

RESEARCH ARTICLE

Detection of Polymorphism on Voltage-gated Sodium Channel Gene of Indonesian *Aedes aegypti* Associated with Resistance to PyrethroidsBudi Mulyaningsih^{1,*}, Sitti Rahmah Umniyati¹, Tri Baskoro Tunggul Satoto¹, Ernaningsih¹,
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Abstract

BACKGROUND: *Aedes aegypti* is a vector of several pathogens including dengue virus. Vector control is an effective way to break the transmission but unfortunately constant use of insecticides ultimately causes vector resistance. Pyrethroids have been used for about 15 years to combat *Ae. aegypti* in Yogyakarta Province, Indonesia. Single amino acid substitutions in the voltage-gated sodium channel associated with pyrethroid resistance constitute one of the main causative factors of knockdown resistance (kdr). The aim of this study is to detect resistant status to cypermethrin and polymorphism on the voltage-gated sodium channel gene of *Ae. aegypti* from 2 dengue endemic areas in Yogyakarta Province (Yogyakarta city and Sleman district).

METHODS: Pyrethroid resistance in *Ae. aegypti* mosquitoes was detected by using CDC Bottle Bioassay. To detect the polymorphism on the voltage-gated sodium

channel gene of *Ae. aegypti* analyses were conducted by using PCR and direct DNA sequencing with primers AaSCF1 and AaSCR4 for S989P, I1011M (or V), L1014F sites, and AaSCF7 and AaSCR7 for the F1534C site.

RESULTS: According to bioassay, the results for *Ae. aegypti* from Yogyakarta city (93% mortality) and Sleman district (88% mortality) suggest the possibility of resistance to cypermethrin. We observed polymorphism on voltage-gated sodium channel gene on site F1534C (heterozygous).

CONCLUSION: The findings provide early evidence that the use of cypermethrin (pyrethroids) in Yogyakarta city and Sleman district, Yogyakarta Province, Indonesia is reducing its effectiveness to control *Ae. aegypti*. Recommendations include additional tests for confirmation.

KEYWORDS: *Aedes aegypti*, dengue virus, cypermethrin, Bioassay, Yogyakarta

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Introduction

Aedes aegypti (Linnaeus, Diptera: Culicidae) is the urban mosquito that transmits several pathogens including dengue virus in many tropical countries. The incidence of dengue and dengue haemorrhagic fever (DHF) is suspected to continue to increase in the near future.

This mosquito is highly anthropophilic, with markedly endophilic and endophagic behaviors; these characteristics are directly related to its high efficiency as a disease vector. (1,2) In the absence of a vaccine or effective therapeutic medications, vector control remains the only available strategy to control and prevent dengue transmission. (1) Physiological resistance to insecticides often involves metabolic resistance either mutations in the insecticide

target site (target-site resistance). Metabolic resistance to pyrethroid is usually associated with increased cytochrome P450 activity.(3)

Pyrethroids constitute a group of chemicals that are structurally modified by natural pyrethrum, and are composed of two main types. Most of the first generation pyrethroids are knockdown agents, which possess high knockdown activity but low killing activity; the second generation pyrethroids possess high killing activity. The use of knockdown agents such as d-allethrin and metofluthrin, which generally exhibit low stability in the environment as “spatial repellents” is biorational, because it causes low selection pressure on insect populations, thereby minimizing the development of physiological resistance. On the other hand, killing agents such as permethrin, deltamethrin, cypermethrin, and l-cyhalothrin generally exhibit high photostability, and this enables their outdoor use in agriculture and their predominant application as vector control agents.(4) However, the high stability and killing efficacy of pyrethroids has accelerated the development of pyrethroid resistance in vector mosquitoes.(5)

Resistance to photostable pyrethroids is a major problem for the vector control program. Pyrethroid resistance involves two main mechanisms, namely, enhanced metabolic detoxification and insensitivity of target sites. Single amino acid substitutions in the voltage-gated sodium channel (VGSC), known as *kdr* mutations, constitute one of the main causative factors of pyrethroid resistance.(4) This *kdr*-type resistance has been observed in *Ae. aegypti*.(6)

Mutations in segment 6 of domain II of the VGSC (I1011M, I1011V, V1016G and V1016I) were reported to play important roles in pyrethroid resistance of *Ae. aegypti*.(6,7,8,9) A F1534C mutation was detected in segment 6 of domain III in DDT/permethrin-resistant *Ae. aegypti*.(10,11) The F1534C mutation was strongly correlated with resistance to DDT and pyrethroid.(12) The S989P mutation in domain II of the VGSC, which occurs in deltamethrin-resistant *Ae. aegypti*, is another principal *kdr* mutation that regulates pyrethroid resistance in mosquitoes.(13) Yanola, *et al.*, reported the existence of the F1534C mutation in *Ae. aegypti* from Yangon City, Myanmar.(11) Kawada, *et al.*, reported detailed genetic analyses of point mutations in the VGSC of *Ae. aegypti* colonies in Myanmar.(4) However, no other genetic studies of point mutations in the VGSC of *Ae. aegypti* in Yogyakarta Province, Indonesia have been performed.

The objectives of the study were to detect pyrethroid resistance status in *Ae. aegypti* collected from Yogyakarta city and Sleman district, Indonesia, and look for any

polymorphism in the VGSC gene. The targets were the four most frequent amino acid replacements in S989, I1011, L1014 and V1016, all of which are located in the area of segment 6 of domain II and also a recently identified amino acid replacement at F1534, located in the area of segment 6 of domain III.

Methods

Before starting this study, we have obtained ethical approval from the Medical and Health Research Ethics Committee, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia.

Mosquito Collection

Ae. aegypti mosquitoes of indigenous strain of Yogyakarta Province, Indonesia were subjected for the assays. They were collected from one site at two different dengue endemic areas, Yogyakarta city and Sleman district through indoor ovitraps surveys.

Colonization

The collected eggs were colonized in the laboratory to get adult stage. Morphology identification was done to adult mosquitoes to confirm the presence of *Ae. aegypti* species. Colonization of the mosquitoes were continued to obtain the F1 generation of adult stages. The same qualifications were also applied to mosquitoes from the Parasitology laboratory, Medical Faculty, Public Health and Nursing, Universitas Gadjah Mada Yogyakarta, Indonesia as negative control.

Bioassay

The bioassay followed the procedures for the Centers for Disease Control and Prevention (CDC) Bottle Bioassay using adult female mosquitoes. According to this method, the diagnostic dose of cypermethrin was 10µg/bottle, whereas diagnostic time of cypermethrin was 30 minutes. Sample size was 100 mosquitoes for the initial test and 25 mosquitoes for the negative control.(14) The bioassay were replicated 3 times, total numbers of mosquitoes are 375. The bioassays were conducted on the same day, in a room under optimum conditions ($t=26^{\circ}\text{C}\pm 2^{\circ}\text{C}$; $\text{RH}=68\pm 4\%$) for all experiments.

Molecular Assay

To identify the presence of polymorphism at S989, I1011, L1014, V1016 and F1534 in *Ae. aegypti*, we used polymerase chain reaction (PCR) and direct DNA

sequencing. (4) The mosquito samples were lightly dried on a paper towel and placed in a 1.5 mL PCR reaction tube. The DNA was extracted from mosquito pools using DNA Mini Kit Geneaid™ Cat No. #GB100. Lot No. JM02202 following the manufacture's instructions. Amplification was accomplished in 25 µL volumes, using the protocols supplied by Go Taq Green Master Mix (Promega, Wisconsin, USA). The solution was heated at 95°C for 3 minutes and neutralized. Initial amplification was carried out using the primers AaSCF1 (AGACAATGTGGATCGCTTCC) and AaSCR4 (GGACGCAATCTGGCTTGTTA) for S989P, I1011M (or V), L1014F and V1016G (or I); or AaSCF7 (GAGAACTCGCCGATGAACTT) and AaSCR7 (GACGACGAAATCGAACAGGT) for F1534C. The PCR mixture contained 4 mL of REExtract-N-Amp ReadyMix (Sigma), 0.5 mM of each primer, and 1 mL of the DNA template in a total volume of 10 mL. The PCR was performed under the following conditions: initial denaturation at 94°C for 3 minutes; 35 cycles each of 94°C for 15 seconds, 55°C for 30 seconds and 72°C for 30 seconds and a final elongation step at 72°C for 10 minutes. The amplified fragments of the expected size were purified with ExoSAP-IT (USB Corporation, Cleveland, OH, USA) at 37°C for 30 minutes, and then 800C for 15 minutes. The DNA sequencing was carried out by First Base Laboratories. The chromatogram analysis were performed using free software Chromaslite® version 2.6.4 (Technelysium Pty Ltd, South Brisbane, Australia). Sequences were aligned using BLAST nucleotide at <http://www.ncbi.nlm.nih.gov/blast/>.

Results

The results of bioassay with cypermethrin (pyrethroids) at the CDC diagnostic dose and its diagnostic time for *Ae. aegypti* adult mosquitoes from Yogyakarta city and Sleman district are given below in Table 1.

Table 1. The mean of percentage mortalities in the CDC bottle bioassay of adult female of *Ae. aegypti* from Yogyakarta city and Sleman district for evaluating insecticide resistance to diagnostic dose and diagnostic time of cypermethrin.

CDC Bottle Bioassay	Mosquitoes Mortality Percentage (%)	
	Yogyakarta City	Sleman District
I	93	92
II	90	90
III	96	82
Mean	93±2.45%	88±4.32%

*diagnostic dose: 10ug/bottle; **diagnostic time: 30 minutes.

At diagnostic dose of 10 µg/bottle an diagnostic time 30 minutes, mosquitoes mortality percentage were 93±2.45% and 88±4.32% for the mosquitoes from Yogyakarta city and Sleman district respectively. Further study was done as an effort to know the mechanism of the decrease sensitivity of the mosquitoes in Yogyakarta city and Sleman district towards cypermethrin (pyrethroids) using PCR technique followed by sequencing of VGSC gene.

The PCR assay with the primers AaSCF1 (AGACAATGTGGATCGCTTCC) and AaSCR4 (GGACGCAATCTGGCTTGTTA) showed specific DNA bands with the size of 619bp (Figure 1 A) and with primers AaSCF7 (GAG AAC AAC ATG CCG TCG TT) and AaSCR7 (GAC GAC GAA GAA CAG ATC GT) showed specific DNA bands with the size of 748bp (Figure 1 B). The specific DNA band indicates the development of resistance in *Ae. aegypti* mosquitoes to pyrethroid insecticide.

The results of sequencing analysis VGSC gene of *Ae. aegypti* from Yogyakarta city and Sleman district with primers AaSCF1 and AaSCR4 (accession No. AB914689 and AB914690) no polymorphism on sites S989, I1011, L1014 and V1016. Sequencing analysis VGSC gene of *Ae. aegypti* from Yogyakarta city with primers AaSCF7

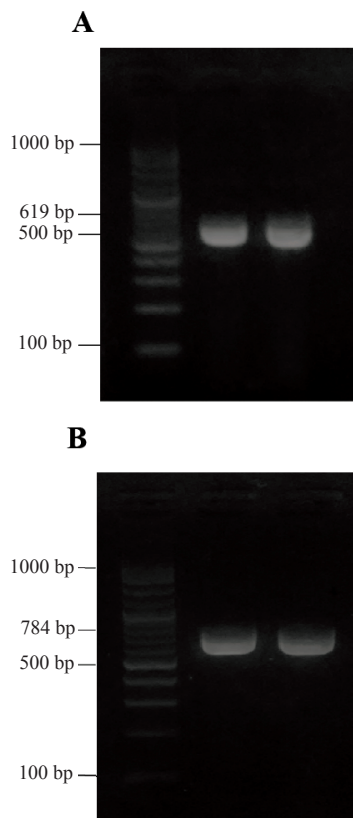


Figure 1. Amplification DNA genom of *Ae. aegypti* from Yogyakarta city and Sleman district, Indonesia.

and AaSCR7 (Accession No. AB914687 and AB914688), showed no polymorphism on site F1534, whereas *Ae. aegypti* from Sleman district showed polymorphism on site F1534 (heterozygous). The data are given below in Table 2.

Discussion

Pyrethroid insecticides were used for dengue control in Yogyakarta Province since about 15 years ago and have been constantly used till now. This intensive use may explain the strong selection pressure toward *Ae. aegypti*, because this mosquito is generally a domestic and endophagic species with a preference for indoor breeding. The widespread use of chemical insecticides has led to the development of insecticide resistance in several insects including mosquitoes.(15)

Insecticide resistance is often a complex dynamic interplay of several mechanisms. The most resistance mechanisms can be divided into two groups, metabolic (alterations in the levels or activities of detoxification proteins), and target site (mutations in the sodium channel, acetylcholinesterase and gamma-aminobutyric acid (GABA) receptor genes). Alone or in combination these mechanisms confer resistance, sometimes at an extremely high level, to all of the available classes of insecticides. (16) Resistance mechanism in *Ae. aegypti* from Yogyakarta city maybe due to metabolic resistance (alterations in the levels or activities of detoxification proteins). Metabolic resistance to pyrethroid is usually associated with increased cytochrome P450 activity.³

The World Health Organization (WHO) recommendations for assessing the significant chance of detected resistance is 98%-100% mortality at the recommended diagnostic time indicating susceptibility, 80%-97% mortality at the recommended diagnostic time

suggesting the possibility of resistance, which needs to be confirmed, and less than 80% mortality at the recommended diagnostic time suggesting some resistance.(14) According to WHO's recommendation, *Ae. aegypti* from Yogyakarta city and Sleman district, Indonesia suggest the possibility of resistance to cypermethrin, which needs to be confirmed. Where <95% mortality occurs at the diagnostic time in bioassays that have been conducted under optimum conditions and with a sample size of >100 mosquitoes, then resistance can be strongly suspected.(14) This study conducted under optimum condition ($t=26^{\circ}\text{C}\pm 2^{\circ}\text{C}$; RH=68±4%), and the sample size were 300 mosquitoes. These data may provide early evidence that cypermethrin (Pyrethroids) is losing its effectiveness.

Yogyakarta city and Sleman district are neighboring districts, and therefore the two areas share environmental, sociological, and biological similarities. As a result, study to clarify polymorphism on VGSC gene between the two *Ae. aegypti* population in Yogyakarta city and Sleman district, which may have exchanged or developed introgression of genes, is required.

The amino acid changes in *Ae. aegypti* mosquitoes were most frequently observed at the points S989, I1011, L1014 and V1016, which are located in segment 6 of domain II. Current identification indicates that the amino acid replacement in F1534 is located in the segment 6 of domain III.4 The existence of the VGSC gene mutation plays a role in the development of resistance.

In this study the VGSC gene polymorphism occur on site F1534 (heterozygous), whereas no polymorphism was seen on sites S989P and V1016G in *Ae. aegypti* from Sleman district. After the first description of the newly identified F1534C point mutation in *Ae. aegypti* collected in Thailand, the same mutation was reported in Vietnam, Brazil, Venezuela, Madeira Island, Portugal and Grand Cayman Island in UK.(4) In that study, findings determined

Table 2. Sequence analysis of segments 6 domain II and III of the voltage-gated sodium channel gene of *Ae. aegypti* from Yogyakarta city and Sleman district, Indonesia.

Polymorphism of the gene	Segments 6 domain II of <i>Ae. aegypti</i> from Yogyakarta city	Segments 6 domain II of <i>Ae. aegypti</i> from Sleman district	Segments 6 domain III of <i>Ae. aegypti</i> from Yogyakarta city	Segments 6 domain III of <i>Ae. aegypti</i> from Sleman district
S989P	S989 / S989	S989 / S989	-	-
I1011M/V	I1011 / I1011	I1011 / I1011	-	-
V1016G/I	V1016 / V1016	V1016 / V1016	-	-
F1534C	-	-	F1534 / F1534	F1534 / F1534C*

*Polymorphism of the gene F1534 / F1534C (heterozygous).

a moderate frequency of this point mutation in Yangon City (21.2%), this frequency was of a comparable level to that recorded in Vietnam (average, 21.6%) but lower than the frequencies recorded in Thailand (59-79%), Grand Cayman Island (68%) and Venezuela (100%).(4) Elucidation of the worldwide distribution of the F1534C mutation in *Ae. aegypti* and *Aedes albopictus* will provide a valuable insight into DHF epidemiology, and yield useful information for vector control programs. In the present study, were detected three patterns of cooccurrence of point mutations, namely, V1016G/S989P, V1016G/F1534C, and V1016G/F1534C/S989P. The S989P mutation has always been linked to the V1016G mutation, however, V1016G has sometimes been found in the absence of S989P.(15) The cooccurrence of V1016G and S989P enhanced the resistance of *Ae. aegypti* to deltamethrin.(17)

The *Aedes* mosquito control unit was established in 1968, and several feasibility studies aimed at the development of *Ae. aegypti* control methodology, including insecticide treatment, biological control by using larvivorous fish, environmental improvement, and health education, were undertaken.(18) However, current information regarding insecticide use for DHF vector control in Indonesia is lacking, and few studies concerning the insecticide resistance of DHF vectors have been published.

Pyrethroids represent one of the most promising countermeasures for controlling malaria, DHF and other arthropod-borne diseases. Currently, there are no suitable chemical substitutes for pyrethroids, and therefore pyrethroid resistance will continue to be a major problem for vector control programs.(19) The development of new chemicals with novel modes of action, which can be substituted for conventional insecticides, is essential. Transitional life-prolonging measures for conventional 1st generation pyrethroids that belong to the photo-unstable knockdown agent groups and are used as “spatial repellents”, rotational use of plural insecticides with different mode of actions, and basic biochemical and genetic research to support the above strategies, are crucial to the effective management of insecticide resistance.(20,4)

Conclusion

Ae. aegypti from Yogyakarta city and Sleman district, Yogyakarta Province, Indonesia are suggests the possibility of resistance to cypermethrin, which needs to be confirmed. The VGSC gene was observed on site F1534 showed polymorphism (heterozygous) in *Ae. aegypti* from Sleman

district. The findings provide early evidence that the use of cypermethrin (pyrethroids) in Yogyakarta city and Sleman district, Yogyakarta Province, Indonesia is losing its effectiveness to control *Ae. aegypti*. Recommendations include additional tests to observe cytochrome P450 activity.

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