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*Article*

# The role of Voltage-Gated Sodium Channel (VGSC) gene mutations in the resistance of *Aedes aegypti* L. to pyrethroid permethrin in Palembang and Jakarta, Indonesia

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**Abstract:** *Aedes aegypti* mosquito is a vector that could transmit various pathogens, such as viruses, bacteria, and parasites. Several human diseases transmitted by *Ae. aegypti* mosquito are dengue fever (DHF), Chikungunya, Yellow Fever and Zika. The occurance of resistance to various insecticides, including pyrethroid, is a current problem faced by various countries. In this research, a WHO bioassay test on Palembang and Jakarta *Ae. aegypti* was conducted using 0.25% permethrin pyrethroid insecticide. VGSC gene fragments associated with pyrethroid resistance (L982, S989, I1011, L1014, V1016 and F1534) of resistant and sensitive strains were amplified and analyzed. The test showed the presence of resistance in *Ae. aegypti* isolates from Palembang and Jakarta. From the results of VGSC gene fragment analyses, it was known that there were mutations (S989P and/or V1016G) on isolates from Palembang and (S989P and/or V1016G) on resistant isolates from Jakarta.

**Keywords:** *Aedes aegypti*; Insecticide Resistance; Pyrethroid; Permethrin; VGSC gene.

## 1. Introduction

*Aedes aegypti* is a mosquito which able to transmit various pathogenic diseases such as viruses, bacteria and parasites which are also called a vector (mosquito borne disease). Various human diseases transmitted through the bite of *Ae. aegypti* mosquitoes is still a health problem in many countries, especially tropical countries like Indonesia. These diseases included dengue fever, Chikungunya, Yellow Fever and Zika. Chemical insecticides were used to suppress the transmission incidence rate in which considered to provide concrete results and short in reducing mosquito population. However, the use of insecticides inappropriately and without any rotation of insecticide types with different modes of action can ultimately have a negative impact, such as causing the death of non-target organisms, causing environmental pollution, and may cause problems of vector resistance to insecticides. Since the case of Organochlorine DDT insecticide resistance in 1947, up until now has reported many resistances to various types of insecticides with different mechanisms of action,including the Pyrethroid [1]. Based on various publications, *Ae. aegypti* known to develop resistant in many countries such as Myanmar[2], Taiwan [3], Thailand [4] and Brazil [5,6]. In addition, resistance to pyrethoride in Indonesia is also reported in Bandung [7,8], Palu, Makassar [8], Central Java [9,10], Central Kalimantan [11], Yogyakarta [12], and Denpasar [13].

The resistances incidence which occurred could be caused by one or several mechanisms of resistance at once. Those mechanisms were including, behavioural resistance, penetration resistance, metabolic resistance and altered target-site resistance [1]. Nowadays, studies of metabolic analysis and altered target-site resistance have been widely conducted. Enzymes are known to play a role

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in *Ae. aegypti* metabolic resistance against pyrethroid is the detoxification enzyme carboxylesterases (CCE), glutathione S-transferases (GST) and cytochrome P450 monooxygenases (P450) [14]. The altered target-site resistance to pyrethroids is known to be similar in mechanism to OC in the presence of single nucleotide polymorphisms (SNPs) in the Voltage-gated sodium channel (VGSC) gene.

Reportedly there were various kdr-mutation on different amino acids on Domain II Segment 6 on Ae. eegypti resistance to pyrethroid. They also appeared in 1016 amino acids which substituted from valine to glycine (V1016G) or isoleucine (V1016I). The next position was in 1011 of the isoleucine substituted into methionine (I1011M) or valine (I1011V) (15,16). In addition, 1023 also reported to occur substitution of valine to glycine (V1023G) [3,15] and in 989 there was a serine substitution into proline (S989P) [17]. In domain II S5 reported a kdr mutation with substitution of glycine into valine in amino acids 923 (G923V). Furthermore, between the attachment of SII and SIII at position 982 there is also known leusin substitution to tryptophan (L982W) [15]. In the Domain III Segment 6 reported substitution of 1534 phenylalanine to cysteine (F1534C) in Ae. aegypti resistan DDT and pyrethroid in Myanmar. Mutations are also found in Thailand in amino acids 1269 and 1552 which is substituted from phenylalanine to cysteine (F1269C & F1552C) [4]. In the adjacent of Domain IV S5 and S 6 on 1794 was known there was a mutation on the aspartate acid substituted into tyrosine (D1794) [3].

## 2. Results

### *2.1. Insecticide susceptibility tests*

*Aedes aegypti* isolates from Palembang and Jakarta did not experience death at the time of observation every 5 minutes for 60 minutes exposure of permethrin insecticide. Furthermore, there was no death at 24 hours recovery time after permethrin exposure. In contrast to them, Laboratory Control Mosquitoes for 60 minutes of permethrin exposure showed individual deaths in all test tubes (mortality = 100%) with varying time. Based on test results interpreted by WHO formula, it is known that Ae. aegypti isolates from Palembang is resistant to permethrin because mortality is <90%, so is the status of *Ae. aegypti* Jakarta. However, in Laboratory Control, mortality reached 100% so it is said to be sensitive to permethrin with a 60-minute death time so it is said to knocked-down (Figure 1).

**Figure 1.** Knockdown assay results in *Aedes aegypti*, Cumulative mortality of *Ae. aegypti* from Palembang, Jakarta, and Laboratory.

### *2.2. VGSC Gene Mutation*

Based on the result of VGSC gene sequencing there is a point change (SNP) at 989 and 1016 codons. At codon 989, there is a mutation point of TCCCCC so that the serine amino acid is substituted into phenylalanine (S989P). Codon 1016 found the GTAGGA point mutation, the change caused the substitution of valine to glycine (V1016G). At codons 982, 1011, 1014 and 1534 no point mutations are found (Figure 2-4). We can see the pattern of mutations in *Ae. aegypti* isolates from Palembang, Jakarta and Laboratory Control (Table 1). On *Ae. aegypti* Palembang, it is known that there is 1 phenotype pattern that is resistance with 3 haplotype patterns that is LSILVF (n = 1), LSILGF (n = 1) and LPILGF (n = 17); while Ae. aegypti Jakarta, there is 1 phenotype pattern that is resistance but there are only 2 haplotype patterns that is LSILG (n = 5) and LSILV (n = 14). In laboratory controlled mosquitoes that have sensitive phenotypes have 2 slightly different patterns in codon 982 that is the heterozygote TTG / TTA and homozygous TTA. From the table it can be seen also that on *Ae. aegypti* that has an LSILVF haplotype will show 90.91% of the phenotype is sensitive to permethrin. In addition, on *Ae. aegypti* phenotypically resistant is known to be 100% of haplotype LSILGF and LPILGF with OR = infinite (Table 2).

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**Figure 2.** VGSC DNA fragment codon 982, 989, 1011, 1014 and 1016 of Ae. aegypti from Palembang (P), Jakarta (J) and Laboratory (L). Bold = exon.

**Figure 3.** VGSC DNA fragment codon 1534 of *Ae. aegypti* from Palembang (P), Jakarta (J) and Laboratory (L). Bold = exon.

**Figure 4.** Electropherogram of the DNA sequencing of VGSC gene fragment from Ae. aegypti. (**A**) indicate S989P mutation and (**B**) indicate V1016G mutation.

**Table 1.** VGSC mutation gene pattern in *Ae. aegypti* from Palembang, Jakarta, and control group.

**Table 2.** VGSC allele and their association with resistance to permethrin.

## 3. Discussion

Mutations which were found at S989P and V1016G on Ae. aegypti resistant to piretroid were in line with the results of previous findings from various countries [5,15–17]. Those findings also aligned and showed the same pattern with findings in Indonesia. Based on study by Sayono et. al, S989P and V1016G mutations were reported in *Ae. agypti* strain of Semarang, Kudus, Jepara and Surakarta. In the study also known that there is a mutation distribution of V1016G which was considerably fast, it occurred within 10 years period of time before the previous findings [9,15]. This was due to continuous exposure to pyrethroids. Also on the study highlighted F1534C mutations in some individuals, and not found in this study. The results of this study had the same pattern with a study conducted in Denpasar. In the study, mutations were found at S898P and V1016G without F1534C mutation. It could be presumed that F1534C mutations were not as widely distributed as S898P and V1016G, but there was a strong correlation to pyrethroid resistance in Indonesia which needed further assessment [13]. Study of VGSC gene mutation in *Ae. aegypti* Palembang has been done previously by Ghiffari et al. with PCR. In that study, it was found the mutation on V1016I and not on V1016G [19]. It showed difference in result with this study, by sequencing, it showed there was a mutation on V1016G which was the substitution of amino acid GTA->GGA. Meanwhile, for the strain of Jakarta there has been no previous research which could add as a reference pattern of piretroid resistance in *Ae. aegypti* in Jakarta.

Mutation of S989P could cause the decreasing of sensitivity level of the VGSC channel due to mutation location which located in the intracellular mouth of the canal according to Srisawat et al. [17] In other research finding, mutation of S989P in Ae.aegypti was always associated with a V1016G mutation, which not always could be associated. There are some findings that stated there was a mutation of S989P, but there was no mutation of V1016G. It could be assumed that mutation of S989P could induce additional mutations therefore the resistance to pyrethroids was also increased [15,17]. However, in this study it was known that the mutation of S989P was always followed by a mutation of V1016G which caused a phenotypic resistance, but there was also a mutation of V1016G without S989P which could lead to phenotypic resistance. The mutation of V1016G was known to play an important role in the process of resistance to pyrethroid [3,15]. This was due to the many finding that showed mutations of V1016G in Thailand [17], thus it could be concluded as the most common mutation happened in *Ae. aegypti* which resistance to pyrethroid. However, this mutation was not found in Latin America. This was most likely due to other mechanism which took place.

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In the other codons which covered by primers 982, 1011 and 1014, no mutations were found. Mutation of the amino acid leucine into phenylalanine was commonly found in codon 982, but in this study, was not found. Mutations of L982W were found in many insects such as *Anopheles gambiae*, but was not reported on *Ae. aegytpi*. This was probably due to substation of amino acid Leu->Phe on the mutation point was impossible to take place[15], but has been reported in Brazil [5,6]. This suggested that the mutation of 1011 did not contribute to the pyrethroid resistance in Indonesia. Mutation of codon 1014 itself was known to occur more frequently in Culex and Anopheles. Based on a study by Syafruddin et al., it was known that there was a mutation of codon 1014 on *An. sundaicus*, *An. aconitus*, *An. subpictus* and *An. vagus* from South Lampung, Indonesia [20].

In this study there were also one mosquitoes which showed a phenotypically resistant, but the mutation was not found on all the analysed codons. This could be due mutation which occurred in different point of mutation which was not reachable by the primer, such as mutations of G923V [15], F1269C [21], D1794Y20 [3,15], V1023G/I [3,12,15], F1565C and S996P [12] or mutations that have not been found on various findings. One of the example, in the study conducted by Wuliandari et al., it was known that the mutation of V1023C was associated with resistance of Ae. aegypti Yogyakarta, Indonesia against type I pyrethroid and mutation of V1023C with S996P associated with resistance to type II piretroid [12]. In addition, there were other resistance mechanisms that could also affected the phenotype of *Ae. aegypti* such as thickening of cuticle hence the penetration of insecticide would be disturbed; and increased of expression of pyrethroid-related detoxification enzymes such as GST, carboxylesterase and P450 [1,14]. Therefore, further study was urgently needed.

## 4. Materials and Methods

### *4.1. Mosquito Collection*

*Aedes aegypti* Palembang obtained by modification of ovitrap as the chosen sampling method. The sampling was done in 100 different points on 26 Ilir urban-village, Bukit Kecil sub-district, Palembang City, South Sumatera Province. Ovitrap installation was carried out at 100 different points which covered four different community neigbourhood, which were number 03, 19, 20, and 21. Point selection was based on previous DHF cases, the position of the house, the cleanliness of the environment, and the cooperation of the homeowner. Ovitrap was installed in mosquito breeding places inside and outside the residents' homes. Aedes aegypti Jakarta obtained from collection of Entomology Laboratory, Department of Parasitology, Faculty of Medicine, University of Indonesia. Aedes aegypti for control group was obtained from the Entomology Laboratory, Faculty of Veterinary Medicine, Bogor Agricultural University.

### *4.2. Insecticide susceptibility tests*

The susceptibility of mosquitoes to pyrethroid insecticides was tested by insecticide-impregnated papers based on the kit and standard protocol issued by WHO [18] which was obtained from Universiti Sains Malaysia. Permethrin paper 0.25% was used for the treatment group and PY Control was used for the control group. Six test tubes were used for each strain which contained 25 F3 female mosquitoes, non–blood feed, 3–5 days old, non-defective wings, able to fly, and the number of legs were still complete. Four tubes were exposed to permethrin paper and two other tubes were used as control. Observation of death due to the treatment was done every five minutes during exposure period and five hours after exposure as well as at the end of recovery time. All the test was done in a conductive room with room temperature ranged between 26-28 Celcius and air humidity ranged from 65-80%. The resistance status was determined by looking at mortality according to WHO guidelines.

### *4.3. DNA Extraction and Amplification*

Homogenate and mosquito DNA isolation was prepared using GENEzol $^{TM}$ Reagent of Geneaid kit. One ml of GENEzole $^{TM}$ reagent was inserted into the microtube which contained the sample

and homogenized using a sterile plastic tissue grinder until disintegrated. The sample than later incubated for five minutes at room temperature which later transferred to a 1.5 mL RNAse-free microtube. Furthermore DNA isolation was performed using standard GENEzol ™ Reagent of Geneaid protocol. Individual DNA was isolated from the mosquito obtained from the bioassay. Amplification of the VGSC gene was performed by PCR method using a VGSC-specific gene primer based on Martins et al. [5], to amplify exons 20-21 with codon coverage of 982, 989, 1011, 1014 and 1016 (Forward AaF20_kdr: 5'-ACAATGTGGATCGCTTCCC-3 'and Reverse AaR21_kdr: 5'-TGGACAAAAGCAAGGCTAAG-3 '; while the codon 1534 on exon 31 was amplified using a specific primer used by Harris et al., (Forward AaEx31P: 5'-TCGCGGGAGGTAAGTTATTG-3' and Reverse AaEx31Q: 5'-GTTGATGTGCGATGGAAATG-3 ') [2]. The 473 bp DNA fragment for 982, 989, 1011, 1014 and 1016 codons were amplified in total reaction of PCR 25 microl using KapaTaq DNA Polymerase (KAPA BIOSYSTEMS), with 2.5 microL DNA templates, 10x PCR Buffer, 50 mM MgCl2, 10mM dNTP, 40 pmol primary forward, 40 pmol primary reverse, 5 U/microL KAPA Taq Polymerase and ddH20. The reaction was carried out with PCR conditioned with exon 20-21 (codon 982, 989, 1011, 1016) at 95C for 5 minutes pre-denaturation; followed by 40 cycles for 30 minutes denaturation at 95C, 30 seconds annealing at 58C, and 30 seconds polymerization at 72C; lastly, 5 minutes post-polymerization at 72C. PCR condition for exon 31 (codon 1534) at 95C for 5 minutes pre-denaturation; followed by 40 cycles for 30 seconds denaturation at 95C, and 30 seconds annealing at 58C. A total of 5 microL of each PCR products of VGSC gene fragment were visualized using 2% agarose gel (SeaKem® LE Agarosa, LONZA) with a 100 pb ladder marker. Electrophoresis was run at 70 V for 45 minutes. Furthermore, agarose gel was visualized using Gel Doc XR (Bio-Rad). Sequencing is performed based on the automated DNA sequencer procedure (ABI 3730xl DNA sequencer 96 capillary). The sequenced DNA sequencing results were analyzed through the BLASTN program to confirm the VGSC gene sequence on Ae. aegypti. Furthermore, the sequence is aligned with the reference sequence of the *Ae. aegypti* VGSC gene that has been published in GenBank (access number KU728155 for exon 20-21 and KM677279 for exon 31) using BioEdit Sequence Alignment Editor ver. 7.2.6.1. program.

## 5. Conclusions

*Aedes aegypti* from Palembang and Jakarta are highly resistant to pyrethroid insecticides, especially permethrin. S989P and V1016G codons mutation on VGSC gene are either alone or a combination which play an important role. The overall findings are expected to provide additional biological information towards the future use of *Ae. aegypti* as a vector control strategy thus the transmission of diseases could be suppressed.

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**Conflicts of Interest:** The authors declare no conflict of interest.

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