Susceptibility Status of *Culex quinquefasciatus* to Malathion in Brebes Regency, Indonesia

Husnatun Nihayah[1(✉)], Budi Mulyaningsih[2], and Sitti Rahmah Umniyati[2]

[1] Faculty of Mathematics and Natural Sciences, University of Jember, Jember, Indonesia
nihayah@unej.ac.id

[2] Departmen of Parasitologi, Faculty of Medicine, Public Health, and Nursing, University of Gadjah Mada, Yogyakarta, Indonesia

Abstract. Brebes Regency is a bancroftian filariasis endemic area. *Culex quinquiefasciatus* mosquito is accused as a vector transmitting bancroftian filariasis. The eradication of disease vector that has been widely performed in Indonesia relies on the use of malathion insecticide. The use of such insecticide in the long term might decrease susceptibility status for the targeted vector. The determination on the susceptibility status in *Cx. quinquefasciatus* in Brebes Regency should be pursued to monitor the insecticide use. The research was to elucidate the susceptibility status of *Cx. quinquefasciatus* on malathion insecticide and to review the activity of non-specific esterase enzyme *Cx. quinquefasciatus* that have been collected from endemic (Ketanggungan district) and nonendemic (Paguyangan district) bancroftian filariasis in Brebes, Central Java, Indonesia. The determination on the susceptibility status was performed by CDC bottle bioassay method on malathion with the diagnostic dose 400 μg/bottle. Then, the biochemical assay was conducted by performing Lee method with substrate α-naphthyl acetate to test the presence of activity on non-specific esterase enzymes. *Culex quinquefasciatus* mosquitos that had been collected from endemic area and non-endemic area were still susceptible to malathion; percentage of mortality is 100%. In addition, the biochemical assay showed increasing activity on non-specific esterase enzymes on both locations. This preliminary research reflects that there is decomposition of insecticides that enter the body which may lead to resistance. Therefore, further molecular research needs to be done to determine genetic mutation in *Cx. quinquefasciatus* in Brebes.

Keywords: Indonesia · Cx. quinquefasciatus · Malathion · Non-specific esterase enzyme

1 Introduction

Filariasis or elephantiasis disease is one of the vector infection diseases that have been found in many tropical areas throughout the world. In relation to the statement, Brebes Regency has been one of the filariasis-endemic areas in the Province of Central Java. The Incidence Rate (IR) of filariasis in 2013 has 1.35/100.000 population, it has increased from 2012, 1.32/100.000 population. The results of the microscopical test by the Brebes

A. Lelono et al. (Eds.): ICOLIB 2021, ABSR 27, pp. 560–571, 2023.
https://doi.org/10.2991/978-94-6463-062-6_57

Public Health Office in 2017 has found 37 patients of filariasis bancrofti. The region that has been categorized as the endemic area of filariasis bancrofti is Ketanggungan District with the number of patients that reaches approximately 10 people. On the other hand, the region that has been categorized as the non-endemic area of filariasis is Paguyangan District [1, 2].

Culex quinquefasciatus mosquitos (*Cx. quinquefasciatus*) is the main vector of *Wuchereria bancrofti* parasitic worm, the main cause behind filariasis bancrofti. The main habitat of *Cx. quinquefasciatus* is outdoor locations such as rice fields, pools, water channels, and water-containing wood or bamboo pieces [3]. *Culex quinquefasciatus* mosquitos are nocturnal; despite their main habitat is outdoor location, the biting habit of these mosquitos are also mostly found inside the house [4].

Vector control is one of the main preventive efforts toward the spread of vector infection disease including filariasis. Vector control that has been widely performed in Indonesia relies on the use of insecticide. Malathion insecticide has been in use since 1970; then, another insecticide that has also been in wide use is permethrin insecticide [5]. In Brebes Regency, organophosphate and pyrethroid-type insecticide have also been in wide use as agricultural insecticide [6]. The irregular use of insecticide in the long term might decrease the susceptibility status of the mosquito toward the insecticide.

Several researchers in relation to the susceptibility status in *Cx. quinquefasciatus* have been conducted. *Cx. quinquefasciatus* from Benin has shown resistance toward permethrin, deltamethrin, and DDT. In addition, the same finding has also shown the existence of mutation on the gen kdr and ace1 [7]. *Culex quinquefasciatus* from Yogyakarta have also experienced increasing activity on esterase enzyme that hydrolyzes α/β naphthyl acetate; as a result, these mosquitos become resistant [8]. In Brebes Regency, research in relation to *Culex* sp. Has just been recently conducted with regards to the prevalence of microfilaria on both the mosquitos and the individuals; the study took place in Dukuhturi Village, Bumiayu District, Brebes. A further study in relation to the state of *Cx. quinquefasciatus* susceptibility within the endemic areas and the non-endemic areas of filariasis bancrofti throughout Brebes Regency should be performed as one of the efforts to control the vector of filariasis bancrofti. In relation to the statement, the objective of the research is to review the susceptibility status in *Cx. quinquefasciatus* on 400 μg/bottle malathion insecticide and also to review the activity of non-specific esterase enzyme in *Cx. quinquefasciatus* larva that have been gathered from the endemic areas of filariasis bancrofti (Ketanggungan) and the non-endemic areas of filariasis bancrofti (Paguyangan).

2 Materials and Methods

2.1 Selection of Sampling Site

This study has obtained research permition by ethics committee of Faculty of Medicine, Public Health and Nursing Universitas Gadjah Mada with number KE/FK/0074/EC/2018. The sample *Cx. quinquefasciatus* mosquitos were collected from the endemic area of filariasis bancrofti namely Ketanggungan District; the representative villages from the district area were Ketanggungan Village, Dukuhturi, Karangmalang, Tanggungsari, Baros, and Cikeusal Lor, amount 70 ovitrap. This location is mostly rural

area except Ketanggugan village, its downtown. On the contrary, the sample *Cx. quinquefasciatus* were also collected from the non-endemic area of filariasis bancrofti namely Paguyangan District; the representative villages from the district area were Pakujati Village, Winduaji, and Kretek, its rural area. The number of ovitrap is 90 ovitrap, its more than endemic area to anticipate the small number of mosquitoes in this area.

2.2 Egg Collection

This study used mosquito egg ovitrap made of black glass. This ovitrap was contained atractan to *Culex* sp, one of these attractants contain *Eleuisina indica* grass fermentation. The fermentation of *Eleuisina indica* grass was created by mixing 30 g of fresh *Eleuisina indica* grass into 2 L of tap water; then, the solution was kept in 27 ± 2 °C for seven days [9]. These ovitraps were put outdor, o the edge f the house that protected from rain and human activities. These ovitrap was left for three days. Egg collection was carried out in March – June 2018. The eggs that had been trapped then were transported into the Laboratory of Parasitology Faculty of Medicine, Public Health and Nursing University of Gadjah Mada for undergoing colonization identification test.

2.3 Rearing of Field Caught Population of Cx. quinquefasciatus

The larva that had been gathered from the endemic area of filariasis bancrofti were fed by fish pellets, it contains fish meal, soybean flour, corn flour, wheat flour and bran. Meanwhile the larva that had been gathered from the non-endemic area of filariasis bancrofti were fed by chicken's food, it because endemic-area. The adult mosquitos were fed by sucrose solution 10% and rat blood. The rats were put in a small cage and left overnight in the mosquito cage. The colonization was conducted until the research attained the second generation of these mosquitos.

2.4 Biological Assay

Biological assay was conducted by performing CDC bottle bioassay method [10]. The dose of malathion insecticide that had been administered was 400 μg/bottle [11]. The biological assay was initiated by preparing five 250 ml-size glass bottles with their lids. Four bottles were contained by 1 ml of test solution with diagnostic dose and the remaining one bottle was contained by 1 ml acetone as control dose. The bottles that had been contained with the solution were rolled in order that the solution would widely distribute to the overall surface of the bottles. These bottles then were put in steady position overnight. The next day, these bottles were ready for being implemented in the test of susceptibility test. The biological assay was performed by putting 15 subject-test mosquitos from the second generation into each bottle. In this study use *Aedes aegypti* srain as control because unavailability of susceptible *Culex* sp. in the laboratory. *Aedes aegypti* wes treated with 50μg/bottle of malathion in 30 min. The diagnostic time for malathion insecticide was 45 min. Observation and record were performed every 5 min during the diagnostic time and were continued for the next 2 h in order to ensure there had not been any knockdown resistance. After 2 h, these mosquitos were transported into the recovery bottles and were abandoned for the next 24 h with the administration of sucrose solution 10%. The biological assay was repeated three times.

2.5 Biochemical Assay on the Enzymes' Activity of Non-specific Esterase

Biochemical assay was conducted by performing Lee method [12] using initial instar IV larva from the second generation (F2). The initial larva was crushed individually into homogenate and the crushed larva were solved into 0.5 ml Phosphate Buffer Saline (PBS 0.02 M solution). The homogenate was taken by using micropipette and the amount that had been taken was 50 μl; the homogenate that had been taken then were transported into microplate. Afterward, the homogenate was added by 50 μl substrate α-naphthyl acetate that contained 3 mg α-naphthyl acetate. The homogenate that had been added by the naphthyl acetate then was solved into 0.5 ml acetone and 100 μl of the homogenate was taken and was put into PBS until the benchmark 10 ml had been met. The homogenate then was put in a steady position for 60 s. Then, 50 μl coupling reagent material (30 mg fast blue B salt (o-dionisidine, tetrazoid, and sigma) into 7 ml SDS 5%) and 3 ml distilled water were added into each microplate and each microplate was abandoned for 10 min. The color gradually changed from red into blue and the reaction was stopped by adding 50 μl acetate acid 10% into each microplate. The final color that had been apparently described the activity on non-specific esterase enzyme qualitatively. The homogenate then was read by using microplate reader on the wavelength 450 nm.

2.6 Data Interpretation

The susceptibility status of *Cx. quinquefasciatus* was determined based on the percentage of mosquito mortality rate after the test of susceptibility status had been performed (10). The percentage of mortality rate achieved 98%–100% belonged to the "susceptible" category (SS), 80%–97% belonged to "Moderately Resistant/Tolerant" category (RS), and less than 80% belonged to "Resistant" category (RR).

The susceptibility status of *Cx. quinquefasciatus* based on biochemical assay on the enzymes' activity of non-specific esterase was determined based on the absorbance value (AV) score. Cut of point was attained from AV mean value of susceptible mosquitos + 3SD and a mosquito is considered RR if AV is > cut of point AV + 6 SD [13].

3 Results and Discussion

3.1 Egg Collection

The results that had been attained from the installation in the endemic area of filariasis bancrofti (Ketanggungan District) were as follows: there was 4,28% positive ovitrap units that had been found in Karangmalang village, Tanggungsari and Baros. *Culex* sp. Egg from Tanggungsari and Baros there was did not succeed hatch so in the next test just use Karangmalang mosquito. On the other hand, the percentage of positive ovitraps in nonendemic area is 7.78% that had been found in Pakujati and Winduaji (Table 1).

3.2 Biological Assay

The results of bioassay showed that *Cx. quinquefasciatus* mosquitos both from the endemic area (Karangmalang) and the non-endemic area (Pakujati and Winduaji) of

Table 1. Percentage (%) of egg-positive ovitrap from endemic and nonendemic area

No	Location	Number	Egg-positive ovitrap
1	Endemic area	70	3 (4.28%)
2	Nonendemic area	90	7 (7.78%)

filariasis brancrofti had still been susceptible toward malathion. The mortality rate of test-subject mosquitos achieved 100% (Table 2). Bioassay on susceptible mosquitoes aim to find out the insecticides used for biological testing are still of good quality (not expired). Therefore, susceptible mosquito biological assay can be carried out on susceptible mosquitoes of other types of the same insecticides that are used for mosquito bioassay testing of research samples.

Bioassay on susceptible mosquitoes in this study was conducted on susceptible *Aedes aegypti* mosquitoes from the parasitology laboratory of faculty of medicine, public health and nursing University of Gadjah Mada conducted by Triana [14] in the same year. The insecticides used for the test also came from the same insecticide, so the results of the tests carried out could be used by the researcher as a negative control. The test results are mosquito deaths reaching 98% (susceptible). This means that the malathion insecticide carried out for testing is still suitable for use.

3.3 Biochemical Assay on the Enzymes Activity of Non-specific Esterase

The results of biochemical assay for the activity on non-specific esterase enzyme 100% had shown the presence of increasing activity (Fig. 1; Table 3). The results of biochemical assay for the activity of non-specific esterase enzyme on the sampled larva from the three locations showed the presence of increasing activity on the enzyme. The increasing enzymes'activity was shown by the AV mean score that had been read. The AV mean score from Karangmalang (the endemic area of filariasis bancrofti) was equal to 1.304; the AV mean score from Pakujati (the non-endemic area of filariasis bancrofti) was equal to 1.005; and the AV mean score from Winduaji (the non-endemic area of fiilariasis bancrofti) was equal to 1.168. The AV mean scores from the three areas had been greater than the cut of point score that had been defined (0.1); as a result, the *Cx. quinquefasciatus* mosquitos from the three areas belonged into "Resistant" or have increased non-specific esterase enzyme activity.

Several researches that detected the susceptibility status in *Cx. quinquefasciatus* to malathion had been performed. The results of research by Kumar et al. [15] showed that *Cx. quinquefasciatus* mosquitos that were native of the endemic area of filariasis in Northern Ireland had been resistant toward malathion 5%. Yanola et al. [16] also reported the presence of resistance in Northern Thailand. Norris et al. [17] who conducted research on the state of *Cx. quinquefasciatus* mosquitos in Macha on malathion 100 μl/bottle by using CDC bottle bioassay method. The research by Norris et al. showed that the presence of resistance in the area Macha; in her research, Norris and her colleagues also found the presence of *kdr allele* on the population *Cx. quinquefasciatus* mosquitos.

Table 2. Susceptibility status of *Cx. quinquefasciatus* from endemic and nonendemic area on malathion

Location	Generation	Number of mosquitoes	Mortality after exposure (%)		Susceptibility status
			45 min	2 h	
Karangmalang (endemic)	F 2	180	100	100	SS
Pakujati (Nonendemic)	F 2	180	100	100	SS
Winduaji (Nonendemic)	F 2	180	100	100	SS
(Laboratory)*	F 1057	125	98	100	SS

* Using *Aedes aegypti* strain with same insecticide in 50 μg/bottle in 30 min

Kudom et al. [18] also reported that *Cx. quinquefasciatus* mosquitos and *Cx. Decans* mosquitos from Ghana had been highly resistant on deltamethrin 0.05% and DDT 4.00% but had been poorly resistant on permethrin 0.75%. The results of their study also displayed the presence of high activity on α-esterase enzyme and β-esterase enzyme, glutathione S-transferase enzyme, and also monooxygenase enzyme. Another researcher toward *Cx. Pipiens* that were native of Tehran, Iran, had been resistant to DDT 4.00% and several insecticides from pyrethroid type such as Lambda-cyhalothrin 0.05%, deltamethrin 0.05%, and cyfluthrin 0.15% [19].

Research by Chakim et al. [20] stated that moderate resistance to permethrin had already occurred among *Cx. quinquefasciatus* mosquitos that were native of Grobogan Regency but high resistance toward permethrin occurred among *Cx. quinquefasciatus* mosquitos that were native of Semarang Regency, Demak Regency, Jepara Regency, and Pekalongan Regency. A report from the research by Kumar et al. [15] stated that *Cx. quinquefasciatus* mosquitos had been resistant to permethrin 0.75%. Similar results were also reported from research in Benin that had been conducted by Yadouleton et al. [7]. Yanola et al. [16] also reported the presence of resistance in the area of Northern Thailand.

Resistance in general is divided into two categories namely behavioural resistance and physiological resistance. Behavioural resistance is defined as the behavioural changes on the population of a special as an individual response from the act of avoiding insecticide. On the other hand, physiological resistance is defined as the biochemical defensive mechanism within the body of an insect when the insect is in contact with the insecticide [21]. There are several mechanisms of resistance on insecticide. Liu [22] mentioned that there are two main mechanisms that cause the resistance on insecticide and these mechanisms are: increasing the metabolic detoxification of insecticide through gene overexpression or amplification and gen P450 structural mutation, esterase enzyme, and glutathione S- transferase (GST); and decreasing the sensitivity of

Table 3. AV mean score from the activity on non-specific esterase enzyme on α-napthyl acetate substrate

Location	Frequency (%) on non-specific esterase activity (AV)[a]		
	AV < 0,1[b]	AV = 0,1–0,115[c]	AV > 0,115[d]
Karangmalang	0	0	100
Pakujati	0	0	100
Winduaji	0	0	100
Susceptible strain	88,89	11,11	0
Resistant strain	0	0	100

a: A 450 nm; b: insecticide susceptible; c: possibly insecticide resistant that need to be confirmed; d: insecticide resistant

target protein namely AChEs receptor (organophosphate and carbamate), sodium channels (DDT and pyrethroid), and γ-aminobutyric acid/GABA (cyclodine and fipronil) on insecticide through modification or mutation.

Culex quinquefasciatus mosquitos from both areas are still susceptible to malathion 400 μL/bottle (mortality rate has achieved 100%) and it is enabled because the application of fogging has not been in use for more than 10 years.

As a result, although malathion insecticide has been in use in Indonesia since 1970s (> 45 years) fogging is still more effective to implement in the research site. However, the research toward malathion insecticide with graded concentration should be conducted to identify knockdown dose on *Cx. quinquefasciatus* mosquitos from each area. A study by Norris et al. [17] showed that *Cx. quinquefasciatus* mosquitos have been resistant toward malathion insecticide 100 μl/bottle that has been tested by using CDC bottle bioassay method. *Culex* spp, include *Cx. quinquefasciatus*, in the word has been known resistant to malathion [17, 23]-[27] but some areas know still susceptible to malathion [28].

Data from the Public Health Office in 2017 shows that Paguyangan district does not include endemic areas of bancroftian filariasis. But the result of the egg collection obtained *Cx. quinquefasciatus*, so that Paguyangan district does not include filariasis endemic area is possible due to absent of filariasis agent factor (*W. bancrofti*). *Culex* spp known find in agricultural area [29], swamp area [30] and also associated with turbid water, [31, 32]. However, Paguyangan district and Ketanggungan district are agricultural area, especially rice. However, based on community-based interviews at the sampling sites, non-endemic local communities filariasis bancrofti (Paguyangan district) also use household insecticide to control mosquitoes as well as local communities of endemic bancroftian filariasis. It is possible to cause resistance to *Cx. quinquefasciatus* in non-endemic bancroftian filariasis area (Paguyangan district) as well as in endemic bancroftian filariasis area.

In addition, the pyrethroid-type insecticide has also been widely used as agricultural insecticide so that there is undeliberate exposure by the agricultural insecticide. One of agricultural insecticides that have been widely used in the area of Brebes Regency is

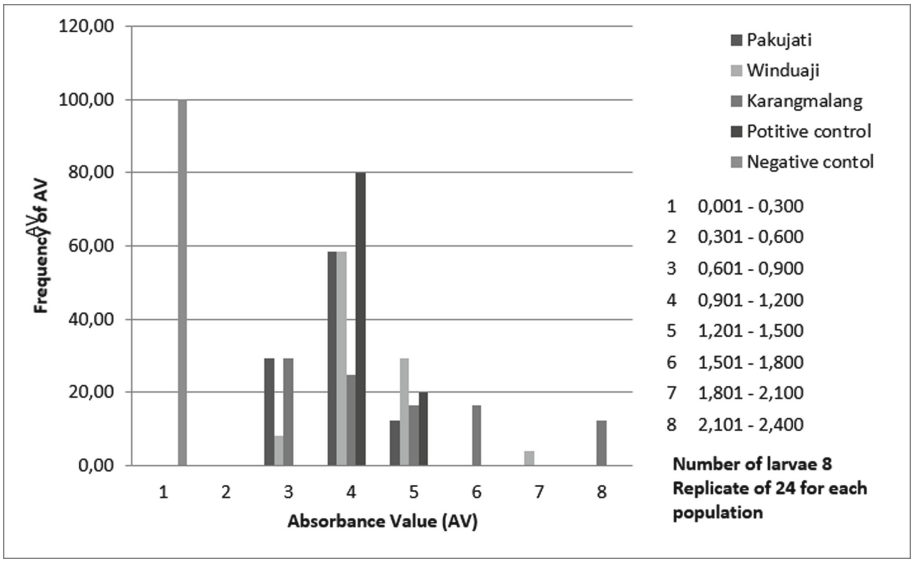

Fig. 1. Variation of AV values based on non-specific esterase enzyme activity against α naphthyl acetate subtrate of each larva.

the pyrethroid-type insecticide. The percentage on the use of land as agricultural site in 2016 based on the data from the Central Bureau of Statistics Brebes Regency [33, 34] in the endemic area of filariasis bancrofti (Karangmalang Village) is 34.97% of total village area width and in the non-endemic area of filariasis bancrofti (Pakujati Village and Winduaji Village) is 8.84% and 35.34% of total village area width respectively.

An increasing activity of non-specific esterase enzymes in this study could be due to hydrolyzing activity of other insecticides, besides malathion. Because sometimes non-specific esterase enzymes are also related to insecticides bendiocarb or permethrin. Like Widiarti et al. [35] research for the vector of malaria in the Province of East Java. The results of their research suggest that *Anopheles sundaicus* mosquitos have experienced increasing activity on non-specific esterase enzyme. The results of cross-sectional test under the impregnated paper WHO standards suggest that increasing activity on non-specific esterase enzyme has been heavily associated with bendiocarb 0.10% and malathion 0.50% insecticide. Different results are found in *Anopheles aconicus* mosquitos that have experienced increasing activity on non-specific esterase enzyme. The results of cross-sectional test under the impregnated paper WHO standards suggest that increasing activity on non-specific esterase enzyme has been heavily associated with permethrin 0.75% insecticide. However, from both species the presence of AChE insensitivity has not been found; thus, in other words, both species are still susceptible [35].

Another research was conducted by Pethuan et al. [36] in relation to biochemical assay on *Aedes aegypti* and *Aedes albopictus* in Thailand. The results of their research in general suggest the presence of increasing activity on MFO for the sample that has been resistant on pyrethroid. Several samples have shown resistance on pyrethroid-type

insecticide (deltamethrin and permethrin) and have also shown increasing activity on non-specific esterase enzyme.

The factors that might influence resistance are genetic, operational, and biological factors. Genetic factors are shown by the frequency of multiple factor gene and the frequency of specific gene. Operational factors are shown by the method of insecticide application. Last but not the least, biological factors are shown by size and characteristics of population from the vector [37].

The limitation of the study is the sample of *Cx. quinquefasciatus* mosquitoes that have been sampled for the testing are only taken from one endemic area and consequently the sample cannot be fully representative for Brebes Regency. The biological assay has also been conducted toward the field sample without any positive control sample and negative control sample. However, the results of this study provide new information that can be used for further research or policy consideration.

Culex quinquefasciatus mosquitoes from Brebes Regency Indonesia have still been susceptible to malathion, but there has been increasing activity on non-specific esterase enzyme. As part of suggestions from the research, it is expected that further research using WHO impregnated paper technique with variation dose to malathion and other insecticide.

Acknowledgments. The researchers would like to thank Brebes Public Health and Brebes Agricultural Office for their assistance through the provision of secondary data. The researchers would also like to thank the staff of Parasitology Laboratory of Faculty of Medicine, Public Health, and Nursing Universitas Gadjah Mada that have been helpful in the conduct of administration test.

Authors' Contribution. H.N., B.M., and S.R.U designed the experiment. H.N. performed the experiments and analyzed the data. H.N. wrote the first draft of manuscripts and B.M wrote the final version. All authors read and approved the final manuscript.

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