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***Aedes aegypti* vector resistance status on malation and activity of non specific esterification enzymes in Tembalang district, Semarang city**

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ABSTRACT

Background: Controlling of dengue vectors around dengue haemorrhagic fever cases is often conducted by using insecticides, including a malathion insecticide in the city of Semarang. A research on susceptibility status of *Ae. aegypti* to insecticides, used for fogging and based on a high number of hemorrhagic fever cases in Tembalang district, Semarang city, needs to be observed.

Objective: This study aimed to determine resistance status of *Ae. aegypti* mosquitoes based on bioassay and biochemical tests of esterase non-specific enzyme activity in the Tembalang District.

Methods: This study used a cross sectional design, it was conducted in June-September 2018 in a population of *Aedes* sp. from Tembalang District, Semarang City. Samples of F2 generations of female *Ae. aegypti* were obtained by ovitraps in the Tembalang District consisting of 12 urban villages, and their resistance status were tested by impregnated paper bioassay with malathion at a diagnostic dose of 0.8%. Esterase enzyme activity in the *Ae. aegypti* mosquito body of this population was tested biochemically to prove an increase in a non-specific esterase enzyme on the α -naphthyl acetate substrate.

Results: The bioassay results showed that mortality rates of *Ae. aegypti* at the 12 villages in Tembalang district after 24 hour test by impregnated paper indicated a range of 0-62%. Mechanism of resistance indicating an increase of non-specific enzyme esterase activity on α -naphthyl acetate at Rowosari, Kedungmundu, Sambiroto, and Meteseh could not be seen, it began to be seen in a low percentage (15%) at Sendang Mulyo, Tandang, Sendangguwo and Bubusan, and in a moderate percentage (45-75%) in Tembalang, Jangli and Mangunharjo. The mechanism of high esterase enzyme activity could be seen in the *Ae. aegypti* population at Kramas with AV \geq 0,700-0,900 by a percentage of 20%, and AV \geq 0,900 by a percentage of 80%.

Conclusion: This study provided information about some *Ae. aegypti* mosquitoes from Tembalang District that showed resistance to the malathion insecticide with an elevation of non-specific esterase enzyme activity on α -naphthyl acetate substrate in several villages except Kelurahan Rowosari, Kedungmundu, Sambiroto, and Meteseh.

Latar Belakang: Pengendalian vektor dengue di sekitar kasus Demam Berdarah Dengue selalu dijalankan dengan menggunakan insektisida malation di kota Semarang. Penelitian status kerentanan *Aedes aegypti* terhadap insektisida yang digunakan untuk fogging berdasarkan tingginya kasus Demam Berdarah Dengue di kecamatan Tembalang perlu dilakukan.

Tujuan: Penelitian ini bertujuan untuk mengetahui status resistensi nyamuk *Aedes aegypti* dengan uji bioassay dan biokimiawi enzim esterase non-spesifik di kecamatan Tembalang.

Metode: Penelitian menggunakan desain cross sectional dilakukan pada bulan Juni – September 2018 pada populasi nyamuk *Aedes sp* dari kecamatan Tembalang kabupaten Semarang. Sampel *Ae. aegypti* betina generasi F2 hasil penangkapan menggunakan ovitrap di kecamatan Tembalang yang terdiri dari 12 kelurahan telah diuji status resistensinya dengan metode impragnater paper bioassay dengan malation pada dosis diagnostik 0,8%. Aktivitas enzim esterase pada tubuh nyamuk dari populasi tersebut diuji secara biokimiawi untuk membuktikan adanya peningkatan enzim esterase non-spesifik terhadap substrat α -naftil asetat.

Hasil: Hasil bioassay menunjukkan bahwa mortalitas nyamuk *Ae. aegypti* dari 12 kelurahan di kecamatan Tembalang 24 jam paska pengujian impragnater paper berkisar antara 0-62%. Mekanisme resistensi yang berupa peningkatan aktivitas enzim esterase non-spesifik terhadap α -naftil asetat di kelurahan Rowosari, Kedungmundu, Sambiroto, dan Meteseh belum terlihat, dan mulai terlihat di kelurahan Sendang Mulyo, Tandang, dan Sendangguwo dengan persentase yang rendah (15%), dan kelurahan Bubusan, Tembalang, Jangli dan Mangunharjo dengan persentase sedang (45-75%). Mekanisme aktivitas enzim esterase yang tinggi ditunjukkan pada populasi nyamuk *Ae. aegypti* dari kelurahan Kramas dengan $AV \geq 0,700-0,900$ sebanyak 20%, dan $AV \geq 0,900$ sebanyak 80%.

Kesimpulan: Penelitian ini memberikan informasi bahwa sebagian nyamuk *Ae. aegypti* dari kecamatan Tembalang menunjukkan status resisten terhadap insektisida malation dengan mekanisme peningkatan aktivitas enzim esterase non-spesifik terhadap substrat α -naftil asetat di beberapa kelurahan kecuali kelurahan Rowosari, Kedungmundu, Sambiroto, dan Meteseh.

INTRODUCTION

Until now, dengue hemorrhagic fever (DHF) is an inevitable health problem in communities. The DHF is caused by dengue viruses, which transmits through bites of *Aedes aegypti* mosquitos.¹ The World Health Organization (WHO) reported that a number of dengue cases each year increased from 0.4 to 1.3 million in 1996-2005, 2.2 million in 2010 and 3.2 million in 2015.²

Based on existing reports, the DHF has been endemic in 33 provinces of Indonesia and 436 districts or cities, 605 sub-districts, and 1800 villages or sub-districts. Central Java province is one of the dengue-endemic areas in Indonesia with a number of 14,376 cases, an incidence rate (IR) of 42.26 / 100,000 and a case fatality rate (CFR) of 1.48% beyond the Indonesian CFR standard of 0.78%.³ Semarang is one of dengue-endemic cities, especially at Tembalang district which consists of 12 urban villages and has the highest dengue cases. The Tembalang district has 2 primary health cares, namely puskesmas Rowosari and puskesmas Kedungmundu. Incidences of the DHF in Tembalang district in 2016 reached IR of 70.68 / 100,000 populations and decreased to 36.63 / 100,000 populations in 2017.⁴

A DHF control, namely a controlling vector, is necessary to prevent and stop the dengue-endemic transmission. One of the DHF vector eradication programs usually uses insecticides.³ Insecticides are the largest group of pesticides used in vector eradication programs for pests and diseases, as well as various types of intruding insects around houses.⁵ Results of interviews with the P2P Department of Health of Semarang City reported that insecticides used for fogging in 2011 were lambda-cyhalothrin; in 2014-2015 was a malathion insecticide; in 2016 was synthetic pyrethroid (Cynof); and in 2017 until now is d-d trans cyphenothrin 50 g / l.

Biochemical testing is one of insect susceptibility tests on the insecticides in addition to the WHO standard tests using impregnated paper and molecular tests. This test is to examine

mosquito resistance on an essential insecticide based on quantification of enzymes responsible for its resistance process. Advantages of the biochemical tests is that the susceptibility status information can be obtained more quickly and can show its resistance mechanism that is measured individually using the esterase enzyme non-specific on α -naphthyl acetate.⁶

Based on a previous research on *Aedes sp* resistance, there was a 77.8% monooxygenase activity caused by a detoxification enzyme mechanism; therefore, *Aedes sp.* is a high-risk vector for transmitting dengue viruses in Semarang City.⁷ Another research conducted by Soenjono et al. examining vector *Ae. aegypti* susceptibility test on malathion in Tomohon City showed that *Ae. aegypti* was resistant to the malathion insecticide.⁸ It is also similar to *Ae. aegypti* mosquitos at the Port of Tanjung Emas Semarang which showed resistance to malathion.⁹

In Tembalang district, there is a tendency for resistance on organophosphate insecticides. *Ae. aegypti* mosquitos as a primary vector need to be examined to determine levels of susceptibility on the insecticides. Based on the discussion above, Purposes of this study is to examine resistance status of *Aedes aegypti* mosquitos on malathion and to analyze increases of non-specific esterase enzymes on α -naphthyl acetate of *Ae. aegypti* at each village in the Tembalang district.

METHODS

This study used analytic observational research and cross-sectional study design. It was conducted on June to November 2018. Its independent variables were exposures of malathion insecticides through impregnated malathion of 0.8% for 1 hour in *Ae. aegypti* in the Tembalang district area, while the dependent variables were resistance status of *Ae. aegypti* and increased activities of the esterase enzyme non-specific on α -naphthyl acetate.

Mosquito sample collections

Aedes agypti mosquitos as research subjects were obtained by ovitraps. Ovitrap installation

was used to catch eggs of the *Aedes sp.* The obtained eggs were bred in the Undip Entomology laboratory and the FK-KMK UGM Parasitology laboratory.

Ae. aegypti larvae survey in residential areas

An entomological survey in residential areas was conducted at some villages of the Tembalang district, which consisted of 117 urban villages with a total number of 36,371 houses. These study locations were only at 12 villages of the Tembalang district, specifically Tembalang, Bulusan, Kramas, Mangunharjo, Jangli, Tandang, Sambiroto, Meteseh, Kundumundu, Sendangmulyo, Sendangguwo, and Rowosari. Samples of population were larvae *Ae. aegypti* from water reservoirs at residents' homes at the 12 villages, Tembalang district, Semarang city.

Referring to a calculation of a Lemeshow cross-sectional formula, this study had 40 samples in each 12 villages, so that the total samples in this study were 480 houses.

Bioassay

Standard kits for vulnerability testing consisted of 4 test tubes and a pair of control tubes. Each tube was filled with 25 healthy female mosquitoes with a total number of 125 mosquitoes. The tests were conducted at the 12 villages in which each village had 125 mosquitoes; therefore, 12 villages consisted of 1,500 mosquitoes. The tests used impregnated paper containing 0.8% malathion insecticides, while in control groups the tests used mineral oil of impregnated paper which did not contain insecticides. The mosquitoes were contacted for 60 minutes in a contact tube, and the numbers of dead mosquitos were recorded. Then the dead mosquitos were transferred in to a collector tube and were placed in fresh air for 24 hours (holding) by giving sugar water. The number of the dead mosquitoes after 24 hours in a recovery tube was calculated.

Criteria to determine resistance status were established according to WHO standards in 2016: percentage of deaths $\geq 98\%$ = susceptible

(SS), 90-97% = tolerant (RS) and needs to be confirmed molecular resistance mechanism, <90% = resistant (RR) and does not need to be confirmed with other bioassay methods if a minimum number of 100 mosquitoes test.⁹

Biochemical Test

Early third instar larvae of the F2 generation crushed individually to be made homogenate and dissolved with 0.5 ml of phosphate buffer saline (PBS) solution. 0.02 M homogenate, 50 μ l by micropipette and put into the microplate wells. For each well, then 50 μ l of prepared substrate materials was added, specifically: 3mg of α -naphthyl acetate was dissolved into 0.5ml acetone, and then 100 μ l was taken to be added to BPS until a volume of a substrate solution became 10ml. The solution inside a microplate well was left for 60 seconds.

Each microplate well containing 50 μ l homogenate and 50 μ l substrate was added by 50 μ l coupling reagent (coupling reagent = 30 mg fast blue and 7 ml SDS 5% + 3 ml aquadest). Then it was allowed to stand for 10 minutes while observing color changes arising from red color to be blue gradually. This reaction was stopped by adding 50 μ l of 10% acetic acid solution. The non-

specific enzyme activity (absorbance value) was then read with an ELISA reader at a wavelength (λ) of 450 nm. Relationships of resistance status and resistance mechanism based on an increase in the activity of the esterase enzyme non-specific on α -naphthyl acetate were grouped as the following: absorbance value (AV) <0,700 (very susceptible/ SR); AV \geq 0,700-0,900 (medium resistance/ RS); AV > 0,900 (high resistance).

Ethical Clearance

No. 131/EC/H/FK-UNDIP/XII/2018, Ethics Committee of Faculty of Medicine, Universitas Diponegoro, Semarang

RESULTS

Bioassay results

Test results of *Ae. aegypti* mosquito susceptibility based on the impregnated paper method containing 0.8% malathion active ingredients were presented in Table 1. Table 1 demonstrated that the highest percentage of dead mosquitos observed for 24 hours after treatment was at Sendangmulyo (49%), while the lowest was at Sambiroto (0%). *Ae.aegypti* from all 12 villages were resistant to the malathion insecticides.

Table. 1. Resistance status of *Aedes aegypti* mosquitoes at 12 villages in Tembalang district on malathion based on an impregnated paper test with a diagnostic dose of malathion (0.8%)

Villages	Numbers of tested mosquitos *	Mosquito mortality 24 hours after treatment		Resistance Status
		Numbers of death	Percentages (%)	
Sendangmulyo	100	49	49	Resistant
Bulusan	100	41	41	Resistant
Tembalang	100	39	39	Resistant
Keramas	100	33	33	Resistant
Tandang	100	16	16	Resistant
Sendangguwo	100	61	61	Resistant
Rowosari	100	2	2	Resistant
Jangli	100	13	13	Resistant
Mangunharjo	100	32	32	Resistant
Kedungmundu	100	19	19	Resistant
Sambiroto	100	0	0	Resistant
Meteseh	100	8	8	Resistant

* Four replications, 25 mosquitoes for each sample in each district

Biochemical Test Results

Results of biochemical test of the non-specific esterase enzyme activity on α -naphthyl acetate

substrate in the *Ae. aegypti* larvae population in 12 villages in the Tembalang district were presented in Table 2 below.

Table 2. Non-Specific Esterase Enzyme Activity On A-Naphthyl Acetate Substrate In *Aedes aegypti* mosquito population in Tembalang district based on a biochemical test by reading Elisa Reader = λ 450 nm

Villages	Absorbance Levels					
	2 (AV<0,700) Low		(AV≥0,700-0,900) Middle		(AV≥0,900) High	
	Numbers	%	Numbers	%	Numbers	%
Sendangmulyo	17	85	1	5	2	10
Bulusan	11	55	6	30	3	15
Tembalang	10	50	5	25	5	25
Kramas	0	0	4	20	16	80
Tandang	17	85	2	10	1	5
Sendangguwo	17	85	1	5	2	10
Rowosari	20	100	0	0	0	0
Jangli	7	35	9	45	4	20
Mangunharjo	5	25	10	50	5	25
Kedungmundu	20	100	0	0	0	0
Sambiroto	20	100	0	0	0	0
Meteseh	20	100	0	0	0	0

Note: 2 repetitions of 20 larvae each in every tube

Table 2 indicated that an increase in the activity of the non-specific esterase enzyme on α -naphthyl acetate is not a resistant mechanism of the *Ae. aegypti*. Those were resistant to the malathion insecticides were at Rowosari, Kedungmundu, Sambiroto, and Meteseh due to their low percentage (100%). The mechanism of increasing esterase enzyme activity was seen in Sendang Mulyo, Tandang and Sendangguwo with a low percentage (15%), and Bulusan, Tembalang, Jangli and Mangunharjo with average percentages (45-75%). The mechanism of high esterase enzyme activity was shown in the *Aedes* mosquito population in Kramas with AV≥0,700-0,900 by a percentage of 20%, and AV> 0,900 by a percentage of 80%.

DISCUSSION

The results of the resistance test on the malathion insecticides indicated that *Ae. aegypti*

at the 12 villages in Tembalang district were resistant to insecticides. These results were in line with the *Ae. aegypti* at Rukukan, Tomohon city which were resistant to the malathion insecticides.

The observation results of esterase enzyme activity of mosquito populations at the 12 villages showed that the most significant percentage of the mosquitos increasing in the enzyme esterase activity was in Kramas, with moderate (20%) and high (80%) level of the activity.

This result was in line with the percentage of the dead mosquitos in the bioassay results. The resistance status meant that the resistance status of *Ae. aegypti* on the malathion insecticides in Kramas could be associated with an increase in the esterase enzyme activity. The increase of the activity indicated a metabolic detoxification mechanism in the insect's body. Esterase is one of the detoxification enzymes known to play

roles in the mechanism of insect resistance to insecticides from organophosphate groups. The esterase is one of the hydrolase enzyme groups, a large group of enzymes that catalyzes hydrolysis reactions of aliphatic compounds, aromatic esters, choline esters and organophosphorus.¹⁰

This information is essential to be used as a basis to select replacement insecticides when evaluating the insecticide replacement. Moderate-high esterase enzyme activity in the *Ae. aegypti* population at some villages in Tembalang district can allow resistance to insecticide compounds beside the malathion, which also has an ester group. Organophosphates are esters of phosphoric acid or thiophosphoric acid so that all insecticides from the organophosphate group have esters. In addition to organophosphates, insecticides from the carbamate group also contain an ester group because carbamate insecticides are esters of N-methyl carbamic acid.¹¹

At four other villages (Rowosari, Kedungmundu, Sambiroto, Meteseh), each of the four village had a low level of esterase activity, so it was not in line with the percentage of the number of the dead mosquitos in bioassay results indicating resistance status. This condition indicated that the resistance status of *Ae. aegypti* of malathion insecticides at the four villages was not related to an increase in the activity of the enzyme esterase, meaning there was no resistance to the malathion.

In the villages that did not show presence of esterase enzyme activity, there might have changes in its carboxylesterase enzyme. However, this study did not assess whether there was specific mechanism. The carboxylesterase enzyme could hydrolyze one or both carboxylate groups making up the malathion compounds. If the carboxylic group making up the malathion compounds changed, this insecticide compounds would lose their functions. Resistance caused by enzyme activity usually occurs when the enzyme blocks the insecticide compounds from reaching their targets.¹²

Tackling problems of the resistance in addition to controlling uses of the insecticides in a controlled and directed manner is also necessary to replace uses of chemical insecticides such as organophosphate with bioinsecticides and mosquito breeding programs (PSN) that are encouraged by the Indonesian government (drain, cover, bury) and are supported by public awareness to maintain hygiene and health.³

CONCLUSION

This research provides information of *Ae. Aegypti* at 12 villages in Tembalang district that showed resistance status to malathion insecticides with a mechanism of increasing non-specific esterase enzyme activity on α -naphthyl acetate substrate in the villages except Rowosari, Kedungmundu, Sambiroto, and Meteseh.

CONFLICT OF INTEREST

None declare.

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