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Short Communication: Genotyping of *kdr* allele in insecticide resistant-*Aedes aegypti* populations from West Sumatra, Indonesia

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Abstract. *Hasmiwati, Supargiyono. 2018. Short Communication: Genotyping of* kdr *allele in insecticide resistant-*Aedes aegypti *populations from West Sumatra, Indonesia. Biodiversitas 19*: *502-508. Aedes aegypti*, the primary vector of Dengue fever, is widely distributed in Indonesia including in West Sumatra. Chemical insecticide is an effective way of shedding the chain of Dengue fever transmission. Long-term applications of insecticides have resulted in the development of resistance in *Ae. aegypti* populations. Knockdown resistance (*kdr*) allele as a result of point mutations has been found in Voltage-Gated Sodium Channel (*VGSC*) gene. This study aimed to design allele-specific primers to detect the *kdr* allele mutations. *Ae aegypti* samples were collected from several dengue endemic areas in West Sumatra. The susceptibility of adult mosquitos to insecticides was determined by bioassays using impregnation paper test with 0.18% malathion and 0.75% permethrin. In this study, we successfully confirmed that the A. populations have point mutations in the *VGSC* gene corresponding to S989P and V1016G amino acid substitutions. To genotype S989P and V1016G alleles, we designed *kdr* allele-specific primers based on the sequence of *VGSC* gene (NCBI Accession number No. EU399179.1. PCR analysis using the *kdr* allele-specific primers successfully identified the genotype of *Ae. aegypti* populations resistant to malathion and permethrin in West Sumatra.

Keywords: *Aedes aegypti*, resistance, primer design, AS-PCR, *kdr* mutation

INTRODUCTION

Dengue Hemorrhagic Fever (DHF) case always increases every year in West Sumatra, Indonesia, indicating the unsuccessful program in controlling *Aedes aegypti* as the dengue vector. In Padang, there were 1.626 cases of DHF in 2012 with a Case Fatality Rate (CFR) of 0.6% or equivalent to 10 deaths. In 2013-2014, dengue case decreased from 998 to 666 cases, but then elevated to 1126 cases in 2015, noted that Padang city as the region with the highest case of DHF compared with 18 other endemic cities/districts in West Sumatra in 2006-2015 (Health Office of West Sumatra Province 2015).

Appropriate dose, drug, target and coverage of insecticides application can control the dengue vector. Conversely, improper dosage and timing of application cause resistance to the vector (Ministry of Health Republic of Indonesia, 2010). Setiawan and Fikri (2014) suggested that the 30-years long usage of insecticide Temephos might contribute to the dengue vector resistance toward the insecticide. Determination of insecticide resistance involves bioassays, fixed concentrations of insecticides, and calculation of time exposure; the data was reported as a percentage of insect death or Knock Down effect (KD). World Health Organization (WHO) established a standard dose of diagnosis for each insecticide (Corbel and N'Guessan, 2013) in which if an insecticide application only results in<80% mortality of experimental insects of an insect population then such condition is categorized as an insecticide resistance and the insecticide cannot be used for vector control (IRAC 2011).

The widespread presence of disease vectors increases the difficulty for controlling vector-borne diseases. Resistance mechanism to insecticides has a biochemical basis: (i) Target site resistance occurs when the insecticide can no longer bind the target. (ii). Detoxification enzyme-based resistance occurs as a result of increased enzyme activity for example esterase, oxidase, or glutathione-S-transferase (GST) degrade insecticides prior to reaching the target site (Brogdon et al. 1998).

Early detection of vector susceptibility status against insecticides is important in selecting the insecticide to control a local specific vector. Vector resistance to insecticides can be detected by (i) susceptibility test as a conventional detection method using the WHO standard, (ii) biochemical or enzymatic assay, and (iii) molecular assay. The principle of molecular assay to detect resistance vector is by detecting mutations in the insecticide target gene. Important target genes such as *Voltage-Gated Sodium Channel* (*VGSC*) and *Acetylcholinesterase* (*AceI*) genes occurred due to the selective suppression of organophosphate and pyrethroid insecticides (French-Costant et al. 2004). Pyrethroid insecticides work by binding to voltage-gated sodium channels (*VGSC*) of neurons. They bind preferentially to open channels. Bound sodium channels then remain in the open, activated state

which leads to repetitive nerve firing, which in turn leads to a loss of control and uncontrolled activity. The target insect experiences convulsions and is unable to maintain normal flight behavior. However, if certain point mutations within the *VGSC* gene are present, the resulting amino acid transversion may greatly decrease the sensitivity of the sodium channel to pyrethroid binding. It may also alter the conformation of the sodium channel to an extent that it remained closed and inactivated (Davies et al. 2007).

Mechanism of resistance is important to observe to allow the detection of changes or mutations in the *VGSC* gene. Molecularly, insecticide resistance-associated mutation in *VGSC* gene is a change of one nucleotide. Our study developed an analytical DNA-based method using PCR on specific *VGSC* alleles to determine the presence or absence of mutations in the target vector gene. The purpose of this study was to detect and identify mutations in allele V1016G and allele S989P *VGSC* gene associated with the resistance of *Ae. aegypti* to insecticides in West Sumatra, Indonesia.

MATERIALS AND METHODS

Sample collection

Seven districts and cities in West Sumatra known as endemic areas of dengue were selected as the study location. Larvae and adult *Aedes aegypti* mosquito were collected from100 houses in each location and kept in Parasitological Laboratory, Faculty of Medicine, Andalas University, Padang, Indonesia.

Susceptibility test

The susceptibility test was performed with bioassay method. Temephos postanal (an organophosphate insecticide) 250 mg 97.5%, (Sigma-Aldrich) with 0.02 mg/L dosage of diagnostic standard (WHO recommendation) was applied on 20 larvae (3rd or early 4th instars) in a glass container. Another 20 larvae in the other bottle were treated as control. Larval mortality was recorded 24 h after the treatment. For adult mosquitoes, the susceptibility test is done by treating 20 adult mosquitoes aged two days by an impregnated paper containing0.8% malathion and 0.25% permethrin on that has been full of glucose. Observations were performed for 60 minutes on the impregnated paper, and mosquitoes were transferred into insecticides-free tubes. The assay was repeated four times to minimize measurement error. Larval mortality classification was according to WHO (2016): (i). 99-100% mortality = Vulnerable. (ii) 80-98% mortality = tolerant/needs verification. (iii) Mortality<80% = resistant.

Identification of point mutation in *Ae. aegypti VGSC* gene sequence

To identify point mutations in the *VGSC* gene, the following analysis was conducted: (i) Isolation of DNA genome from *Ae. aegypti* larvae from the previous bioassay test using an isolation kit of Invitrogen following the protocol provided by the manufacturer. (ii) Amplification of *Ae. aegypti VGSC* gene using primers Aed3 F; 5'ACT ACA TCG GAA TGT GGA TCG 3' and Aed2 R: 5'TTG GTG TGG TGC GTT GGC CGT CGG 3' by PCR following the protocol of Marcombe et al. (2012). The positive DNA bands obtained from the PCR were purified for sequencing. DNA Sequences were analyzed by using BioEdit v7.0.5 (Ibis Therapeutics, Carlsbad). An SNP analysis was done using Geneious version 7.0.6 (Biomatters Ltd, Auckland, New Zealand) to identify the mutation of the allele on *VGSC* gene.

Design of *kdr*allele-specific primers

Primers for the specific mutated *kdr*alleles of *Ae. aegyptiVGSC* gene was designed using Geneious's version 7.0.6 based on *Ae. aegypti VGSC* reference sequence (NCBI Accession EU399179.1).

Detection of mutation by *kdr* allele-specific primer

The *kdr* allele-specific primers designed were tested to detect the respective point mutations in *VGSC* gene identified in this study.

RESULTS AND DISCUSSION

Larvae bioassay

Aedesaegypty larvae from populations collected in seven districts and cities in West Sumatra have been resistant to temephos, malathion, permethrin, and alpha-cypermethrin. Larvae bioassay results were conducted to identify resistant mosquitoes by detecting mutations in gene encoding *VGSC*, a target of pyrethroid insecticides.

In the bioassay, larvae and adult mosquitoes exhibiting<80% mortality rate upon insecticides treatment were established as resistant. (Health Office of West Sumatra Province (2015) reported that *Ae. aegypti* populations have been resistant to insecticides Bukittinggi and Pesisir Selatan, but they were still tolerant to temephos in Padang city. Different results of larval bioassay in seven locations in West Sumatra may be due to many aspects; different typology of the endemic area, different kind of insecticides and larvicides used in each area, and varied application of insecticides with different intensity for adult mosquitoes.

Table 1. Resistance status of *Ae. aegypti* against to various types of insecticides in several districts/cities in West Sumatra

Location	Mortality rate (%)			
	Temephos (%)	α Sipermetrin (%)	Permetrin (%)	Malathion (%)
Dharmasraya	73***	10***	97,5*	33***
Batusangkar	100*	100*	10***	0***
Solok	100*	90**	68,33***	0***
Pariaman	75***	100*	20***	15***
Payakumbuh	100*	90**	66,7***	15***
Padang	86,7***	22,5***	5***	0***
Bukittingi	5***	5***	0***	0***
Control	100*	5***	20***	0***

Note: Larval susceptibility criteria: (i). 99-100% mortality = Vulnerable (*). (ii) 80-98% mortality = tolerant/needs verification (**). (iii) Mortality <80% = resistant (***)

Temephos is a larvicide of organophosphate group. Malathion, permethrin, and alfa-cypermethrin are insecticides of pyrethroid group, for adult mosquito. These insecticides are widely used by people and government for mosquito control program. Uncontrolledutilization of insecticides developed resistance in the mosquito. The mechanism of resistance caused by organophosphate is metabolic resistant; insecticide is degraded by enzymes before reaching its target site. Resistance to pyrethroid is due to the presence of mutation in the gene encoding for*VGSC*, the target of pyrethroid. Mosquito resistance data from different locationsareneeded from time to time for improving the vector control management strategy, and sequencing data are available to confirm mutations in *Ae. aegypti VGSC* gene in several districts/cities in West Sumatra.

Molecular assay

Isolation of larval and adult mosquitoes DNA fragment was done by using a pair of primer; Aed3 F and Aed2R, yielded 579 bp *VGSC* gene fragment. Electrophoresis of the PCR products in 1.5% agarose gel is presented in Figure 1. The DNA sequence analysis obtained showed two point mutations; at codon 898, the 2985th nucleotide, a change from TCC (Serine) to CCC (Proline), of *VGSC* peptide sequence (S989P mutation), and at codon 1016, the 3067th nucleotide, a change from GTA (Valine) to GGA (Glycine) of *VGSC* protein, a V1016G mutation. The sequences of the amplified VSGC gene from *Ae. aegypti* analyzed in this study are shown in Figure 2.

Our result was in line with the findings of Marcombe et al. (2012) in Martinique, France, Kuswah et al. (2015) in India, and Li (2015) in Latin America, Southeast Asia, and China which described *Ae. aegypti VGSC*T-to-C and T-to-G point mutations corresponding to S989P and V1016G amino acid substitutions. Different results were reported by Widiarti et al. (2012) in Simongan, Semarang city, who detected a point mutation that resulted in a substitution in 1014th amino acid from leusin (TTA) to phenylalanine (TTT), and Ghiffari et al. (2013) in Palembang who identified a V1016I point mutation. These different results are due to the use of different specific primers and different sequence targets. Several point mutations have been found in *Ae. Aegypti VGSC*: I1011M (ATA to ATG) (Daborn et al. 2002), I1011V (ATA to GTA) (Yanola et al. 2011), F1552C (TTC to TGC) (Kawada et al. 2009), F1534C (TTC to TGC) (Yanola et al. 2011), V1023G (GTA to GGA) (Lima et al. 2011), V1016G (Srisawat et al. 2010; Kasai et al. 2011), and F1023C (TTC to TGC) (Kasai et al. 2011).

Our findings corroborated the previous reports on different point mutations in *VGSC* gene. Primer design is important to identify the knockdown resistance (*kdr*) allele using specific primers by allele-specific PCR (AS-PCR) method.

Detection of mutation by *kdr* allele-specific primers

Based on the sequencing analysis, we identify two point mutations in *VGSC* gene of *Ae. aegypti* population tested in this study, i.e., S989P and V1016G. We then designed specific primer pairs for the respective mutations. The length of amplification products and characteristics of the primers are shown in Table 2 and Table 3.

VGSC sequences primers are designed by using Bioinformatic version 7.0.6. Details on the primers including the primer attachment position and SNP position are presented in Figure 4. These primers were used for the AS-PCR for *VGSC* gene. Primers covered a portion of exon region containing the two SNPs; S989P (TCC to CCC) and V1016G (GTA to GGA). The length of amplification product was specific for *kdr* allele.

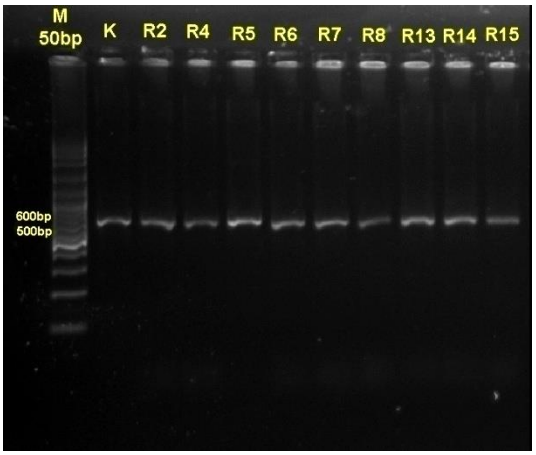
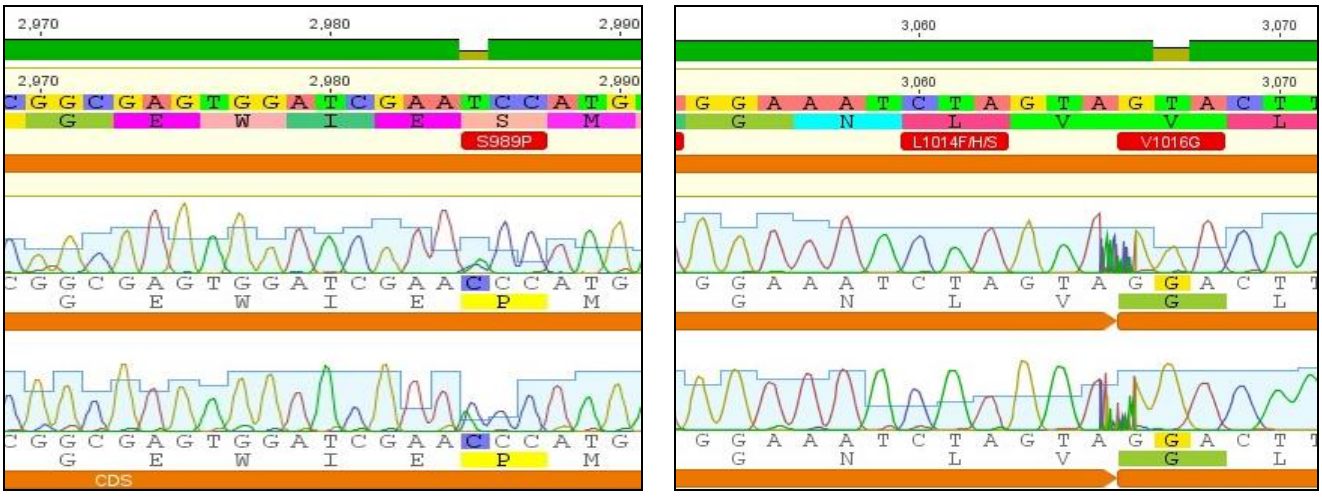


Figure 1. Electrophoresis of *VGSC* PCR products in 1.5% agarose gel. The amplified DNA band size are between 500-600bp. (M, DNA ladder; K, control group; R, samples).

Table 2. Results of primer design and size of amplification product for *VGSC* Gene

Mutation	Forward	Reverse	Allele	Size (bp)	Sequences
S989P	*VGSC* For	S989-Rev	T	108	GGACAAAGACCTGCCACG AGCATACAATCCCACATGGA
	S989P For	V65C-Rev	C	405	CGGCGAGTGGATCGAAC CGACAGCGAGGATGAACC
V1016G	V6SC For	V1016-Rev	G	445	GGACAAAGACCTGCCACG GCAAGGCTAAGAAAAGGTTAACTC
	V1016G Rev	V1016-For	T	74	CGAAGGCTAAGAAAAGGTTAAGTC TTTCCCACCCGCACAGGT



A B

Figure 2.A. Electrophoregram of *VGSC* gene segment from several *Ae. aegypti* populations. A change of base sequence from TCC to CCC corresponding to S989P mutation. B. Electrophoregram of *VGSC* gene segment from several *Ae. aegypti* populations. A change of base sequence from GTA to GGA corresponding to V1016G mutation

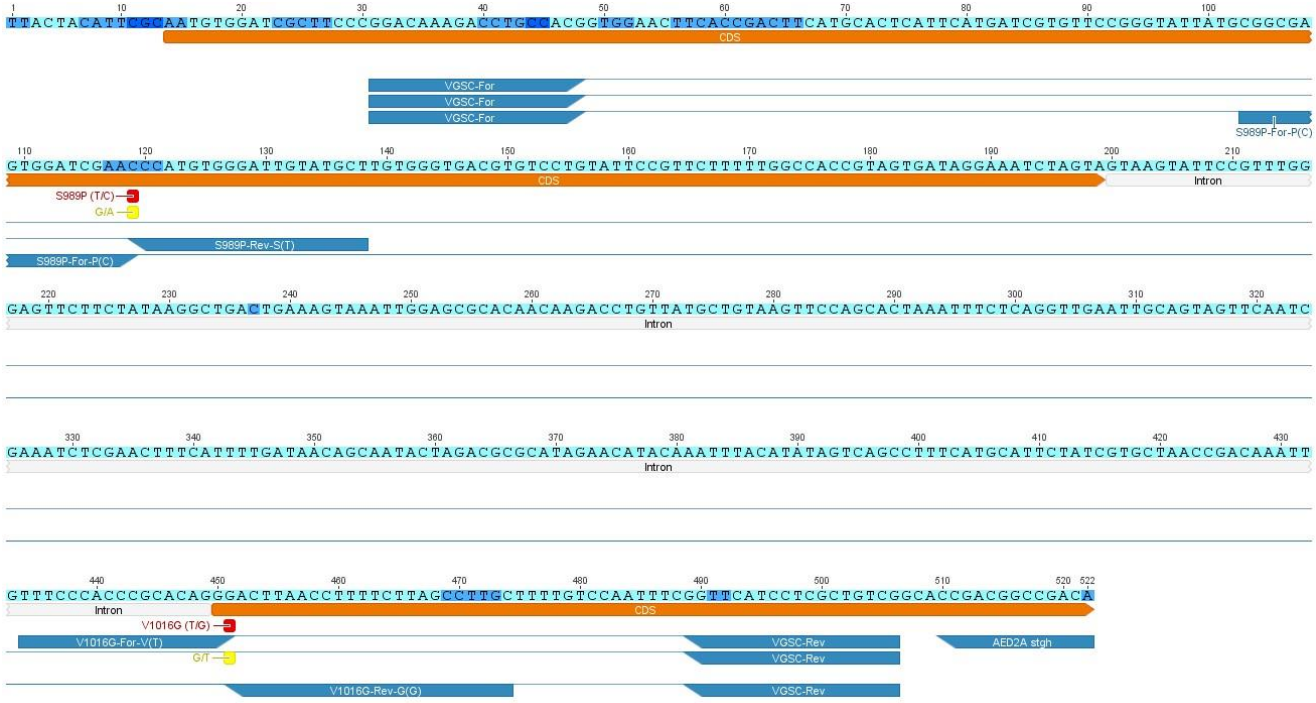
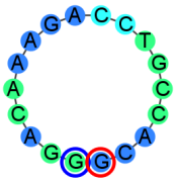
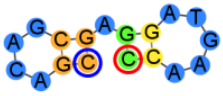
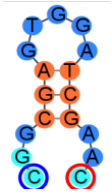
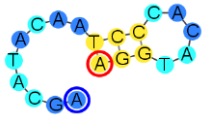
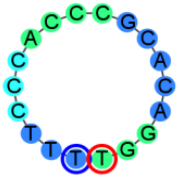
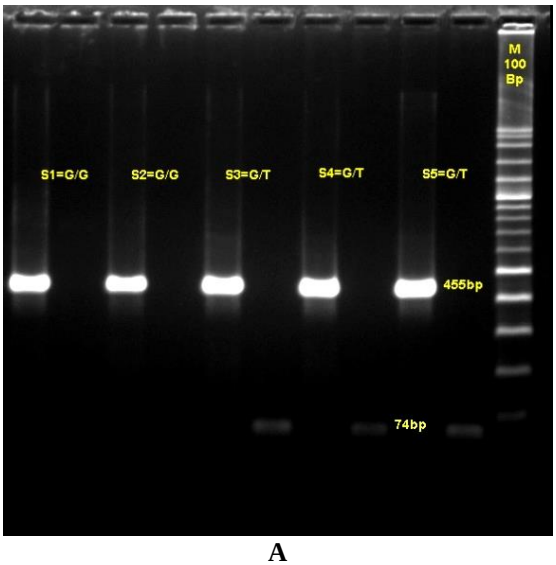


Figure 4. Primer attachment of *kdr* allele-specific primers (S989P and V1016G mutations) in *Ae. Aegypti* VSGS gene. Amplification results were separated by electrophoresis to verify the size of PCR products. The exact product consisted of two fragments of 445 bp and 74 bp for V1016G, and three fragments of 477 bp, 405 bp and 108 bp for S989P. PCR amplification using the specific primers successfully detected S989P (TCC to CCC) and V1016G (GTA to GGA) *kdr* alleles of *Ae. Aegypti* VSGS gene

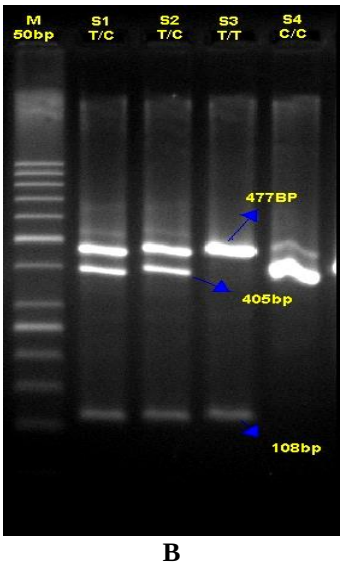
Table 4 presents the percentage of the genotype frequency of the insecticides-resistant *Ae.aegypti* in all the study locations. Higher frequency in heterozygous and homozygous mutants in comparison with that of the wild-type suggested that the occurrence of resistance will arise more quickly in the future because of the protective mechanism of the mosquitoes. Protective mechanism depends on genetic factor; single or recessive, semi-dominant or dominant, in the process of offspring. If the heterozygous alleles appear, the initial resistance process rarely occurs in a population, but survived-heterozygous when mating with other heterozygous generates mutant homozygous mutant with stronger resistance to insecticides. If the homozygous mutant becomes dominant, the resistance spreads rapidly in a population a; it is due to the ease of *Ae.aegypti* to adapt to its environment (David and Gilles 2002).

Tabel 3. Characteristics of the primers covering S989P and V1016G mutations in *VGSC* gene

Characteristic of forward primer	DNA folding	Characteristic of reverse primer	DNA folding
VGSC-For Sequence (5'-3'): GGACAAACACCTGCCACG Type: Primer Length: 18 Created by: primer3 %GC: 61.1 Tm: 58.3 Hairpin Time: None Self Dimer Time: None		*VGSC*-rEV Sequence (5'-3'): CGACAGCGAGGATGAACC Type: Primer Length: 18 Created by: primer3 %GC: 61.1 Tm: 57.9 Hairpin Time: 40.8 Self Dimer Time: None	
S989P-For-P (C) Sequence (5'-3'): CGGCGAGTGGATCGAAC Type: Primer Length: 17 Created by: primer3 %GC: 64.7 Tm: 57.9 Hairpin Time: 55.6 Self Dimer Time: None		S989P-Rev-S (T) Sequence (5'-3'): AGCATACAATCCCACATGGA Type: Primer Length: 20 Created by: primer3 %GC: 45.0 Tm: 56.6 Hairpin Time: 50.5 Self Dimer Time: None	
V1016G-For-V (T) Sequence (5'-3'): TTTCCCACCCGCACAGGT Type: Primer Length: 18 Created by: primer3 %GC: 61.1 Tm: 61.5 Hairpin Time: 42.2 Self Dimer Time: None		V1016G-Rev-G (G) Sequence (5'-3'): GCAAGGCTAAGAAAAGGTT AAGTC Type: Primer Length: 20 Created by: primer3 %GC: 41.7 Tm: 58.3 Hairpin Time: None Self Dimer Time: None	



A



B

Figure 5. Electrophoresis of PCR products amplified using mutation-specific primers S989P (TCC to CCC) (A) and V1016G (GTA to GGA) (B)

Table 4. Genotype frequency of insecticides-resistant *Ae. aegypti* in West Sumatra using *kdr* allele-specific primers for S989P (TCC to CCC) mutation

Ae. aegypti resistant to	Genotype (%)			
	Wild-type (TT)	Hetero-zygous mutant (CT)	Homo-zygous mutant (CC)	Total
Malathion (n=61)	27.8	21.32	50.81	100
Permethrin (n=20)	5	55	40	100
Alphacypermethrin (n=35)	17.15	60	22.85	100
Temepos (n=24)	20.83	58.34	20.83	100

Table 5. Percentage of *VGSC* genotype in *Ae. aegypti* VSGC gene allele S989P (TCC to CCC) with malathion treatment from several districts/cities in West Sumatra

Types of allele	Location of sampling		
	Darmasraya (%) (n=10)	Pariaman (%) (n=10)	Laboratory (%) (n=10)
Mutant (CC)	70	30	90
Heterozygous (CT)	20	40	10
Wild-Type (TT)	10	30	0
Total	100	100	100

Genotypes of the tested *Ae. aegypti* identified using our primer-specific PCR assay were normal homozygous T/T (wild-type), homozygous C/C mutant, and heterozygous C/T (Table 4). The T-to-C mutation (S989P) causes a substitution from Serine into Proline of the VSGC peptide sequence, and renders the mutant resistant to insecticides.Pyrethroidhijacked the Voltage-Gated Sodium Chanel (*VGSC*), interfering mosquito nervous system (Davies et al. 2007). Previous reports have shown that point mutations in the gene encoding *VGSC* are associated with the *Ae. aegypti* resistance toward pyrethroid (Stenhouse et al. 2013). In this present study, we successfully detected the *kdr* allele of *VGSC* gene from *Ae. aegypti* populations in West Sumatra using AS-PCR technique.

Discussion

Currently, the control of *Ae. aegypti* larvae by the government are done by using temephos for larvae, malathion, cypermethrin and alpha-cypermethrin for adult mosquitoes, especially in towns and in dengue-endemic areas in West Sumatra. A technical meeting of *The National Resistance Monitoring of Ae. aegypti* (MoReNAa) in 2006 has determined that vector control programs in cities with *Ae. aegypti* resistance ratio >3 should use alternative insecticides with different mechanism of action to reduce the pressure of exposure selection (Ministerio da Saude 2006). For adult mosquitoes, insecticides for dengue vector eradication program are 0.8% malathion, 0,05% cypermethrin, lambda-0,05% cyhalothrin, 0,1% bendiocarb, 0,05% deltamethrin, 0,5% etofenprox, 0,25% and 0,75% permethrin, 0,05% deltamethrin, 4% DDT, and 0,005% alpha-cypermethrin (National Health Research and Development Agency 2013).

Our AS-PCR could effectively distinguish homozygous mosquitoes from the heterozygous ones by specifically amplifying the *kdr* alleles of S989P and V1016G of *VGSC*, a gene that encodes the voltage-gated sodium channel, the target of pyrethroid insecticides. The length of PCR product amplified using these genotyping primers were 445bp and 74bp for the V1016G allele; and 477bp, 405bp, and 108bp for the S989P allele. Diagnostic AS-PCR to detect V1016G and S989P mutations by using these primer sets has been verified and a sequencing process was necessary to verify the nucleotide substitution occurred in the amplified products. *Ae. aegypti* populations should be monitored continuously to evaluate the resistance of the mosquito to all insecticides. By diagnostically genotyping the resistance genes of *Ae. aegypti*, we can select and recommend effective insecticides to control a particular *Ae. aegypti* population in a specific location. In addition, the society needs to continuously improve their life style to allow a better control of *Ae. aegypti* and the whole area effectively.Further study needs to be done to detect other possible resistance mechanisms in *Ae. aegypti*.

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