes in some strains as compared to controls (Table 4 and 5). As an example, Bogor and Garut strains showed a significant increase (U test, $P<0.001$) in the esterase levels when exposed to temephos (2-fold) and Malathion (3-fold), respectively. In addition, Bogor (2-fold), Garut (4-fold), and Sumedang (17-fold) strains also showed significant increase when exposed to permethrin.

After exposure to temephos, there was significant increase (*t* test, $P<0.001$) in MFO activity in three out of five field strains, i.e.,Garut (1-fold), Tasik (18-fold) and Semarang (3-fold). Interestingly, the level was 3 times higher in VCRU control (susceptible reference strain) as compared to the ones treated with temephos. Two out of five field strains showed significant increase in MFO when exposed to Malathion, i.e., Tasik (13-fold) and Semarang (19-fold). However, only one strain, Sumedang (4-fold) that showed a significant increase of MFO when exposed to permethrin.

Table 4: Esterase B activity observed in *Ae. aegypti* larvae treated with temephos, malathion, and permethrin from five field strains and the VCRU strain

Strain	Esterase B (µmol/min/mg protein)							
	N	Control (±SE)	N	Abate (±SE)	N	Malathion (±SE)	N	Permethrin (±SE)
Vcru	20	0.019(0.01)	20	0.015(0.003)	39	0.0207(0.001)	20	0.020(0.001)
Sumedang	24	0.025(0.006)	55	0.016(0.009)	11	0.014(0.002)	16	0.419(0.13)*
Garut	21	0.005(0.004)	13	0.004(0.003)	12	0.016(0.002)*	14	0.019(0.009)*
Bogor	20	0.014(0.007)	19	0.022(0.01)*	19	0.017(0.004)	34	0.024(0.006)*
Tasik	12	0.024(0.005)	13	0.109(0.01)	14	0.020(0.005)	0	DNA
Semarang	17	0.026(0.01)	32	0.049(0.03)	24	0.02(0.009)	0	DNA

DNA: Data not available; *) Significantly increase in Esterase B activity. Esterase B activity was compared between control and treatment with significant value at $p<0.05$

Table 5: Mixed-Function Oxidase activity observed in *Ae. aegypti* larvae treated with temephos, malathion, and permethrin from five field strains and the VCRU strain

Strain	Mixed-Function Oxidase ($x10^{-6}$ unit per mg protein)							
	N	Control (±SE)	N	Abate (±SE)	N	Malathion (±SE)	N	Permethrin (±SE)
Vcru	20	5.11(0.28)	15	1.64(0.65)	33	2.47(0.5)	14	1.96(0.56)
Sumedang	9	12.19(4.5)	51	14.32(1.46)	4	3.18(2.07)	9	52.07(6.66)*
Garut	22	1.66(0.17)	14	2.40(0.33)*	7	2.10(1.11)	14	2(1.06)
Bogor	14	5.55(0.65)	5	5.22(1.32)	16	1.00(0.14)	26	4.46(0.90)
Tasik	10	1.89(2.85)	7	33.87(10.89)*	14	24.61(2.99)*	0	DNA
Semarang	11	4.10(0.76)	19	12.94(4.36)*	7	77.17(10.6)*	0	DNA

DNA: Data not available; equivalent unit = µmol cytochrome C P_{450} / min *) significantly increase in MFO activity. Mixed Function Oxidase activity was compared between control and treatment with significant value at $p<0.05$

Many studies have suggested that the increased levels of detoxifying enzymes such as oxidases, esterase A, and esterase B, may be related to the development of resistance to pyrethroids and organophosphate insecticides in mosquitoes [4]. This finding suggests that activities of esterase B correlated well with temephos-resistance strain from Bogor and malathion-resistance strain from Garut. In addition, there is a possibility that the low resistance of Sumedang, Garut and Tasikmalaya strains to both Malathion and temephos may be cross resistance, which is resistant to different insecticide which have similar mode of action. In fact, cross resistance between Malathion and temephos in *Ae. aegypti* has been previously reported in India [28].

Exposure to permethrin increased the activity levels of esterase enzymes of all field strains, e.g. Sumedang (17-fold), Garut (4-fold) and Bogor (2-fold). High level of Esterase B activity was probably inherited from adult mosquitoes as pyrethroid resistance is prominent in Indonesia [4]. Esterase B, itself, detoxified pyrethroid by hydrolysis of ester bond of pyrethroid [15] and well documented in numerous resistant insects including houseflies [14], blowflies [9], planthopper [26], and Lepidoptera [27]. However it is also known that not all hydrolysis process involved in resistance resulted in enhanced esterase activity [23].

This finding was not expected, and would appear to exclude any possible contribution for MFO involvement in the resistance of some strains to temephos, including Bogor strain which had lower MFO after exposure to Malathion (Table 5). However, in other strains, treatment with Malathion increased the levels of MFO to 13 and 19 times higher than controls in Tasikmalaya and Semarang strains respectively. Results with permethrin showed that MFO levels increased significantly in one out of three field strains, i.e., Sumedang (4 fold), suggesting the role of MFO in the development of resistance to permethrin.

The present study shows the importance of MFO as the

predominant enzyme responsible for temephos, malathionand permethrin resistance. In general, our findings are similar to those reported earlier [8, 12, 20, 21]. Whereas for permethrin resistance, our study demonstrated that both MFO and esterase conferred permethrin resistance in three out of four field strains. The involvement of esterase and MFO in permethrin resistance might cause cross resistance to Malathion and temephos as shown in some strains as mentioned before. Although the results of this study indicated that larval resistance to temephos, Malathion, and permethrin is still low, or incipient resistance, no control failures had been reported. In addition, based on these data, since the availability of insecticide to control *Ae. aegypti* is limited, it is imperative to do continuous resistance monitoring both for larvae and adults to preserve the susceptibility against the existing insecticides, or to implement the resistance management strategy, should high level of resistance occurred to prevent control failure that may lead to severe DHF and DF outbreak in the future.

4. Conclusion

In conclusion, this study shows that resistance level of *Ae. aegypti* larvae to commonly used insecticide is still low (RR_{50}>1 to ≤ 5), suggesting that the existing insecticides commonly used in Indonesia to control *Ae. aegypti* larvae are still effective. However, special attention has to be given to some Garut strains that already developed moderate resistance to permethrin (RR_{50} 7.40). Biochemical analysis showed, in general, detoxifying enzymes were involved in the mechanism of resistance to insecticide in majority of strains, although results also suggest other possible mechanism (s) might also involve. Thus, continuous and systematic monitoring of the development of insecticide resistance levels as well as its possible underlying mechanisms of resistance on mosquito population in wider area in Indonesia is needed in order to develop effective and efficient mosquito control to prevent severe DHF and DF outbreak in the future especially with possibility of increasing mosquito habitat range from Indonesia to upper geographic regions due to climate change.

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