

Molecular Resistance Status of *Aedes aegypti* to the Organophosphate and Pyrethroid Insecticides in Central Sulawesi and East Nusa Tenggara Provinces, Indonesia

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Abstract— Currently, dengue has become a major public health problem in Indonesia. *Aedes aegypti* is confirmed as the main dengue vector. The organophosphate and pyrethroid have been used in vector control program for more than 3 decades. Insecticide resistance evidences and mechanisms are essential to find the current status of insecticide susceptibility of dengue vectors. In this study, we analyzed the molecular principles of resistance to pyrethroid and organophosphate insecticides on mosquitoes collected from Palu, Central Sulawesi, and Belu and Ende, East Nusa Tenggara. Single-step polymerase chain reaction (PCR) method was conducted to detect amino acid mutations in paratype voltage-gated sodium channel (VGSC) gene and Acetylcholine esterase-1 (AChE) gene of *Ae. Aegypti* mosquitoes. The result showed that V1016G mutations of VGSC gene were detected from the wild-caught *Ae. Aegypti* mosquito from Palu, Belu and Ende. In contrast, G119 wild type allele of AChE gene was found from all *Ae. Aegypti* of all study sites. These evidences suggest that *Ae. aegypti* from Palu, Belu and Ende have developed multiple resistance towards pyrethroid insecticides. Based on prior susceptibility test, *Ae. aegypti* from all study sites were possibly developing resistance to organophosphate in other mechanisms. New strategies are needed, especially insecticide rotation in this area to achieve efficient vector control.

Keywords: *Aedes aegypti*, resistance, VGSC, AChE, Central Sulawesi, East Nusa Tenggara

I. INTRODUCTION

Dengue is one of the most important mosquito-borne viral diseases in the world. The disease has rapidly spread in more than 100 countries with 390 million dengue cases reported yearly[1]. Currently, Asia-pasific region has been reported to have the highest burden of dengue among other regions in the world. The south East Asia is one of the most impacted regions. At least 451 thousand dengue cases (14.11% of global cases) were reported from this region in 2015 [2].

Indonesia is one of the highest dengue endemic countries in the world. In the past 45 years, annual Incidence rate of DHF increased significantly from 0.05/100,000 in 1968 to 78.8/100,000 in 2016. In contrast, the case fatality ratio of DHF decreased considerably from 41% in 1968 to 0.96% in 2016. The areas affected by the disease by 2016 included 90% of the total 463 of districts/municipalities in the country[3]. All of dengue virus (DENV) serotypes are endemic in most of the large cities in the country with DENV-3 being frequently associated with large outbreaks[4]. *Aedes aegypti* is the principal dengue vector, while *Aedes albopictus* is the secondary vector. Both of them breed extensively in all the islands[5]. These mosquitoes breed in various breeding sites, such as disposed water-holding vessels, water containers, small containers, discarded tyres and waste disposal areas [6–8]. Rapid urbanization due to economic and industrial development has created slum areas with inadequate water and sanitation

facilities, as well as poor solid-waste management. These situations have resulted in the abundance of *Aedes aegypti* and *Ae. albopictus* breeding sites and led to rapid spread of dengue leading to dengue epidemics in this country[5,9].

In the absence of specific medications against dengue and an effective vaccine still under research, the control of mosquito vector is the sole strategy to prevent and control the transmission of dengue [4,10]. The government of Indonesia, through the Ministry of Health and local governments, have carried out vector control activities for more than five decades. In the early 1990s, the Ministry of Health issued a series of legislations concerning the National DF/DHF prevention and control program and the implementation of the control program. In 2000, the national dengue control strategy has been focused on the community participation in the reduction of *Aedes* breeding places through 3M program, i.e. *Menutup* (covering water containers); *Mengubur* (burying discarded container) and *Menguras* (cleaning water containers)[5]. Although the strategy is one of the best solutions to reduce Dengue vector density and DHF cases, various obstacles still have to be faced. Weak community participations in many DHF endemic areas, the community saturation factor after the implementation of this program for many years, and a lack of cross-sectoral roles have led to a less successful implementation of the 3M program. In the recent years, the government of Indonesia continues to strengthen the vector control strategy through: strengthening surveillance of DF/DHF case and its vector; disease management; and changing behaviour of communities and building partnership in order to reduce the abundance dengue vector [5,11]. However, the results are still not as what are expected.

Effective and efficient vector control is important as an effort to reduce dengue transmission. Insecticides still play as an essential role in dengue vector control program [12]. In Indonesia, the organophosphates (OP) has been used as a larvicide and adulticide for more than 30 years[13]. In addition, the Pyrethroids (PY) was introduced in 2000s for the control of adults[14]. However, insecticide resistance has become a serious problem for dengue vector control efforts. Currently, vector resistance against different classes of insecticides has been developed and recorded throughout the country.

Aedes aegypti insecticide resistance basically arises through four mechanisms. The first mechanism is an increased activity of metabolic detoxification enzyme known as metabolic resistance. The second mechanism is mutations in insecticide target proteins in the central nervous system that leads to these protein to susceptibly decrease to corresponding insecticides [15–18]. The third and fourth are behavioral changes and thickening of the cuticle, respectively [10,16,17]. The first two mechanisms are most studied [10,18,19]. Increased production of three main metabolic enzymes, i.e. cytochrome P450 monooxygenases or mixed-function oxidases (P450 or MFOs), glutathione S-transferases (GST), and esterases are reported to be associated with metabolic resistance [10,15,16,20]. The most common target site resistance mechanism is associated with mutations of a target protein at the voltage-gated sodium channel (VGSC) gene, which is the target of pyrethroid (PY) insecticide and point mutation at the

acetylcholinesterase (AChE) gene that is the target of OP and carbamate insecticides. Currently, several point mutations of insecticide knockdown (*kdr*) have been identified to be associated with the occurrence of pyrethroid resistance of *Aedes aegypti* [21]. These mutations of VGSC gene were reported to play roles in pyrethroid resistance of *Aedes aegypti*, especially the mutations at domain II (V410L); domain III (G923V, L982W, S989P, I1011M, I1011V, V1016G, V1016I); and domain III (T1520I, F1534C, D1763Y) [17,21–25]. *Aedes aegypti* *kdr* mutations with high allele frequency have been detected in many countries, including, US, India, Malaysia, Brazil, Myanmar, Thailand, Mexico, and Indonesia[17,21,22,26–29].

Acetylcholinesterase (*AChE*) gene has been described as a key enzyme of the cholinergic system, by regulating the level of acetylcholine and terminating nerve impulses by catalyzing the hydrolysis of acetylcholine [30,31]. The use of OP and carbamate insecticides inhibits acetylcholinesterase, which cause the death of insects. Target-site resistance against acetylcholinesterase due to *Ace-1* gene mutations has been reported in several species of mosquitoes, i.e. *Anopheles albimanus* [32], *Culex pipiens* [33], and *Aedes aegypti* in India [34].

Palu, Belu, and Ende are considered as high dengue endemic areas in Indonesia with IR of 176.86, 16.13 and 42.9 per 100,000 population, respectively. The frequent usage of physical and chemical approaches on the vector control efforts are reported to be very high in order to control dengue vector mosquitoes in these areas, particularly for *Ae. aegypti*. Besides physical approaches by 3M program, vector control measures against *Aedes* mosquitoes are mostly based on chemical application of ultra low volume space spray (ULV) and thermal fogging of malathion (OP) and cypermethrin (PY), and temephos (OP) as the main larvicide. Several reports on insecticide resistance status of *Ae. aegypti* as a principle dengue vector are available from these locations, but were very limited. Based on these reasons, a regular surveillance of insecticide resistance and its resistance mechanisms are needed and important to evaluate the effectiveness of the insecticides used. The present study was conducted to expound the mechanisms of pyrethroid and organophosphate resistance in *Aedes aegypti* collected from man-made containers in Palu city, Central Sulawesi province and Belu and Ende city, East Nusa Tenggara Province. In this study, we analyzed molecular resistance by identifying mutations in the genes of pyrethroids and organophosphates in the target site, and then determining the mutations role in malathion, cypermethrin and temephos resistance in both of these cities.

II. METHODS

A. Study areas and mosquitoes collection procedures

The study was conducted in three urban areas with high dengue incidence in Indonesia, namely Palu, the capital of Palu municipality, Central Sulawesi province; Atambua, the capital of Belu district, East Nusa Tenggara province, and Ende, the capital of Ende district, East Nusa Tenggara from March to June 2016.

During the field entomology survey, larvae and pupae were collected from artificial containers in and around the

residential areas and other buildings from selected locations. At least 100 houses from selected villages in every location were surveyed. The collected immature stages of *Aedes* sp. mosquitoes were then immediately transported to the insectary laboratory, NIHRD-IVRCRD, MoH Indonesia and reared under standar insectary conditions. After emerging, the adult mosquitoes were identified using illustrated keys of Rueda (2004)[35].

B. DNA isolation, Ace-1 and kdr mutation detection

The DNA was extracted from individual adult mosquito using DNA Purelink genomic DNA Minikit by following the manufacturer’s instructions (Invitrogen, USA). DNA isolation products were then stored at -80°C until further analysis. PCR identification was conducted using two different primer sets targeting amino acid of the Ace-1 gene and domain II of amino acid loci of the VGSC gene, as described previously by Kawada et al., 2016 and Hamid, et al., 2017. To identify ACE-1 gene mutation, PCR amplifications were carried out in the final volume of 25µl where 5 µl DNA isolation product was used as a template.

Genomic DNA was amplified using the Ace1 primers, AceF (5’-CGATAACGAATGGGGAACG -3’) and AceR (5’-TCAGAGGCTCACCGAACACA -3’). PCR was performed using SimpliAmp™ Applied Biosystems thermal cycler (Perkin Elmer, Branchburg, NJ, USA) under the following conditions: initial step of denaturation was carried out at 94° C for 3 minutes, followed by 35 cycles of amplification at 94° C for 1 minute, 58° C for 1 minute, and 72° C for 2 minutes, with a final elongation step at 72° C for 10 minutes. PCR products were purified and then directly sequenced in both directions with the same primer for PCR amplification, at the position of G119S.

To identify *kdr* mutation, PCR was done using specific primers targeting domain II of the VGSC, *vgscF*(5’-GGTGGAACTTCACCGACTTC 3’) and *vgscR* (5’-GGACGCAATCTGGCTTGTTA 3’). Twenty five micro litres of DNA mix were amplified, and PCR reaction was performed with an initial step of denaturation at 94° C for 10 minutes, followed by 40 cycles of amplification at 94° C for 1 minute, 63° C for 45 seconds, and 72° C for 1 minute, with a final elongation at 72° C for 7 minutes.

staining and were run for 60 minutes at 90 V in TAE buffer to check the quality of PCR products. The PCR product was purified using ExoSAPI-IT™ Applied Biosystems™ (Affymetrix inc, CA USA) and was then amplified using BigDye™ terminator v3.1 cycle sequencing kit. Subsequently, BigDye®Xterminator purification kit was added directly to the finished sequencing reactions. The sequencing analysis was then conducted with Applied Biosystems 3500 series genetic analyzer. All sequences were deposited in GenBank. For *Ace-1*, the accession numbers were MK896349, MK896350, MK896351, MK896352 and MK896353; For *kdr*, the accession numbers were MK896354, MK896355 and MK896356. Locations of the collected sample can be see in Table 1.

C. Data analysis

DNA sequences were edited by using sequencing analysis 5.2.0 Applied Biosystems. Mutations and allelic variants were determined by sequence alignment analysis with MEGA 6 and Bioedit v7.0.5. The results were then compared with national report of insecticide resistance evaluation which was conducted by multicenter study team, NIHRD MoH Indonesia to provide information of current

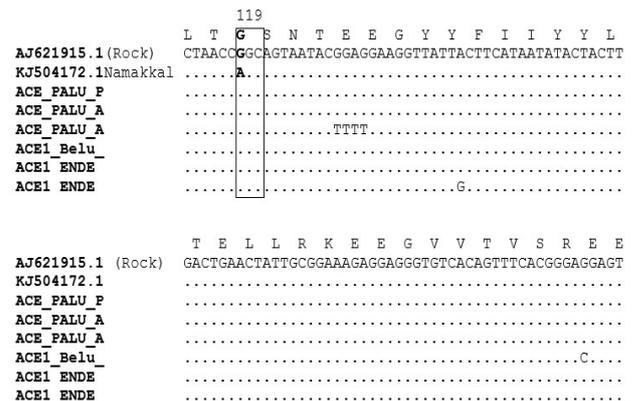


Figure 1. Alignment of Ace-1 gene sequences (Palu, Ende and Belu). The sequences from three locations were aligned with the Ace-1 gene wild type Rockefeller strain G119 (acc. number AJ621915.1) and G119S mthusamy strain (acc. Number KJ504172). Wild Type GGC encoding Glycines were found in all locations.

resistance status of *Ae. Aegypti* against organophosphate and pyrethroid insecticides.

III. RESULTS

A. Detection of Ace-1 mutation

Ace-1 genes were succesfully analysed among 5 mosquitoes. The 384 bp fragment of the *ace-1* were amplified from the three populations studied. Insertions or deletions were not identified in all these sequences. All three populations at position 119 were represented (119 G/G). Accordingly, we only found individuals that expressed the wild type (GGC) at this position and point mutations were not found in all sequences (Fig 1).

TABLE 1. LOCATION OF *Aedes aegypti* COLLECTION

| Acc. Number | Gene Target | Species | Location |
|-------------|-------------|--------------------|--------------------------|
| MK896349 | ACE1 | <i>Ae. aegypti</i> | Palu, Central Sulawesi |
| MK896350 | ACE1 | <i>Ae. aegypti</i> | Palu, Central Sulawesi |
| MK896351 | ACE1 | <i>Ae. aegypti</i> | Belu, East Nusa tenggara |
| MK896352 | ACE1 | <i>Ae. aegypti</i> | Ende, East Nusa tenggara |
| MK896353 | ACE1 | <i>Ae. aegypti</i> | Ende, East Nusa tenggara |
| MK896354 | VGSC | <i>Ae. aegypti</i> | Palu, Central Sulawesi |
| MK896355 | VGSC | <i>Ae. aegypti</i> | Belu, East Nusa tenggara |
| MK896356 | VGSC | <i>Ae. aegypti</i> | Ende, East Nusa tenggara |

All PCR amplification products were then loaded into a 2% agarose gel electrophoresis following SYBR safe Invitrogen



Figure 2. Alignment of VGSC gene sequences (Palu, Ende and Belu). The sequence of three locations were aligned with the VGSC gene Kawada strain with point mutation, V1016G (acc. Number AB914689) and wildtype (acc. Number AB914690). Nucleotides in capital letters are exon, and in lowercase are intron.

B. Detection of *kdr* mutation

A total of 3 *Ae. aegypti* samples were tested to identify the *kdr* mutation. All samples were successfully amplified by a single step and sequenced PCR. Sequencing revealed the presence of the point mutation within domain II at position 1016 from valine to glycine (V1016G). However, another point mutation at domain II of VGSC gene where both had a correlation with resistance status to pyrethroid (1011 and 1014) was not found (Fig 2).

IV. DISCUSSION

The insecticide application by using ultra-low volume and thermal fogging are known as the important methods to control adult *Ae. aegypti* population. In addition, larvicide plays an important role to manage and control larval stages of *Ae. aegypti*[10]. The selection of active ingredients with an appropriate dose is very important for this purpose. Temephos and malathion, two members of the organophosphate (OP), have been used as the selected active ingredients of larvicides and adulticides of *Aedes* mosquitoes for more than 30 years in Indonesia. In addition, cypermethrin, a member of phyrethroid (PY), has been used as another active ingredient of adulticides for more than 2 decades. We conducted molecular resistance study to identify three of the insecticide resistance status of *Ae. aegypti*; malathion (organophosphate), temephos (organophosphate), and cypermethrin (phyrethroid) and polymorphisms in *Ace-1* and *kdr* genes among *Ae. Aegypti* collected from three different municipalities/ districts in Indonesia.

A. *Ace-1* mutation analysis

Recently, organophosphate resistance in *Ae. Aegypti* has been reported to be quite extensive in this country. In Palu, *Ae. Aegypti* was reported to be resistant to Malathion. However,

this species was still reported to be susceptible to Temephos. In East Nusa Tenggara, *Ae. Aegypti* was reported to be resistant to Malathion and Temephos [36]. Therefore, the monitoring of molecular mechanisms of these insecticides are essential to determine the long-term strategy for dengue vector control program in these areas.

The mutations on the *ACE1* gene have also been reported in several mosquito species, i.e. *Culex tritaeniorhynchus*, *Cx. pipiens* and *An. gambiae*. Mostly, this resistance is associated with OP resistance [37–39]. However, although the increase of the themepos resistance was reported in Belu and Ende, and the malathion resistance was reported in all of study sites (Palu, Belu and Ende), the molecular mechanisms of acetylcholinesterase were not clearly shown. The mutation of G119S was not detected in all the samples studied. With the absence of *Ace-1* gene mutations in *Ae. Aegypti*, this resistance to the organophosphate is most likely to occur through metabolic mechanisms.

Globally, studies to identify *ACE1* mutations related to the resistance of *Ae. Aegypti* against organophosphate and carbamate insecticides are relatively limited. Muthusamy (2015) reported a mutation in the G119S codon in the *Ae. Aegypti* mosquito from Namakkal area, India. Although organophosphate and carbamate has been used for more than two decades, the mutation of G119S codon has never been reported in Indonesia and Southeast Asia.

B. *Kdr* mutation analysis

Several mutations in *kdr* gene of *Ae. Aegypti* have been reported to be associated with pyrethroid resistance. Two *kdr* mutations of V1016G and F1534C within domain II are common in *Ae. Aegypti* in Asia [23,25]. The pyrethroid resistant *Ae. Aegypti* has been widely reported in Indonesia. The mutation in the V1016G codon is most strongly associated with target site resistance. Pyrethroid works by binding the sodium channel gate (VGSC) to inhibit the gate. This will cause the sodium channel to be flooded with positive charges, so the mosquito will die. Mutations at this gate will cause pyrethroid insecticides not to be attached to the sodium channel gate (VGSC), and mosquitoes will not die [40]. This mutation in V1016G has been widely reported in Indonesia, particularly in several areas of Central Java[14], Palembang and Jakarta [41], and in Thailand [42]. In Brazil, mutations related to phyrethroid resistance were found related to the mutation in codon I1014M, whereas in codon V1016, there were no mutations [15]. The other study showed that V1016G was strongly associated with resistance to pyrethroids, and mutations were also found in F1534C in Myanmar [22]. In Indonesia, insecticide resistance of *Ae. Aegypti* against Cypermethrin, Lambdasihalotrin, and Deltamethrine have also been reported in several areas [36].

In this study, mutations in VGSC gene at V1016G were found in all of study areas, whereas mutations of 1011 and 1014 were not found. These findings indicate that the resistance of *Ae. aegypti* against pyrethroid is likely to occur due to the target site mutation in VGSC gene, whereas organophosphate resistance is metabolic resistance due to the presence of the esterase enzyme.

Although the application of pyrethroid insecticide is relatively new compared to organophosphate in Indonesia, the potential for its resistance is quite high. Macorist et al. (2007) reported that the continuous application of pyrethroid for 10 years in Sao Paulo, Brazil caused a high resistance to pyrethroids [43]. Pyrethroids are also widely used for household insecticides because they are cheap and safe to use. This has caused pyrethroid's contact to *Ae. Aegypti* to occur more intensively [44]. The Gray experiment showed that the use of household insecticides against *Ae. Aegypti* caused a decline in mosquito mortality. The use of spray insecticides reduces mortality from 99% to 44%, and residual spray to 50%. The use of residual spray of insecticide has also increased the occurrence of mutations in V1016G gene, which is responsible for *kdr* resistance [45]. Agriculture insecticides also plays a role in the occurrence of insecticide resistance. Previous study in Burkina Faso reported the role of the use of agricultural insecticides to trigger VGSC (L1014F, L1014S) and ACE1 (G116S) mutation in *An. gambiae* [46].

An understanding of resistance and its mode of action, including molecular resistance as one of the main mechanisms of resistance, will enable assistance in the management of insecticide resistance for vector control efforts [47–49]. In the insecticide resistance management, mosquitoes with no-mutation in the target of VGSC and ACE1 genes are valuable resource and must be attempted to preserve by limiting their exposure with insecticide. One of the most effective recommendations and activities in resistance management is rotating the pesticide with different modes of action [47,49].

In this study, we make suggestion on the role of metabolic resistance in *Ae. aegypti* against organophosphate. Due to the inactive metabolic enzyme, insecticides will be able to reach the target site and effectively kill the mosquitoes. With the discovery of molecular resistance in VGSC gene, but no mutations in ACE1 gene, it is necessary to provide a synergist to be added to organophosphate and karmabat type of insecticides. One of the potentials to overcome this metabolic resistance is by adding synergist in the use of insecticides. Synergist is an insecticidal property, which can inhibit the enzyme that normally acts to detoxify the insect system. This mechanism will enhance the potential capacity of insecticide [50]. The use of this synergist is a reasonable choice, given the mode of insecticide action in health vector control compared to agriculture.

V. CONCLUSION

The result showed that V1016G mutations of VGSC gene were detected from the field collected in *Ae. Aegypti* mosquitoes in Palu, Belu and Ende. In contrast, G119 wild type allele of AChE gene were found from all *Ae. Aegypti* of all study sites. These evidences suggest that *Ae. aegypti* from Palu, Belu and Ende have developed multiple resistance towards pyrethroid insecticides. Based on susceptibility test result in the previous study, *Aedes aegypti* from all study sites are possibly developing resistance to organophosphate in other mechanisms, particularly metabolic resistance.

The susceptibility of used organophosphate in target gene should be monitored regularly to identify the emergence of OP resistance among *Ae. Aegypti* population. A high level resistance to pyrethroid among *Ae. aegypti* population was also noticed. Replacement of pyrethroid with other class of insecticides is very necessary in the study areas.

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