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V1016G Point Mutation: The Key Mutation in the Voltage-Gated Sodium Channel (*Vgsc*) Gene of Pyrethroid-Resistant *Aedes aegypti* (Diptera: Culicidae) in Indonesia

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Abstract

Resistance to pyrethroid insecticides is widespread in Indonesian *Aedes aegypti* (Linnaeus), the primary vector of dengue viruses. This study aims to investigate the mutations in the voltage-gated sodium channel (*Vgsc*) conferring pyrethroid resistance against *Ae. aegypti* populations from Indonesia. Molecular genotyping of mutations using polymerase chain reaction assay and direct DNA sequencing were performed at positions 989 and 1,016 in IIS6 region, and 1,534 in IIIS6 region of the voltage-gated sodium channel (*Vgsc*) in nine populations of Indonesian *Ae. aegypti*. The V1016G and S989P genotyping identified the RR genotype to be predominant in six out of nine populations of *Ae. aegypti*, whereas the SS genotype occurred only in minority. Interestingly, co-occurrence of the V1016G and S989P mutations was detected in the aforementioned six populations with high frequency. Genotyping of F1534C showed all nine populations exhibited the SS genotype, with merely two individuals from a population were heterozygous (RS). Significant correlations were demonstrated between the allele frequencies of the V1016G mutation and the survivability rates as well as resistance ratios in pyrethroid adult bioassays. This signifies the V1016G can contribute more to the insensitivity of *Vgsc* than the F1534C. Homozygous 1016G mosquitoes were likelier to survive pyrethroid exposure. Identification of underlying mechanisms resulting in insecticide resistance is advantageous in developing effective mosquito control programs in Indonesia.

Key words: mosquito, Indonesia, knockdown resistance, pyrethroid, voltage-gated sodium channel

The life-threatening mosquito *Aedes aegypti* (Linnaeus) is the primary vector of dengue fever, with an ~2.5 billion people reside in dengue-endemic countries. The absence of anti-dengue drugs and vaccine lay stress upon the importance of chemical insecticides as the most effective resort in vector control in the course of a dengue outbreak. To suppress the populations of dengue vector below the threshold level in causing disease transmission, heavy reliance of chemical insecticides in several forms such as household insecticide products or space sprays are indispensable. Leading the insecticide market with a high share, pyrethroids constitute the majority of chemical insecticides used by the government or households in Indonesia (Ahmad et al. 2007). Due to the quick knockdown effect on insects, low mammalian toxicity and fast degradation rate in the environment (Katsuda 1999), pyrethroids are routinely used even when diagnosis of an outbreak is unavailable. The role of pyrethroids in vector control is indisputably dominant in the country. However, numerous dengue-endemic regions are combating the issue of pyrethroid resistance because of rigorous use of these chemicals. Pyrethroid resistance in *Ae. aegypti* highly threatens public health when this issue has been documented worldwide including Southeast Asia (Amelia-Yap et al. 2018a). Elucidating the insecticide resistance mechanism is utterly crucial in designing a scheme of vector control which stresses on long-term sustainability.

The issue of insecticide resistance development is aggravated when local reports accentuated escalating resistance in *Ae. aegypti* to a wide range of pyrethroids, including permethrin, lambda-cyhalothrin, cypermethrin, delta-methrin, and D-allethrin in Indonesia (Brengues et al. 2003; Wuliandari et al. 2015; Hamid et al. 2017a,b; Amelia-Yap et al. 2018b). Since metabolic detoxification did not comprehensively explain the pyrethroid resistance in some Indonesian *Ae. aegypti* from a past study (Amelia-Yap et al. 2019), this study aimed to fill the research gap. One of the most well-studied resistance mechanism in pyrethroid-resistant *Ae. aegypti* is the amino acid substitution in the voltage-gated sodium channel (*Vgsc*), the target site of pyrethroids. This mechanism, known as knockdown resistance (*kdr*), causes resistance by exerting insecticidal effect from pyrethroids to interrupt the function of *Vgsc* in mosquitoes and stop repolarizing of action potentials, which subsequently results in incapability of maintaining normal flight behavior. There are at least 10 mutations have been identified in sodium channel of *Aedes* that cause channel insensitivity to insecticides (Du et al. 2016, Amelia-Yap et al. 2018a, Lien et al. 2018). In Indonesia, previous studies have identified the V1016G mutation (valine to glycine transversion) as the most common *kdr* mutation within the *Ae. aegypti* gene, which triggered pyrethroid resistance. Since resistance to insecticides is affected by environment (Lima et al. 2011), the extensive geographical area of Indonesia (34 provinces) spanning Indian and Pacific oceans may cause differences in susceptibility profile of *Ae. aegypti* against pyrethroids. However, screening of *kdr* in *Ae. aegypti* from past research efforts has largely focused on populations from Java Province.

In this study, we investigated the mutations in the *Vgsc* that might be important in conferring resistance against pyrethroids in nine populations of *Ae. aegypti* from Indonesia via molecular approaches (polymerase chain reaction [PCR] and direct DNA sequencing). This would thereby acknowledge the prevalence of the three commonly occurred amino acid substitutions in Southeast Asia, namely the V1016G, S989P, and F1534C mutations in knockdown resistance (*kdr*) gene in different strains of Indonesian *Ae. aegypti*. The results obtained were used to assess the associations between the frequency of resistance alleles and the degree of resistance phenotype of *Ae. aegypti* from Indonesia (Amelia-Yap et al. 2018b).

Materials and Methods

Mosquito Strains

Details of the sampling sites and toxicological assays have been described elsewhere (Amelia-Yap et al. 2018b). There were a total of nine sampling sites, including Kuningan, Padang, Samarinda, Pontianak, Denpasar, Mataram, Dompu, Manggarai Barat, and Sumba Timur (Fig. 1). Mosquitoes used for the toxicological study were randomly selected to screen for *kdr* mutations.

Genomic DNA Isolation

The abdomens were dissected and removed from mosquito specimens before carrying out genomic DNA isolation to avoid contamination. The mosquito genomic DNA was isolated using a commercial kit named G-spin Total DNA Extraction Kit (iNtRON Biotechnology, Inc., Gyeonggi-do, Korea), according to the manufacturer protocol.

Direct DNA Sequencing

Genotyping of the V1016G, S989P, and F1534C mutations were performed by direct DNA sequencing based on a previous publication (Yanola et al. 2011). For V1016G and S989P genotyping, a total of 100 mosquitoes were sequenced using the same pair of primers designed, encompassing domain II segment 6 of *Ae. aegypti Vgsc* gene. Amplification was performed using IIP_F as a forward primer (5′-GGT GGA ACT TCA CCG ACT TC-3′), whereas IIS6_R as a reverse primer (5′-GGA CGC AAT CTG GCT TGT TA-3′). A subset of 43 samples from the V1016G and S989P genotyping was subjected to the detection of the F1534C mutation, performed using Ge-IIIS6_F as a forward primer (5′-GCT GTC GCA CGA GAT CAT T-3′), whereas IIIS6_R as a reverse primer (5′-GTT GAA CCC GAT GAA CAA CA-3′) designed to include domain III segment 6 of *Ae. aegypti Vgsc* gene (Yanola et al. 2011). Amplification of the sodium channel region was performed in a final volume of 20 µl for each sample, comprising 1 µl of 10 pmol of each forward and reverse primer, 12.5 µl of GeNet Bio ExPrime Taq Premix (Global Gene Network, Daejeon, South Korea) and 25–50 ng of genomic DNA of mosquito.

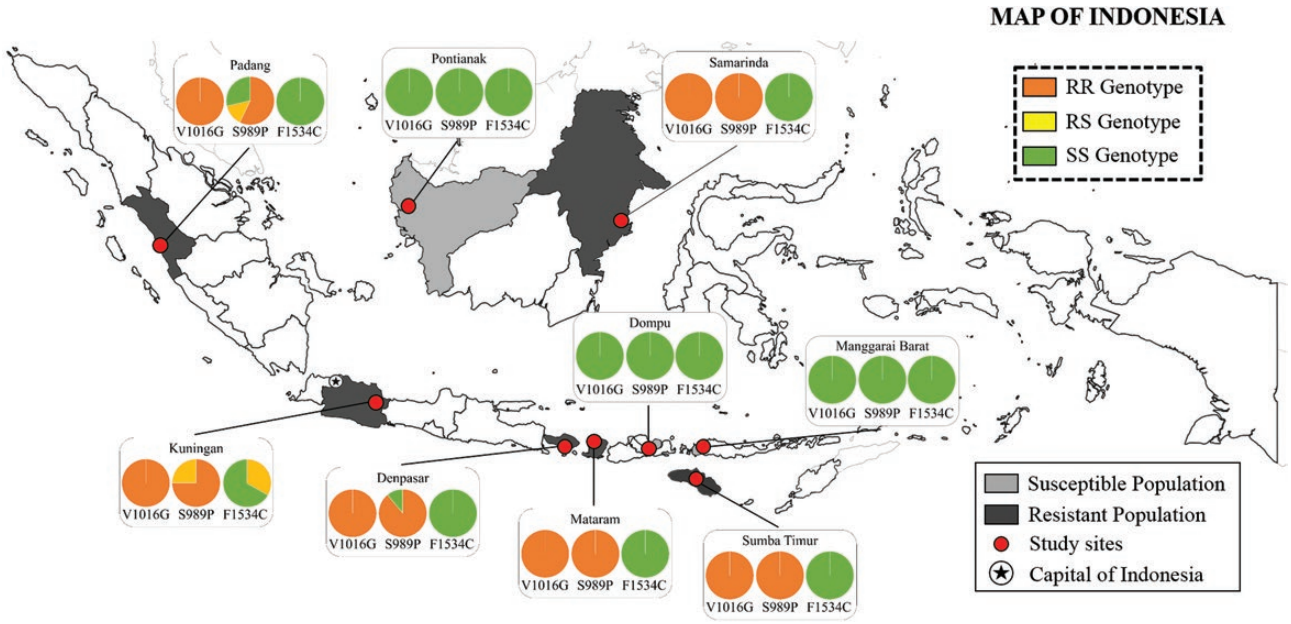

Fig. 1. Sampling sites displaying the distribution of *kdr* mutations (V1016G, S989P, and F1534C) in *Ae. aegypti* from Indonesia.

PCR amplifications for both of the V1016G and F1534C mutation regions were carried out using an Applied Biosystems Veriti 96-well thermal cycler (Thermo Fisher Scientific, Inc., Waltham, MA), with an initial denaturation of 95°C for 2 min, followed by 35 cycles of 95°C for 30 s (denaturation), 63°C for 30 s (annealing), 72°C for 30 s (extension), and a final extension at 72°C for 2 min. PCR amplicons were subjected to gel electrophoresis with the use of a 2% agarose gel prestained with SYBR Safe (Invitrogen, Carlsbad, CA) in Tris-Acetate-EDTA buffer.

The PCR amplicons were then delivered to a commercial company for DNA direct sequencing. Samples were sequenced using BigDyeH Terminator v3.1 Sequencing Kit (Thermo Fisher Scientific, Inc.) and analyzed on ABI PRISM 377 Genetic Analyzer (Thermo Fisher Scientific, Inc.).

Data Analyses

Heterogenous mutations were quantified based on generated sequences: heterozygous genotype (RS) exhibits double peaks in the mutation point, whereas homozygous genotype (RR/SS) exhibits only one specific peak (Simsek et al. 2001, Low et al. 2013a).

The frequencies of the resistant and susceptible *kdr* alleles for each of the point mutation were determined by the Hardy–Weinberg Equilibrium using an online calculator (Rodriguez et al. 2009). To examine the correlation between genotype and phenotype, we adopted Spearman's rank-order correlation analyses (Low et al. 2013a,b). Correlation analyses between the allele frequencies of the point mutations and the survivability rates as well as resistance ratios of all active ingredients tested in our previous study (Amelia-Yap et al. 2018b) were performed using the computer software SPSS (version 21).

Results

To screen for sodium channel mutations in the wild populations of Indonesian *Ae. aegypti*, the direct DNA sequencing method validated the presence of the V1016G, S989P, and F1534C mutations (Fig. 1). Since the sequences of 37 samples were noisy with low-quality scores on the trace chromatogram, only 63 female *Ae. aegypti* mosquitoes collected from suburban areas in nine regencies were included for analysis. In the V1016G genotyping, the RR genotype was identified to be in major among the study sites (6 out of 9) with 46 individuals from a total sample size of 63 mosquitoes (Table 1). In these six study sites, namely Denpasar, Mataram, Kuningan, Padang, Samarinda, and Sumba Timur, the frequencies of the resistant 1016 allele were all equal to 1.0. Out of nine populations, merely three populations (i.e., Pontianak, Dompu, Manggarai Barat) demonstrated the presence of SS genotype with a total of 17 individuals recorded. The frequencies of the susceptible 1016 allele were also documented to be 1.0 in these three populations. None of the RS genotype was detected in the 1016 position across any tested populations in Indonesia. Thus, only two genotypes at the position 1016 were presented (46 RR and 17 SS).

The direct DNA sequencing method also confirmed the presence of serine-proline transversion at the position 989 within domain II (S989P) along with the V1016G mutation. The genotypes detected from the S989P *kdr* mutation in the wild populations of *Ae. aegypti* were summarized in Table 2. The RR genotype being the most predominant genotype was found in six out of nine populations, with a record of 41 individuals of adult female *Ae. aegypti* from a sample size of 63. It was subsequently followed by the SS genotype with 20 individuals, whereas only 2 individuals were detected as the RS genotype.

To examine the role of another frequently detected *kdr* mutation that might presumably induce pyrethroid resistance, 43 individuals from all of the populations genotyped earlier for the V1016G/ S989P mutations were used to determine for the presence of the F1534C mutation. Through verification, all the nine populations exhibited the SS genotype in the 1,534 position as shown in Table 3. Two individuals were found exhibited heterozygous, but no individuals exhibited the mutant homozygous.

Spearman's rank-order correlation showed significant positive correlations between the allele frequencies of the V1016G mutation and the survivability rates in adult bioassays of d-allethrin (r = 0.825, P = 0.006), transfluthrin (r = 0.836, P = 0.005); and metofluthrin (r = 0.836, P = 0.005). In addition, significant positive correlations were also recorded between the allele frequencies of the V1016G mutation and the resistance ratios of d-allethrin (r = 0.822, P = 0.007), transfluthrin (r = 0.822, P = 0.007), and metofluthrin (r = 0.822, P = 0.007). With respect to the S989P and F1534C mutations, no significant correlations were detected for all of the active ingredients assayed on the adult female *Ae. aegypti*.

Co-occurrence of the V1016G and S989P mutations in individual *Ae. aegypti* was detected in six out of nine populations (i.e., Kuningan, Padang, Samarinda, Denpasar, Mataram, Sumba Timur) with a high frequency of 68.25% (43 out of 63 individuals; Table 4).

Discussion and Conclusions

The S989P, V1016G, and F1534C mutations are the most commonly detected *Vgsc* mutations found in pyrethroid-resistant *Ae. aegypti* in

Table 1. The V1016G genotyping and frequency of *kdr* alleles in Indonesian *Ae. aegypti*

Regencies	n	Genotype			Allele frequency	
		SS	RS	RR	S	R
Kuningan	4	0	0	4	0.00	1.00
Padang	7	0	0	7	0.00	1.00
Samarinda	9	0	0	9	0.00	1.00
Pontianak	6	6	0	0	1.00	0.00
Denpasar	9	0	0	9	0.00	1.00
Mataram	7	0	0	7	0.00	1.00
Dompu	4	4	0	0	1.00	0.00
Manggarai Barat	7	7	0	0	1.00	0.00
Sumba Timur	10	0	0	10	0.00	1.00
Total	63	17	0	46	0.27	0.73

Table 2. The S989P genotyping and frequency of *kdr* alleles in Indonesian *Ae. aegypti*

Regencies	n	Genotype			Allele frequency	
		SS	RS	RR	S	R
Kuningan	4	0	1	3	0.13	0.88
Padang	7	2	1	4	0.36	0.64
Samarinda	9	0	0	9	0.00	1.00
Pontianak	6	6	0	0	1.00	0.00
Denpasar	9	1	0	8	0.11	0.89
Mataram	7	0	0	7	0.00	1.00
Dompu	4	4	0	0	1.00	0.00
Manggarai Barat	7	7	0	0	1.00	0.00
Sumba Timur	10	0	0	10	0.00	1.00
Total	63	20	2	41	0.40	0.60

Table 3. The F1534C genotyping and frequency of *kdr* alleles in Indonesian *Ae. aegypti*

Regencies	n	Genotype			Allele frequency	
		SS	RS	RR	S	R
Kuningan	6	4	2	0	0.83	0.17
Padang	4	4	0	0	1.00	0.00
Samarinda	4	4	0	0	1.00	0.00
Pontianak	5	5	0	0	1.00	0.00
Denpasar	6	6	0	0	1.00	0.00
Mataram	3	3	0	0	1.00	0.00
Dompu	5	5	0	0	1.00	0.00
Manggarai Barat	4	4	0	0	1.00	0.00
Sumba Timur	6	6	0	0	1.00	0.00
Total	43	41	2	0	0.98	0.02

Table 4. Co-occurrence of the V1016G and S989P mutations in Indonesian *Ae. aegypti*

Regencies	n	Co-occurrence of V1016G and S989P (*n*)	Percentage
Kuningan	4	4	100.00
Padang	7	5	71.42
Samarinda	9	9	100.00
Pontianak	6	0	0.00
Denpasar	9	8	88.88
Mataram	7	7	100.00
Dompu	4	0	0.00
Manggarai Barat	7	0	0.00
Sumba Timur	10	10	100.00
Total	63	43	68.25

*Co-occurrence denoted whether an individual carries both point mutations showing the same genotypes (RR/RR) or a mutation showing heterozygosity (RR/RS).

Southeast Asia (Amelia-Yap et al. 2018a). The sampling locations encompassed larger geographical expansions to screen for *kdr* in *Ae. aegypti* relative to past studies completed in the country, making the present study to be the largest in respect of geographical coverage in Indonesia thus far. In accordance with the mosquito coil bioassays accomplished earlier (Amelia-Yap et al. 2018b), most of the Indonesian *Ae. aegypti* populations revealed high levels of resistance against all the pyrethroids assayed, namely, d-allethrin, transfluthrin, and metoflurthrin. With the emergence of such alarming issue, scientific findings from Southeast Asia (i.e., Malaysia) still displayed the effectiveness of pyrethroid-based mosquito coils against the field strains of *Ae. aegypti* (Chin et al. 2017). In our study, mosquitoes from some localities showing somewhat low *kdr* mutation frequency were in accordance with their low-resistance phenotype.

Based on the results, three mutations, V1016G, S989P, and F1534C, were detected in Indonesian *Ae. aegypti*. Direct DNA sequencing of IIS6 region of *Vgsc* of adult *Ae. aegypti* collected across some of the regencies in Indonesia demonstrated the presence of both V1016G and S989P mutations. The presence of the V1016G mutation was within expectation when this statement can be supported by a fund of evidence through which it was commonly discovered in Southeast Asia, namely, Malaysia (Ishak et al. 2015), Thailand (Brengues et al. 2003), Vietnam (Kawada et al. 2009), Singapore (Rajatileka et al. 2008), and Myanmar (Kawada et al. 2014). Previous studies in some parts of Indonesia such as Semarang (Brengues et al. 2003), Yogyakarta (Wuliandari et al. 2015), Jakarta (Hamid et al. 2017a), and Denpasar (Hamid et al. 2017b) also concurred with the findings from the current study. This point mutation, on the other hand, is yet to be reported in South American *Ae. aegypti*, despite its prevalence in Southeast Asian countries (Brengues et al. 2003, Saavedra-Rodriguez et al. 2007, Rajatileka et al. 2008).

From the findings of this study, the V1016G mutation revealed that the RR genotype to be the most predominant through the extensive dispersal across all of the study sites involved, whereas a low frequency of the SS genotype was shown. The absence of the heterozygosity in the V1016G mutation could be because of polymorphism, as reported by Martins et al. (2013) that other than amino acid changes, nucleotide and insertion or deletion polymorphisms can also be involved in a gene duplication event. Since heterogeneous (RS) duplications had an intermediate phenotype with lower

resistance and fitness cost which is in opposed to homogeneous (RR) duplications that enabled increment in both pesticide resistance fitness costs (ffrench-Constant and Bass 2017), the frequency of the RS genotype may reduce rapidly after a few generations when strong selection pressure exerted. Thus, the gene duplication of the mutant homogeneity in the *Vgsc* of *Ae. aegypti* may be arisen in response to adaptation due to the extensive use of pyrethroids in the country. This aids in maintaining the genotype with high fitness cost in order to enhance such trait for the continuity of the development of insecticide resistance and decrease the likelihood of deleterious effects concerning fitness cost.

Additionally, great differences were detected between number of the mosquitoes being the 1016G homozygous mutant and the 1016V wild-type homozygous. It was also discovered that the highest frequency of *kdr* resistance allele in *Ae. aegypti* populations were those from Kuningan, Padang, Samarinda, Denpasar, Mataram, and Sumba Timur. This result corresponded to the result of the mosquito coil bioassays performed previously (Amelia-Yap et al. 2018b), indicating those categorized as resistant were with 1.0 resistant allele frequency, whereas those grouped as susceptible were with 1.0 susceptible allele frequency. In this case, it can be concluded that homozygous mutant females were resistant, whereas wild-type homozygous were susceptible. This is indeed true when Wuliandari et al. (2015) verified 1016G homozygote was more largely related to the resistance of both Type I and Type II pyrethroids rather than other genotypes.

Inversely, among all of the adult mosquitoes selected for the F1534C mutation screening, SS genotype was discovered in majority. The F1534C mutation was the least frequently found relative to the S989P and V1016G mutations in this study. This outcome conformed to the past studies conducted in Jakarta (Hamid et al. 2017a) and Denpasar (Hamid et al. 2017b) but in contrast to studies performed in other parts of Southeast Asia such as Thailand (Yanola et al. 2011), Vietnam (Kawada et al. 2009), Malaysia (Ishak et al. 2015), and Singapore (Pang et al. 2015). The discrepancies from these findings may very much linked to a more recent emergence of the F1534C mutation in Indonesian *Ae. aegypti*, when gene flow occurs through the occurrence of migration events. Interestingly, such incongruities in result can also be attributed to the extensive geographical distribution of Indonesia with geographical expansion extending 5,120 km from east to west and 1,760 km from north to south, causing the difference in findings across Southeast Asia countries when gene expression is mediated in response to environmental dissimilarity. Thus, this point mutation is thought to be yet significant in leading to the development of pyrethroid resistance in *Ae. aegypti* populations from Indonesia.

By comparing the relationships between the allele frequencies of the V1016G mutation and the pyrethroid survivability rates as well as resistance ratios to all of the active ingredients examined, significant correlations were detected. This points out the higher the frequency of the V1016G mutation, the greater the level of resistance to d-allethrin, transfluthrin, and metofluthrin in these tested Indonesian *Ae. aegypti* populations. In contrast, no significant correlations were demonstrated between the allele frequencies of the S989P as well as F1534C mutations and the status of the insecticide susceptibility mosquito coil bioassays. Such lack of significant correlations may indicate the minor role of both S989P and F1534C mutations in the development of pyrethroid resistance. This may also suggest only the V1016G mutation is closely associated with pyrethroid resistance in Indonesian *Ae. aegypti* populations. Nevertheless, the possibility of the involvement of other resistance mechanisms (i.e., metabolic detoxification mechanisms) in *Ae. aegypti* should be taken into consideration and thoroughly investigated.

Brengues et al. (2003) pointed out knockdown resistance against pyrethroids in *Ae. aegypti* may possibly be conferred by one or more point mutations in the *Vgsc* locus. In the present study, a pattern of co-occurrence of point mutation, specifically V1016G/S989P was detected. The co-occurrence of the S989P and V1016G mutations were often found in pyrethroid-resistant *Ae. aegypti* populations, indicating that they may have resulted in an increment of the insensitivity of *Vgsc* to pyrethroids. Although the S989P mutation was detected in all of the study sites, it may not stand alone as the main cause in leading to the occurrence of insecticide resistance. This is because the S989P mutation has always been associated with the V1016G mutation, whereas the S989P mutation has yet to be found occurring alone (Kawada et al. 2014, Ishak et al. 2015). However, the V1016G has been discovered in spite of an absence of the S989P mutation in Thai *Ae. aegypti* (Brengues et al. 2003, Rajatileka et al. 2008). Therefore, it can be inferred that the S989P mutation may likely result in synergizing mutation, escalating the effect of pyrethroid resistance. Du et al. (2016) further proved that the S989P mutation did not show any additive effect to the V1016G mutation, but later, a contradictory study showed that the existence of the S989P mutation can highly decline the sensitivity to pyrethroid (Hirata et al. 2014). It is also worth mentioning the high frequency of the co-occurrence of the V1016G and S989P mutations is in line with past studies in Indonesia. The presence of the debating concern if the S989P mutation was responsible in causing additive role in pyrethroid resistance development in *Ae. aegypti* is of controversial. Thus, it is of critical need to conduct further studies with regards to the additive effect of the S989P mutation to the V1016G mutation.

Many controversies were shown on the accuracies of both direct DNA sequencing and PCR in detecting the point mutations in the *Vgsc* of *Ae. aegypti*. Despite the availabilities of allele-specific PCR (AS-PCR) and multiplex PCR that enable the detection of few point mutations in one or two reactions at our conveniences, the sensitivity and specificity of these detection methods may not be ideal. In the present study, there were deviations in the results when scored visually on gel electrophoretograms. Multiplex PCR showed inconsistency on the presence of internal control bands, whereas negative control bands were observed for AS-PCR throughout the entire span of optimization process, although the protocols were strictly adhered to. Thereafter, direct DNA sequencing was opted for to obtain the results in the present study. The use of the direct DNA sequencing was further in approval when incongruence of results from both direct DNA sequencing and AS-PCR were documented in previous study in *Ae. aegypti* (Yanola et al. 2011) and *Culex quinquefasciatus* (Low et al. 2013b).

The genotyping of mutations with regard to insecticide resistance can contribute as an advantageous surveillance tool in tracking resistance and involving in the intervention of novel chemical insecticides for mosquito control. With such, the V1016G, S989P, and F1534C mutations in *Vgsc* were detected in Indonesian *Ae. aegypti*. Being the first study consisted of large biogeographical areas in Indonesia, the current report has documented the V1016G mutation as the predominant genotype in Indonesian *Ae. aegypti*. This situation requires immediate attention before all of the control tactics were compromised.

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