BIOCHEMICAL DETECTION AND CHARACTERIZATION OF INSECTICIDE RESISTANCE IN DENGUE VECTOR *AEDES AEGYPTI* (L.) FROM AREAS AROUND UNIVERSITAS GADJAH MADA CAMPUS, YOGYAKARTA, INDONESIA

Budi Mulyaningsih[1], Sitti Rahmah Umniyati[1], Paulin Surya Phillabertha[2], Riyo Pungki Irawan[2] and Michael Aaron Romulo[2]

[1]Department of Parasitology, Faculty of Medicine, Public Health and Nursing; [2]Undergraduate Program of Medicine, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia

Abstract. In Yogyakarta, as in other areas of Indonesia, dengue hemorrhagic fever (DHF) is an important disease, especially in densely populated areas. The primary strategy to control DHF is by reducing the vector population using organophosphate and pyrethroid groups of insecticides. However, applications of insecticides becomes a contributing factor in the development of vector resistance. Insecticide resistance and its possible mechanism in *Aedes aegypti*, a dengue vector from areas near Universitas Gadjah Mada campus with over 50,000 students residing within a relatively small area. Larvae were collected from domestic breeding sites from Pogung, Sekip and Sendowo. Larvae from the same locality were pooled and reared to reach adult stage, which then were morphologically identified to confirm the presence of *Ae. aegypti*. Based on a CDC Bottle Bioassay mosquitoes from Sekip were susceptible to cypermethrin (10 μg) and malathion (50 μg), while those from Pogung and Sendowo were marginally resistant to cypermethrin but susceptible to malathion. However, in biochemical tests (non-specific esterase and mixed function oxidase activities), all mosquitoes from Pogung, Sekip and Sendowo indicated susceptibility to cypermethrin and malathion. Nevertheless, continued testing for insecticide resistance in dengue vectors is recommended in all areas of the country together with implementation of appropriate preventive measures, especially during the early rainy season.

Keywords: *Aedes aegypti*, insecticide resistance, non-specific esterase, mixed function oxidase, Yogyakarta, Indonesia

Correspondence: Budi Mulyaningsih, Department of Parasitology, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta 55281, Indonesia.
Tel: +62 274 546215, mobile: +62 878 39193356; Fax: 062 274 546215
E-mail: budi.mulyaningsih@ugm.ac.id; budimulyaningsih@yahoo.com

INTRODUCTION

Asia and Pacific countries are areas with a high prevalence of mosquito-borne dengue hemorrhagic fever (DHF). Accordingly, the World Health Organization (WHO) announced Strategic Plans in these areas with the aim to reduce the burden of contagious diseases, one plan

being the identification of disease vectors and monitoring of insecticide resistance (WHO, 2012). In Indonesia, DHF has a high incidence, with Yogyakarta Special Region (YSR), which includes five provinces, having the highest morbidity rate in 2015 (MOH, 2016). During 2011-2016 there was an increase of dengue cases in Sleman, a district of YSR, with the highest number of cases reaching 880 in 2016 and a mortality of 9 in 2015 and 2016 (Sleman District Health Office, 2016). Pogung, Sekip and Sendowo areas are geographically located very close to Universitas Gadjah Mada (UGM) campus, the largest university in Indonesia and located in Sleman District, YSR. In recent years these areas have become densely populated because the residential areas are occupied by many UGM students who come from outside Yogyakarta. Some of the determinating factors that trigger the emergence of dengue cases are population growth and unplanned and uncontrolled urbanization (MOH, 2010).

As there is currently no effective vaccine or drug for dengue, vector control remains the most effective strategy to overcome this disease. Up to now, use of chemical insecticides is still the main method to reduce occurrence of the disease, even though environmental management, educational programs and elimination of breeding sites of its main vector, *Ae. aegypti*, continues to be implemented. However, use of insecticides for vector control faces many challenges due to the development of insecticide resistance in dengue vectors, especially *Ae. aegypti* (Ranson *et al*, 2011). Control of mosquitoes using insecticides becomes more difficult with the appearance of resistance to chemical insecticides. According to Hemingway *et al* (2004), the most common mechanism of mosquito resistance to insecticides is metabolic changes, such as decreases in level or activity of detoxifying proteins and mutations in target sites, such as sodium channels, acetylcholinesterase and GABA receptors. The preferred method to assess the mechanism of resistance to insecticides is by a biochemical approach, which can now be achieved with high accuracy (Muthusamy *et al*, 2014).

Aedes aegypti in YSR (Bantul, Sleman and Yogyakarta), Central Java (Blora, Jepara, Magelang, Purwokerto, Salatiga, Semarang, Surakarta, and Tegal), West Java (Bandung and Cimahi), East Java (Surabaya); Jakarta (Mampang Prapatan and Tanjung Priok), South Sumatra (Palembang), and Central Sulawesi (Palu) have been reported resistant to cypermethrin and malathion (Ahmad *et al,* 2009; Zulhasril and Lesmana, 2010; Widiarti *et al*, 2011; Mulyatno *et al*, 2012; Sayono *et al*, 2012). Increased urbanization is associated with continuous insecticidal exposure to *Ae. aegypti* population and causes insecticide resistance due to deleterious modifications of the mosquito detoxification mechanisms (Poupardin *et al*, 2008; Riaz *et al*, 2009; David *et al*, 2010).

Hence, the purpose of this study was to detect resistance status against cypermethrin and malathion insecticides and its underlying cause(s) in *Ae. aegypti* population from Pogung, Sekip and Sendowo, Sleman District, YSR.

MATERIALS AND METHODS

Mosquito collection sites

Samples of *Ae. aegypti* larvae and pupae were collected from houses and schools in three locations, Pogung, Sekip and Sendowo, Sleman District, YSR in July 2017. Positive control UGM laboratory-reared *Ae. aegypti* consisted of F 310 strain, resistant to (50 μg/bottle) malathion (64%

mortality), and F 87, resistant to (10 μg/bottle) cypermethrin (70% mortality). Negative control, F 1057 strain from the Institute for Medical Research, Kuala Lumpur, Malaysia is highly susceptible to cypermethrin and malathion (100% mortality to both insecticides).

This study was approved by the Ethics Committee for Medical and Health Research, Faculty of Medicine, Public Health and Nursing, UGM, Yogyakarta (reference no. KE/FK/643/EC/2017).

Colonization

In the laboratory, larvae from the same place were colonized together to reach adult stage (Service, 1996; Umniyati *et al*, 2008). Adult mosquitoes were identified to confirm the presence of *Ae. aegypti* (WHO, 1972). Female mosquitoes were maintained at 26 ± 2°C under 12:12 light:dark cycle and relative humidity of 68 ± 4%, with 10% sucrose as feed. Females were blood-fed on mice and adults obtained from F2 progeny were used for bioassays and biochemical studies.

Bioassay

Bioassays were performed following the protocol of CDC Bottle Bioassay (CDC, 2010) conducted on the same day in a room at 26 ± 2°C and relative humidity of 68 ± 4% for all experiments. In brief, 25 female mosquitoes from each location were placed in a glass bottle (250 ml) pre-coated with 10 μg of cypermethrin or 50 μg of malathion (both dissolved in acetone). Each test consisted of 4 experimental bottles and a negative control bottle. After 30 minutes the numbers of live and dead mosquitoes in each bottle were recorded. Criterion of mosquito mortality is difficulty in flying or attaching on the bottle surface.

Enzyme assays

For assay of non-specific esterase activity (Lee, 1991; Mardihusodo, 1996), individual mosquito was homogenized in 0.5 ml of 20 mM phosphate-buffered saline solution pH 7.0 (PBS) and 50 μl aliquot was placed in a well of a 96-well flat bottom microtiter plate. Then 50 μl of freshly prepared substrate solution (60 μg/ml α-naphthyl acetate in PBS) was added and the mixture incubated for 60 seconds at 27°C. A 50-μl aliquot of coupling reagent (3 mg/ml Fast Blue B salt in 3.5% SDS), incubated for 10 minutes before the reaction was terminated by the addition of 50 μl of 10% acetic acid. $A_{450\,nm}$ was measured using a Bio-Rad Benchmark Microplate Reader (Bio-Rad, Hercules, CA). Each experiment was conducted in triplicate.

For mixed function oxidase activity assay (Lee and Nazni, 2007), mosquitoes were placed in a plastic cup and kept at 4°C for 5 minutes. Then individually mosquito was homogenized in 200 μl of PBS and 20-μl aliquot of clear supernatant was placed in a well of a 96-well flat bottom microtiter plate, followed by 80 μl of PBS. A 200-μl aliquot of 10 mg of tetramethyl benzidine in 187.5 mM sodium acetate buffer containing 25% (v/v) methanol followed by a 25-μl aliquot of 3% hydrogen peroxide were added and the mixture was incubated at 27°C for 30-60 minutes. $A_{595\,nm}$ was measured as described above. Each experiment was conducted in triplicate.

Statistical analysis

A one-way analysis of variance (ANOVA) was used to compare the enzyme activity between collected mosquitoes tested to UGM strain, with significance accepted at $p<0.05$.

RESULTS

Insecticidal susceptibility bioassay of adult *Ae. aegypti*

Employing CDC Bottle Bioassay

(CDC, 2010) and following the WHO (1998) criteria, namely, susceptible if mosquito mortality is 98-100%, resistant but needs confirmation if mosquito mortality is 80-97% and resistant if mosquito mortality is <80%, *Ae. aegypti* populations from Pogung, Sekip and Sendowo were susceptible to malathion, and those from Sekip were susceptible to cypermethrin while those from Pogung and Sendowo were resistant but needs confirmation (Table 1).

Non-specific esterase activity of *Ae. aegypti*

Non-specific esterase activities of *Ae. aegypti* collected from Pogung, Sekip and Sendowo were comparable to that of malathion-sensitive IMR F 1057 strain and significantly lower than that of malathion-resistant UGM F 310 strain (Table 2).

Mixed function oxidase activity of *Ae. aegypti*

Mixed function oxidase activities of *Ae. aegypti* collected from Pogung, Sekip

Table 1
Percent mortalities of colonized adult female *Aedes aegypti* collected from Pogung, Sekip, and Sendowo, Sleman District, Yogyakarta Special Region, Indonesia, July 2017 following exposure to cypermethrin and malathion.

Locality	Percent mosquito mortality	
	Malathion[a]	Cypermethrin[a]
Sekip	100 (n = 300)	100 (n = 300)
Sendowo	98 (n = 300)	94 (n = 100)
Pogung	100 (n = 300)	95 (n = 200)
(IMR F 1057 strain[b])	0 (n = 75)	
(IMR F 1057 strain[c])		0 (n = 67)

[a]CDC Bottle Bioassay using 10 and 50 µg of cypermethrin and malathion, respectively per botttle for 30 minutes (CDC, 2010). [b]From Phillabertha (2018). [c]Cypermethrin and malathion sensitive.

Table 2
Non-specific esterase activities of colonized adult female *Aedes aegypti* collected from Pogung, Sekip, Sendowo, Sleman District, Yogyakarta Special Region, Indonesia, July 2017.

Locality	Number of mosquitoes	Non-specific esterase activity Mean $A_{450\ nm}$ (SEM)
Sekip[a]	8	0.194 (0.080)*
Sendowo	16	0.256 (0.036)*
Pogung[a]	16	0.231 (0.057)*
(IMR F 1057 strain[b])	6	0.264 (0.025)*
(UGM F 310 strain[c])	6	0.358 (0.074)

[a]From Irawan (2018). [b]Malathion sensitive. [c]Malathion resistant. *p<0.05, compared to UGM F 310 strain.

and Sendowo were comparable to that of cypermethrin-sensitive IMR F 1057 strain and significantly lower than that of cypermethrin-resistant UGM F 87 strain (Table 3).

DISCUSSION

In dengue endemic areas, detection of mosquito vector resistant to insecticides is important for effective vector control. This study shows mosquito dengue vector *Ae. aegypti* collected from Pogung, Sekip and Sendowo, Sleman District, YSR in July 2017 is still susceptible to malathion and cypermethrin. These results were unexpected as the Basic Health Research data in 2013 reported use of mosquito coils, repellent and insecticides in Sleman Districts was as high as 74% (MOH, 2013).

Georghios and Mellon (1983) reported insect resistance to insecticides generally occurs after a period of 2-20 years of introduction. In Yogyakarta organophosphate insecticides (malathion and temephos) have been used in dengue disease control program since 1974 (Mardihusodo, 1996) and cypermethrin, a synthetic pyrethroid insecticide, for 15 years. A recent research of *Ae. aegypti* from villages in Minomartani, Plosokuning, Sleman, and Yogyakarta, detected resistance to both organophosphate and pyrethroid insecticides (Mulyaningsih *et al*, 2018). Lagunes (1980) found a *Culex quinquefasciatus* strain containing a gene responsible for resistance to three types of insecticides, such as permethrin, propoxur and temephos. However, Georghiou (1983) suggested a rotation approach can inhibit insecticide resistance.

Worldwide, mechanisms of insecticide resistance in mosquitoes are being carefully examined because understanding pathways of resistance development can aid in developing preventive strategies and delaying insecticide resistance (Hemingway *et al*, 2004). There are two main resistance mechanisms, namely, alterations in the target site and metabolic resistance (Miller, 1988). Resistant insects typically exhibit increased activity of esterases as the majority of chemical insecticides have ester linkages susceptible to hydrolysis (Wu *et al*, 2004; Yang *et al*, 2004). The major target of organophosphate insecticides is acetylcholine esterase, which often can undergo mutations that prevent drug binding (Crow

Table 3
Mixed function oxidase activities of colonized adult female *Aedes aegypti* collected from Pogung, Sekip and Sendowo, Sleman District, Yogyakarta Special Region, Indonesia, July 2017.

Locality	Number of mosquitoes	Mixed function oxidase activity Mean $A_{595\ nm}$ (SEM)
Sekip	16	0.151 (0.080)*
Sendowo[a]	8	0.149 (0.036)*
Pogung[a]	16	0.143 (0.053)*
(IMR F 1057 strain[b])	6	0.152 (0.018)*
(UGM F 87 strain[c])	4	0.762 (0.074)

[a]From Romulo (2018). [b]Cypermethrin sensitive. [c]Cypermethrin resistant. *p<0.05, compared to UGM F 87 strain.

et al, 2007). The other major mechanism of insecticide resistance in insects is through detoxification mediated by up-regulation of non-specific esterases (Montella *et al*, 2012). Target sites for pyrethroid insecticides involve ion channels in nerve membranes, and resistance often is due to reduced binding affinities of pyrethroids to voltage-gated sodium channels (Brengues *et al*, 2003). In addition monooxygenases expressed in tissues of the gut, fat, reproductive tract and Malpighian tubes are able to detoxify pyrethroid-based insecticides (Feyereisen, 1999; Poupardin *et al*, 2008).

Aedes mosquitoes are most often present in commercial areas and thus control measures must cover both public and residential properties. High percentage of dengue-infected mosquitoes in public places is especially of concern as the likelihood of transmission is greater in these areas, resulting in the spread of infection to previously disease-free residential areas (Ming *et al*, 2018).

In summary, results of the bioassays indicate *Ae. aegypti* in Sekip were still susceptible to malathion and cypermethrin, while those in Pogung and Sendowo were susceptible to malathion but with possible resistance to cypermethrin, which subsequently was shown by biochemical tests to still be sensitive to this pyrethroid insecticide. Although bioassays are convenient to carry out, where marginal resistance is detected, a follow-up biochemical assays are of benefit to avoid premature discontinuation of compounds that are still effective. In order to reduce the development of insecticidal resistance, it recommended insecticides of different chemical structures be applied in rotation.

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