Unnes Journal of Public Health 7 (2) (2018)

Unnes Journal of Public Health

http://journal.unnes.ac.id/sju/index.php/ujph

Resistance Status of *Aedes albopictus* (Skuse) on Malathion in Bengkulu City

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Info Artikel

Article History:
Submitted January 2018
Accepted February 2018
Published July 2018

Keywords:
Ae; albopictus; bottle bioassay; malathion;

Abstract

Dengue Hemorrhagic Fever (DHF) is a disease caused by dengue virus and transmitted by *Aedes aegypti* mosquito as the main vector and *Aedes albopictus* as secondary vector. In 2016, Bengkulu City was one of 3 cities that experiencing DHF outbreaks in Indonesia. Insecticides malathion have been used since 1990 in bulk in DHF control programs in Bengkulu City and have not had an evaluation report on *Aedes sp*. vulnerability to malathion. The purpose of this research was to know the resistance status of *Ae albopictus* mosquitoes from endemic and sporadic areas of DHF in Bengkulu City to malathion. The sample of the study was the *Ae. albopictus* adult female mosquitoes which collected from endemic and sporadic areas of DHF in Bengkulu City with ovitrap installation. The resistance test was performed by CDC Bottle Bioassay method with malation 96% and 50 µg/ml diagnostic dose of. The results of this study showed 1% mortality on endemic area and 5% on sporadic area. *Aedes albopictus* of both areas were resistant to malathion.

Abstrak

Demam Berdarah Dengue (DBD) merupakan penyakit yang disebabkan oleh virus dengue dan ditularkan oleh nyamuk Aedes aegypti sebagai vektor utama dan Aedes albopictus sebagai vektor sekunder. Pada tahun 2016, Kota Bengkulu merupakan 1 dari 3 kota yang mengalami KLB DBD di Indonesia. Insektisida malation telah digunakan sejak tahun 1990 secara massal dalam program pengendalian DBD di Kota Bengkulu dan belum memiliki laporan evaluasi mengenai kerentanan Aedes sp terhadap malation. Tujuan penelitian untuk mengetahui status kerentanan nyamuk Ae. albopictus dari daerah endemis dan sporadis DBD Kota Bengkulu terhadap malation. Sampel penelitian adalah nyamuk betina dewasa Ae. albopictus yang dikoleksi dari daerah endemik dan sporadis DBD Kota Bengkulu dengan pemasangan ovitrap. Uji kerentanan dilakukan dengan metode CDC Bottle Bioassay dengan malation 96% dan dosis diagnostik 50 µg/ml. Hasil penelitian ini menunjukkan mortalitas 1% untuk daerah endemis dan 5% untuk daerah sporadis. Aedes albopictus kedua daerah telah resisten terhadap malation.

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pISSN 2252-6781
eISSN 2584-7604

INTRODUCTION

Dengue Haemorrhagic Fever (DHF) is a disease caused by dengue virus consisting of four virus serotypes: DENV-1, DENV-2, DENV-3 and DENV-4 and transmitted by *Aedes aegypti* (*Ae. Aegypti*) mosquito as the main vector and *Aedes albopictus* (*Ae. Albopictus*) as a secondary vector. The four dengue viruses infect humans in Africa and Southeast Asia from 100-800 years ago. The dengue virus developed rapidly in World War 2 where the massive spread of mosquitoes occurred along with the delivery of goods contributing to the global spread of DHF (CDC, 2010). DHF disease is still a public health problem because it often leads to outbreak, a broad impact on the quality of life and economy, and can cause death (WHO, 2012).

Dengue hemorrhagic fever (DHF) is an endemic disease that occurs throughout the year and usually occurs in the rainy season because the population of *Ae. aegypti* and *Ae. albopictus* mosquitoes can develop optimally in the community. In the last 50 years, the incidence of DHF has increased 30 times with increased geographic expansion into new countries and expansion from urban to rural areas. An estimated 50 million dengue infections occur each year and around 2.5 billion people live in 128 DHF endemic countries (WHO, 2009).

DHF is commonly found in tropical and sub-tropical regions. Asia ranks first in the world in the number of DHF patients each year. Meanwhile, from 1968 to 2009, WHO recorded Indonesia as the country with the highest DHF case in Southeast Asia. Indonesia is an archipelagic country that has a tropical climate with 17,508 islands, 34 provinces and 447 districts that have optimal environmental supportability for the growth and development of flora and fauna including *Ae. Aegypti* and *Ae. albopictus* mosquitoes.

DHF has been a public health problem in Indonesia for the last 48 years. Since first reported in Surabaya in 1968 with Case Fatality Rate (CFR) was very high that was> 41.3% (58 cases with 24 deaths). Then in 1969 it was reported in Jakarta and Bandung and Yogyakarta in 1972. The first epidemic outside Java occurred in 1972 in West Sumatra and Lampung, in 1973 in North Sulawesi and Bali, in 1974 in South Kalimantan and Nusa Tenggara. In 1997, dengue fever has spread to all provinces in Indonesia and is now endemic in big cities and to rural areas. Since 1968, there has been an increase in the number of provinces and districts/cities from 2 provinces and 2 cities, to 34 provinces infected with DHF from 34 provinces in Indonesia (100%) and 436 districts/cities infected with DHF from 514 districts/cities (90%) in 2015 with the number of 126,675 people and 1,229 of whom were died (WHO, 2012).

The outbreak of DHF has become national disasters and is categorized as outbreak with high mortality and morbidity rates. Ministry of Health data in 2009 showed that the number of cases was 158,912 with the number of deaths of 1,420 people, in 2010 the number of cases was 156,086 people with the number of deaths of 1.358 people, in 2013 the number of cases of people with the number of deaths of 1,358 people. In Indonesia, in 2012 the incidence of DHF reached 37.27% with mortality rate almost 1% (CDC, 2010). Directorate of vector infectious disease control and zoonosis of the Ministry of Health reported that by the end of January 2016 there were outbreaks of dengue disease in 12 districts and 3 cities from 11 provinces in Indonesia including Bengkulu City with the total of DHF patients from 11 provinces as many as 8,487 people and total deaths of 108 people.

Based on data from Health Office of Bengkulu Province 2015, dengue disease was endemic disease and was categorized as the top 10 disease in the community health clinic with cases that were soaring high in 2015 and in early 2016 there was an outbreak. In 2013 there were 443 cases (Incidence rate (IR) of 24.2 per 100,000 population and Case Fatality Rate (CFR) of 0.9%), there were 467 cases in 2014 (IR of 24.3 per 100,000 population and CFR of 2.8%) and by 2015 there were 925 cases (IR of 49.3 per 100,000 population and CFR of 2.1%). By 2015 Bengkulu Province had an IR of 49.3 which was classified as moderate risk category.

In 2014 there were 315 dengue cases in Bengkulu City with 8 deaths (IR of 91,9 per 100,000 population and CFR of 43.2%) so that Bengkulu City was included in high-risk category and contribute in increasing DHF case in Bengkulu Province. Various efforts have been made to suppress the morbidity and mortality rate, but DHF still persists throughout the year and has a tendency to increase the incidence every year. This is supported by the presence of vectors and the availability of habitats for breeding, and the focus of infection is very difficult to control and until now the vaccine for the prevention of dengue infection is still in the process of research and the drug is still not available, so one of the efforts to control DHF is primarily addressed through control the vector to break the chain of disease transmission (Arslan et al., 2016; Lima et al., 2011)

DHF endemic sub-district is a sub-district that for three consecutive years there are cases of DHF. DHF sporadic sub-district is a sub-district that for three consecutive years there are cases of DHF but not sequentially. DHF potential sub-district is sub-district where for three years there are no dengue cases but the number of free from larvae (*ABJ or Angka Bebas Jentik*) is less than 95%. Gading Cempaka sub-district is one of the DHF endemic sub-districts since 2008 there are always cases of DHF. On Gading Cempaka sub-disctrict in 2014 there were 67 cases (IR of 158.66 per 100,000 population and CFR of 14.2%) and was included in high-risk category with IR> 55 per 100,000 population and contributed to the increase of DHF case in Bengkulu City.

Control with natural active ingredients is still under development and the results were not yet satisfactory (Listyorini, 2012: Harfriani, 2014; Khalalia, 2016). This resulted in chemical control is still the most popular for dengue control program although it can cause some harm both from human health and safety as well as causing resistance to mosquitoes. Adult vector control with fogging is still the primary choice for the prevention of DHF. This control effort is effective if targeted mosquitoes are still susceptible to the insecticides used. The use of insecticide for a long time can cause resistance (Heel et al., 2011); Kamgang et al., 2011; Arslan et al., 2016).

Insecticide controls insects by disrupting important processes in their lives. Insecticide has working ways with an effect on insects based on the way insecticide affects the specific target point in the body of insects. Organophosphorus insecticides are all insecticides containing phosphorus in their chemical composition, one example of an organophosphate insecticide is malathion. Characteristic of this insecticide is fast knockdown ability, toxicity to mammals is relatively low, toxic to vertebrates, relatively less stable, corrosive and smelly. Organophosphorus insecticide works by binding the acetylcholine esterase (AChE) enzyme which serves to hydrolyze acetylcholine to choline and acetic acid. Under normal circumstances, acetylcholine serves as a transmitter of nerve impulses, so that when the organophosphate binds to this enzyme there is no hydrolysis but it is phosphorylated (Hell and Hachimi-Idrissi, 2011; El Naggar et al., 2009, Liu et al., 2015)

Malathion is included in organophosphate group insecticides that are widely used in programs for mosquito control, especially dengue vectors in Indonesia. Malathion is characterized by the ability to rapidly disable insects, its toxicity to relatively low mammals and less stable to vertebrates, corrosive, smelly, included as aliphatic group and has short carbon chains. Malathion works as a stomach poison, contact poison and inhaled toxin. The types of insecticides that have the active substance malathion are fumition, gitantion, drexelion, rider, and sinaption. Malathion is a class of parasympathomimetic insecticides that have the properties of binding to the choline esterase enzyme on the insect nerve irreversibly. *Ae. aegypti* and *Ae. albopictus* resistance includes genetic factors, biological factors, and operational factors. When *Ae. albopictus* is resistance against insecticides, the mosquito population will increase so that an area becomes DHF endemic that can even be an outbreak (Wang et al., 2012; Scott et al., 2013).

Insecticides malathion has been used in Indonesia since 1972, but the incidence and prevalence of DHF tend to increase even though the mortality rate of the sufferers tends to decrease, which indicates the beginning of resistance to malathion. Evaluation report on Ae albopictus mosquitoes' resistance to malathion insecticides in Bengkulu and regencies/municipalities has not existed, although malathion insecticides have been used since about 1990. Based on this, it is necessary to conduct research on the resistance status of organophosphate insecticides malathion that has been used for ± 27 years.

METHOD

This study has been approved by the ethics committee of the Faculty of Medicine, University of Gadjah Mada. This research is a quasi-experimental research with post-control group design research. This research was conducted on August 21, 2017 - December 11, 2017. The population of adult Ae albopictus mosquitoes from F0 egg generation from research locations of Sidomulyo Village of Gading Cempaka sub-district and Tanjung Jaya Village of Sungai Serut Sub-district of Bengkulu City. The sampling method of mosquito eggs referred to the number of buildings located in an area defined by Fundacao Nacional de Saunde (FUNASA) as in Table 1.

The number of buildings in Sidomulyo and Tanjung Jaya Villages was <60,000 so that the installation of ovitrap as many as 100 pieces on each Village and the installation of ovitrap in each house as many as 2 pieces that are indoor and outdoor. The number of houses that must be checked to detect the eggs of *Ae. aegypti* referred to the guidance of DBA entomology survey according to WHO criteria by using house number and House Index (HI) data on the location.

House Index in Sidomulyo Village> 5% with 4335 houses, the number of houses to be installed with ovitrap was 58 houses. House Index in Tanjung Jaya Village> 5% with 331 houses, the number of houses that should be installed was 54 houses. The total house that should be installed with ovitrap was 112 houses. The sample in this study used stratified random sampling by stratifying in 2 Villages based

Table 1. Number of ovitrap for each research location

The number of buildings on the location	Number of ovitraps that must be installed
< 60.000	100
60.000 – 120.000	150
120.000 – 150.000	200
> 500.000	300

on the number of DHF cases and the number of vector control activities with fogging focus.

The selection of house location used for the study based on data that in the Village had been a case of DHF within the last 6 months. The previous house data had been obtained from the head of the neighborhood association (*RT or Rukun Warga*) which used to determine the house to be surveyed. The survey started from the house where dengue fever has occurred within the last six months as a starting point, then selected intermittently to a radius of 100 m to the west, north, east and south.

Collection of Mosquito Eggs

Installation of ovitrap was performed on endemic area (Sidomulyo Village) and sporadic area (Tanjung Jaya Village). Ovitrap was made from 250 ml plastic cups that painted in black and labeled according to location, endemic or sporadic. Ovitrap was placed inside and outside the house that was not exposed to direct sunlight and rain as well as moisture place. Ovitrap filled with clear water of 2/3 parts and ovistrip mounted in full circle on the water border for the oviposition of Aedes sp. Ovitrap was installed for 7 days then the ovistrip was carefully dried, labeled and inserted into clear plastic for storage.

Mosquito Colonization

Mosquito eggs were obtained from the research location in the colonization at the Insectarium of the Department of Parasitology Faculty of Medicine, University of Gadjah Mada, ovistrip was soaked into a container of plastic tray size 24x36x6 cm containing water for 1-2 days to hatch into larvae. Maintenance of larvae to survive was fed with chicken liver and water was replaced 2-3 times in 1 week. Pupa was taken with a plastic pipette and put into a plastic cup that had been filled with water and put in a mosquito cage. Mature mosquitoes would appear after 2 days and fed with 10% sugar solution. Test room temperature of 27 ± 2°C, humidity of 75 ± 10% and light period of 12 hours light: 12 hours dark (WHO, 2016). An adult mosquito was identified to determine the Ae albopictus (F0) mosquito.

CDC Bottle Bioassay Test

Resistance Test by CDC bottle bioassay. The number of Ae albopictus adult female mosquitoes aged 3-5 days with satiety condition of 10% sugar was125 heads each testing time. By using a 250 ml glass bottle of 5 pieces with a cover consisting of a bottle cap and gauze. Each bottle was labeled (4 test bottles, 1 bottle of control). The test bottle was filled with 1 ml of 96% 50 μg/ml malathion solution and control bottle filled with 1 ml of acetone. After insecticide or acetone inserted according to the label, the bottle was tightly closed and then rolled so that the solution will be evenly distributed on the wall, bottom and bottle cap. Test mosquitoes were inserted in the test bottle for 30 minutes of diagnostic time and can be continued for up to 2 hours if the test mosquitoes had not died. If the number of controlling mosquitoes that died between 3-10% then

Table 2. Number of houses examined on the location

Number of houses on the location	House Index (HI)		
	>1%	>2%	>5%
100	95	78	45
200	155	105	51
300	189	117	54
400	211	124	55
500	225	129	56
1000	258	138	57
2000	277	143	58
5000	290	147	59
10.000	294	148	59
Infinite	299	149	59

Table 3. Mortality percentage of Ae. albopictus in exposure time of 30 minutes and 2 hours with 50 µg/ml malathion

Location	Generation	The number of test mosquitoes	Mortality (%)		Category
			30 minutes	2 hours	
Endemic Area (Sidomulyo)	F 1	125	1%*	96%	Resistant
Sporadic Area (Tanjung Jaya)	F 1	125	5%*	98%	Resistant
Laboratory	F 1057	125	98%*	100%	Vulnerable

*Resistance Status (CDC, 2010): 98%-100% (vulnerable), 80%-97% (tolerant) and <80% (resistant).

the mortality was calculated with Abbot Formula (CDC, 2010). If the number of control mosquitoes is > 10% death, then the test should be repeated. The Abbot formula is as follows:

Data Analysis

Resistance status of Ae albopictus adult mosquitoes from the location and the mosquitoes from the control group on the insecticides malathion and cypermethrin were performed empirically on the basis of mortality (AK). The percentage of deaths was classified into 3 categories according to WHO: 98% -100% (vulnerable), 80% -97% (moderate/tolerant) and <80% (resistant) (CDC, 2010).

The probit analysis was used to view LT50,90,99 malathion on *Ae. albopictus* from endemic and sporadic dengue areas. To specify the value of ERR 50,90,99, it was computed first LT50,90,99 mosquito malathion from endemic and sporadic area divided by LT50,90,99 mosquitoes from the laboratory. Mosquito is categorized as vulnerable if ERR <5, tolerant if ERR = 5-10 and resistant if ERR> 10 (Selvi et al., 2010). The ERR value determination formula is as follows:

RESULTS AND DISCUSSION

Resistance Status

The diagnostic dose of CDC for insecticides malathion against Aedes sp mosquitoes was 50 µg/ml with a 30-minute diagnostic time, which is, the concentration of 50 µg/ml can kill 100% of the test mosquitoes at exposure for 30 min. The status of resistance by percentage of death is classified into 3 categories according to WHO (CDC, 2010), namely: 98% -100% (vulnerable), 80% -97% (moderately resistant/tolerant) and <80% (resistant). In this study, for 30 minutes, mosquitoes can only kill 1% and 5% *Ae. albopictus* of each population, which resulted in population *Ae. albopictus* from endemic area (Sidomulyo Village) and sporadic area (Tanjung Jaya Village) of DHF in Bengkulu City was categorized as resistant. The result of statistical analysis through the bivariate test with independent T-test was found that there was difference of resistance status based on death rate between endemic and sporadic area with a value of P = 0.018 (P <0.05). So there was no difference in resistance status in endemic area (Sidomulyo Village) and sporadic area (Tanjung Jaya Village) of DHF in Bengkulu City.

Resistance status of *Ae. albopictus* adult female mosquitoes based on biological resistance test results with CDC Bottle Bioassay method on 96% of 50 µg/ml malathion diagnostic dosage and diagnostic time at endemic area and sporadic area of DHF in Bengkulu City can be seen in Table 3.

$LT_{50,90,99}$ and $ERR_{50,90,99}$

Lethal Time is the average time required to cause 50%, 90% and 99% death of Ae albopictus mosquitoes against exposure to 96% insecticides malathion with a diagnostic dose of 50 *µg*/ml. Lethal Time (LT50,90,99) of 96% insecticides malathion with 50 *µg*/ml diagnostic dose in endemic area was longer than LT50,90,99 in sporadic area. As shown in table 4.

Lethal time of *Ae. albopictus* in endemic area was longert than in sporadic area which showed that *Ae. albopictus* populations in endemic area were more resistant to insecticides malathion due to the more frequent exposure of insecticides malathion used in DHF vector control programs through thermal fogging by Bengkulu City Health Office while sporadic area due to there were rarely cases of DHF so the exposure to insecticides malathion through fogging was also less frequent. One of the conditions for the implementation of fogging is that there is a case and spraying is performed at a radius of 100 meters from where the DHF patients live. The result of this study was clarified by the ratio of $ERR_{50,90,99}$ of *Ae. albopictus* in endemic area with sporadic area which indicated that endemic area was included in moderate/tolerant resistant category whereas sporadic area belongs to the vulnerable category.

Table 4. $LT_{50,90,99}$ of Ae. Albopictus on 50 μg/ml insecticides malathion

Location	*Lethal Time* (minutes)			Regresssion Line
	LT_{50}	LT_{90}	LT_{99}	
Endemic Area (Sidomulyo)	87.232 (83.118 – 91.625)	130.630 (120.145 – 147.493)	181.557 (158.558 – 222.484)	Y= 0.8343X – 23.772
Sporadic Area (Tanjung Jaya)	54.960 (52.807 – 56.982)	82.519 (79.210 – 86.505)	114.936 (107.560 – 124.605)	Y= 1.1146X – 15.08
Laboratory	16.263 (15.489 – 17.011)	24.366 (23.108 – 25.932)	33.879 (31.252 – 37.465)	Y= 0.5242X + 55.072

Estimated Resistance Ratio (ERR 50,90,99) is the determination of resistance level of insecticide populations obtained from the quotient results of LT50,90,99 population studied with LT50,90,99 laboratory/control populations. Mosquito is categorized as vulnerable if ERR <5, tolerant if ERR = 5-10 and resistant if ERR> 10 (Selvi et al., 2010). The value of ERR in the endemic area was tolerant, whereas in sporadic area it was categorized as vulnerable. As shown in table 5.

Continuous insecticide use will selectively suppress insects and result in insect resistance to insecticide. Resistance is defined as the ability of an insect population to withstand the lethal effects of insecticides. In each population there are mosquitoes that are still sensitive and resistant, but with continuous insecticide use can cause the number of sensitive mosquitoes to decrease and the remaining ones are insecticide resistant mosquitoes.

Vulnerability status is largely influenced by biological, genetic and operational factors. Genetic factors in the form of genes that resemble the esterase enzyme in the regulation of insecticidal metabolism become non-toxic. Biological factors of insect behavior in terms of migration, isolation, avoidance, and adaptation. Operational factors in the form of continuous insecticide use are not suitable with indication that caused the selection pressure or failure in operation. Things like migration and the amount of insecticide given can affect the number of mosquitoes that survive and affect the evolution of resistance. Differences in resistance status among areas can also be influenced by differences in knowledge and education as well as control efforts that have been made and the frequency of insecticides used for both health and agricultural purposes. Therefore, the pattern of resistance may vary between areas in a country.

The ratio of *Ae. aegypti* and *Ae. albopictus* population from the Sidomulyo Village research area of 65.2% (Ae aegypti) and 34.8% (Ae albopictus), while Tanjung Jaya urban Village was 40% (*Ae. aegypti*) and 60% (*Ae. albopictus*). A similar study was conducted at Havelock Island (Sivan et al., 2016) by comparison of the *Ae. aegypti* of 7.5% while *Ae. albopictus* of 58.3% of the 27 points of sample collection. A similar report was reported from Rawalpindi, Pakistan with *Ae. aegypti* population rtaio by 46% and *Ae. albopictus* of 54%, it was known that *Ae. albopictus* was dominant in many rural areas of forest in Indonesia (Arslan et al., 2016). *Ae. albopictus* population was getting bigger than the population of *Ae. aegypti* which caused *Ae. albopictus* has the potential to become a major vector of DHF.

CONCLUSION

The results showed that *Ae. albopictus* from endemic area (Sidomulyo Village and sporadic area (Tanjung Jaya Village) have started to experience resistance to insecticides malathion in thermal fogging as a DHF vector control program in Bengkulu

Table 5. $ERR_{50,90,99}$ of Ae. aegypti on 50 μg/ml insecticides malathion

Location	Estimated Resistance Ratio			Category
	ERR_{50}	ERR_{90}	ERR_{99}	
Endemic Area (Sidomulyo)	5.364	5.361	5.359	Tolerant*
Sporadic Area (Tanjung Jaya)	3.379	3.387	3.392	Vulnerable*

* ERR value (Selvi et al., 2010): < 5 (vulnerable), 5-10 (tolerant), >10 (resistant).

City through Bengkulu City Health Office which has been used for ± 27 years. Rotation of insecticide use in DHF vector control program is necessary and periodic evaluation to know the status of resistance in order to prevent the occurrence of resistance to insecticide and strategy of DHF vector control becomes more effective.

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