BIODIVERSITAS
Volume 24, Number 1, January 2023
Pages: 300-307

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d240136

Bioassay and molecular detection of insecticides resistance of *Aedes aegypti*, vector of dengue in Central Java Province, Indonesia

SAYONO[1,♥], ULFA NURULLITA[1], WAHYU HANDOYO[2], WINDA SEPTY TYASNINGRUM[3], IRFANUL CHAKIM[1], ANTO BUDIHARJO[4]

[1]Faculty of Public Health, Universitas Muhammadiyah Semarang. Jl. Kedungmundu Raya No. 18, Semarang 50273, Central Java, Indonesia. Tel./fax.: +62-24-76740291, ♥email: say.epid@gmail.com

[2]Provincial Health Office of Central Java Province Government. Jl. Kapten Piere Tendean No. 24, Semarang 50132, Central Java, Indonesia

[3]Kagok Public Health Center of District Health Office of Semarang Municipality Government. Jl. Telomoyo No. 3, Semarang 50252, Central Java, Indonesia

[4]Integrated Laboratory, Department of Biology, Faculty of Sciences and Mathematics, Universitas Diponegoro. Jl. Prof Soedarto, SH, Kampus UNDIP Tembalang, Semarang 50275, Central Java, Indonesia

Manuscript received: 28 November 2022. Revision accepted: 8 January 2023.

Abstract. *Sayono, Nurullita U, Handoyo W, Tyasningrum WS, Chakim I, Budiharjo A. 2023. Bioassay and molecular detections of insecticides resistance of* Aedes aegypti, *vector of dengue in Central Java Province, Indonesia. Biodiversitas 24: 300-307.* The emergence of insecticide-resistant strains among *Aedes aegypti* populations hampered Dengue control programs in the endemic areas. Moreover, to understand the current situation and distribution of insecticide resistance status of *Ae. aegypti* to cypermethrin, malathion, and temephos compounds, we conducted morphological and molecular detection in the Dengue endemic areas in Central Java Province, Indonesia. Mosquito larvae were obtained from thirteen villages of five Dengue endemic areas representing different altitudes. Larval and adult stage of *Ae. aegypti* colony from each village was subjected to a bioassay test based on the WHO procedures. Subsequently, they were sampled and subjected to molecular analysis to identify the 1016G kdr allele using the allele-specific polymerase chain reaction (AS-PCR). Mortality of *Ae. aegypti* after exposure to cypermethrin, malathion, and temephos ranged from 16-86%, 75-100%, and 6-51%, respectively. These findings showed that *Ae. aegypti* populations were resistant to cypermethrin and temephos, although malathion-susceptible strains were found among 23.08% of the different altitudinal localities. The result of the AS-PCR indicated that the homozygous (G/G) and heterozygous (V/G) alleles of codon 1016 of the *AaNav* gene were found throughout the study site altitudes. The development of multiple resistance strains was found among *Ae. aegypti* populations in Central Java Province, Indonesia. The use of cypermethrin and temephos compounds must be delayed for at least five years, while malathion can still be used selectively to control the *Ae. aegypti* population in several areas, namely Karangjati, Gebugan (Semarang District), and Rowosari in Semarang City.

Keywords: *Aedes aegypti*, insecticide resistance, pyrethroid, organophosphate, 1016G kdr allele

INTRODUCTION

Aedes aegypti mosquito is an efficient vector for Dengue, Chikungunya, and Zika virus transmission (Peterson et al. 2016). This species can be found at low to high-level altitudes of more than 1,000 m above sea level (Lozano-Fuentes et al. 2012; Sayono et al. 2017) impacted by the increase in the air temperature average of 30ºC, causing the enhancement of the potential of the Dengue outbreak (Lee et al. 2018; Reinhold et al. 2018). Annually, new dengue infection in the community has been estimated to have as many as 390 million cases per annum in tropical and subtropical regions, including Indonesia (Brady et al. 2012). The incidence rate (IR) of Dengue Hemorrhagic Fever (DHF) in Indonesia was 50.75 from 100,000 inhabitants, and the case fatality rate (CFR) was 0.83% (Ministry of Health of the Republic of Indonesia 2017). The burden of the Chikungunya virus is similar to dengue in areas where *Aedes* vectors are established (Fredericks et al. 2014). Zika virus has rapidly spread intercontinental (Duffy et al. 2009, Musso et al. 2014). Zika virus was first reported in Central Java Province in 1977-1978 (Olson et al. 1981), followed by Jakarta (Kwong et al. 2013), Bali (Leung et al. 2015), and Jambi (Perkasa et al. 2016).

Multiple burdens of those viruses stimulated community efforts to control the diseases actively, focusing on vector control since antiviral medication has not been available yet (Elsinga et al. 2015). The use of insecticides with high intensity in controlling *Ae. aegypti* during the last decades has led to the emergence of strains resistant to neurotoxic insecticides in the Americas, Africa, and Asia (Moyes et al. 2016). The resistance strains of *Ae. aegypti* to different insecticide compounds and classes have also been reported in several parts of Indonesia, such as temephos in Surabaya (Mulyatno et al. 2012; Putra et al. 2016), malathion in Bandung (Ahmad et al. 2009), organophosphate in Jakarta (Hardjanti et al. 2015) and Wonosobo (Widjanarko et al. 2017), α-cypermethrin in Cimahi, West Java (Astuti et al. 2012), permethrin in Bali (Hamid et al. 2017), and several compounds of mosquito coils from several islands in Indonesia (Amelia-Yap et al. 2018a). The resistance of *Ae. aegypti* to two pyrethroid compounds (deltamethrin and permethrin) was found in Yogyakarta (Wuliandari et al. 2015). In addition, the emergence of cross/multiple resistance to some insecticide compounds was reported in some countries (Brengues et al.

2003; Putra et al. 2016; Bharati et al. 2018).

Studies reported the molecular mechanisms of *Ae. aegypti* resistance to pyrethroid in Central Java Province by exploring the *AaNav*-gene polymorphisms of S989P, V1016G, and F1534C, resulting in the kdr alleles of 989P, 1016G, and 1534C (Sayono et al. 2016a). Geographically, the polymorphisms of the codon 1016 *AaNav*-gene have two various amino acid substitutions from valine [V] to glycine [G] or isoleucine [I]. V to G substitution is found consistently in Southeast Asia (Kawada et al. 2014; Li et al. 2015; Widyastuti et al. 2015; Sayono et al. 2016a; Amelia-Yap et al. 2018b), while V to I is only found in Latin American regions (Saavedra-Rodriguez et al. 2007; Harris et al. 2010; Martins et al. 2013; Linss et al. 2014). This phenomenon indicates the correlation between geographic region and genetic change variation. This study aimed to understand the distribution of *Ae. aegypti* resistance status to cypermethrin, malathion, and temephos compounds in the Dengue endemic areas of Central Java Province, Indonesia. Additionally, we apply the allele-specific polymerase chain reaction (AS-PCR) to detect the existence and distribution of 1016G kdr alleles among the *Ae. aegypti* population (Stenhouse et al. 2013) throughout the locality altitudes, the results of this simple method will be recommended to health officers for routine monitoring.

MATERIALS AND METHODS

Study sites, larval collection, and rearing

This research was conducted in the fifteen dengue-endemic areas in four districts, and one municipality in Central Java Province, Indonesia with the highest Dengue incidence rate, Semarang, Pemalang, Tegal, and Kudus districts and Semarang municipality (Figure 1). Therefore, one to two villages were selected in each district and municipality based on the occurrence of new Dengue cases in 2016. Furthermore, only thirteen villages obtained sufficient larvae from the fifteen dengue-endemic areas. Larval collections were conducted from June to August 2016 toward indoor and outdoor water container breeding sites in residents' dwellings in a radius of 50 meters from the house of Dengue patients. The mosquito larvae were aspirated from the container using a larvae aspirator (Figure 2). This device was made from an aluminum pipe with a diameter of 5 mm and a length of 60 cm. This pipe was connected with 2 meters of plastic hose with a similar diameter. Larvae were collected in plastic bottles containing water from the origin habitat separately based on the study cluster and location of the container, indoor or outdoor. Then, the larvae were delivered to be reared in a laboratory using a 20 x 30 centimeters plastic tray and fed with dog food. The average air temperature and humidity were maintained in the range of 29.6-30ºC and 78 to 81 percent, respectively. The pupae emergences were moved into the mosquito cage and classified based on the study cluster. The imagoes were fed with a 10% sugar solution through permeated cotton.

Bioassay test

The susceptibility of *Ae. aegypti* against cypermethrin, one of the most frequently used pyrethroid class insecticides, and two organophosphate compounds, malathion, and temephos, were evaluated. World Health Organization (WHO) standard bioassay test tools and procedures are used to distinguish the resistance status of *Ae. aegypti* using impregnated paper containing 0.05% α-cypermethrin and 5% malathion according to the variance of the concentration of active insecticide compounds produced by WHO (WHO 2016). These sets and materials were obtained from the WHO Vector Control Research Unit at the Science University of Malaysia.

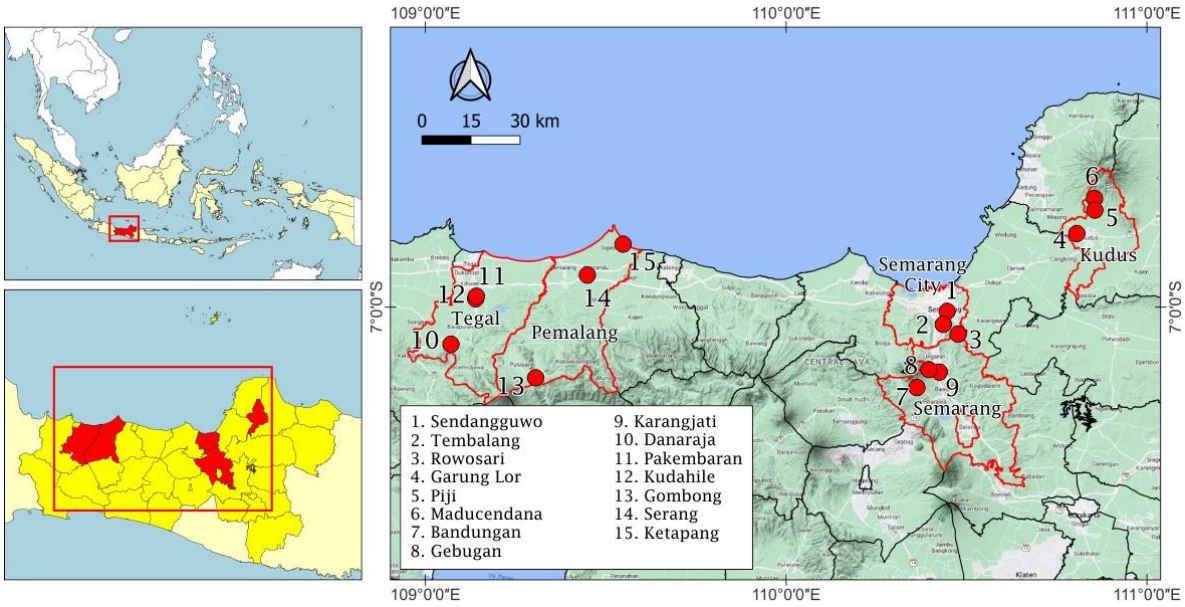

Figure 1. Map of study sites in Central Java province, which included five districts or municipalities, namely Kudus, Semarang, Pemalang, Tegal districts, and Semarang city. They are indicated by the circle line surrounding each cluster of study sites

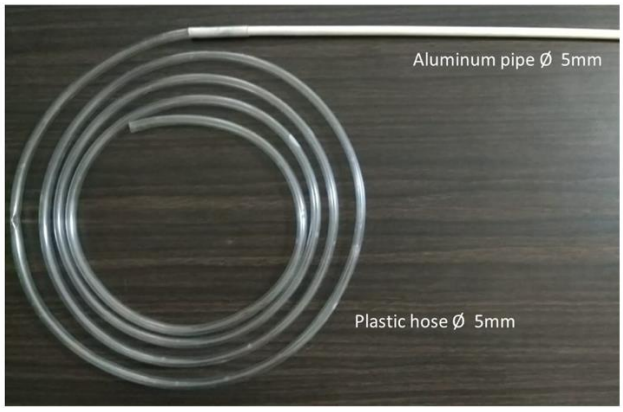

Figure 2. Larvae aspirator device

The research subjects were filial 1 (F1) female mosquitoes fed on sugar and healthy (3-5 days old). A total of 150 mosquitoes from each study site were subjected to a bioassay test with details as follows, an experimental tube (coated with impregnated paper on the inner surface) and two control tubes (without impregnated paper) where each tube contained 25 mosquitoes. The experimental tubes were four times replicated so that the total samples were 150 mosquitoes. Each sample was left in contact with the impregnated paper for 60 minutes. The test was carried out on three consecutive days so that the total sample for each study site was 450 mosquitoes. The number of knockdown mosquitoes was counted every five minutes. After 60 minutes of contact with the impregnated paper, all mosquitoes were carefully transferred to a collection cup for 24 hours of recovery. Then the dead mosquitoes were recorded. Air temperature and humidity were maintained at 27±20ºC and 75±10% during the holding period. To test the susceptibility of larvae to temephos, we prepared 150 *Ae. aegypti* late 3rd or early 4th instars for each study site, so 1,950 larvae were needed for thirteen locations. The larvae were put into five single-use plastic cups containing 0.02 ppm temephos in 100 mL of distilled water and one control cup (distilled water), each containing 25 larvae. The larvae were left in contact with temephos for 24 hours, and the larval mortality was calculated after that. The susceptibility status of the mosquito population to insecticides at the study site was classified into susceptible (S), showing resistance (SER), and resistant (R). The WHO standard bioassay test was used based on the percentage of deaths over 98% (S), 90-97% (SER), and lower than 90% (R), respectively (WHO 2016).

Allele-Specific Polymerase Chain Reaction

Based on the previous bioassay test we obtained the susceptible and resistant mosquitoes and subjected them to the identification of the 1016G kdr allele of the *AaNav* gene using the AS-PCR method, ten resistant and susceptible mosquitoes were taken from each study site (in total, 220 mosquitoes). Genomic DNA was isolated individually from each resistant and susceptible mosquito sample. The concentration and purity of the genomic DNA were measured by Nanodrop 2000 spectrophotometer. DNA amplification was performed in the 25 µL total volume consisting of 1.5 mM MgCl2 and 1X PCR buffer, 0.25 µM forward primer (5'-ACCGACAAATTGTTTC CC-3'), 0.125 µM Gly reverse primer (5'-GCGGGCAGGG CGGCGGGGGCGGGGCCAGCAAGGCTAAGAAAAG GTTAACTC-3') or Val (5'-GCGGGCAGCAAGGC TAAGAAAAGGTTAATTA-3'), 200 µM dNTP mix and 0.2 µL polymerase Taq (Stenhouse et al. 2013). The thermal cycle condition of AS-PCR was started with the pre-denaturation of the DNA template for 2 min at 94°C, followed by 35 cycles for 30 sec at 94°C, 30 sec at 55°C and 30 sec at 72°C, and followed by 72°C of final elongation. The amplification products were run using the gel electrophoresis for 50 min with 100-volt acceleration. Visualization of the electrophoresis product was performed to find the 60 base pairs (valine) and 80 base pairs (glycine) DNA bands using gel documentation imaging (Stenhouse et al. 2013).

Data analysis

The mortality rate of mosquitos and larvae was calculated based on the number of dead mosquitos and larvae after 24 hours of contact. Results of the bioassay susceptibility test were shown in the table frequency. Statistical analysis using a one-way comparison test was conducted to understand the difference in mortality of the pyrethroid and organophosphate-treated mosquitoes. The association between 1016G kdr allele frequency and the resistance status was analyzed using the Chi-Square test.

Ethical statement

Data collection was carried out after obtaining permission from the provincial government and the local health office, and informed consent was obtained from the household. This study did not use human specimens.

RESULTS AND DISCUSSION

Morphological resistance status

Bioassay test showed a knockdown time of 50% (KDT50) of *Ae. aegypti* mosquitoes after exposure to α-cypermethrin, and malathion ranged from 28.33 to 494.29 and 41.63 to 375.83 min, respectively (Figure 3). Furthermore, the comparison revealed that malathion 5% is the most effective insecticide compared to cypermethrin 0.05% and temephos 0.02 ppm, as indicated by a significant level of mortality compared to the two ($p<0.0001$). The second effective line of insecticide compound is cypermethrin ($p=0.0027$ compared to temephos) (Figure 4). The mortality status of malathion was higher than others, and likewise, cypermethrin toward temephos, although in some areas, temephos is still more likely to be effective, namely Kaliwungu and Maducendono. Analysis of differences in mosquito and larvae mortality according to study sites indicated uniformity in resistance status of Cypermethrin-0.05% and Temephos-0.02 ppm and variations in susceptibility to malathion-0.5% (Table 1 and Figure 5.). Mosquitoes from

Tembalang showed the shortest knockdown time after pyrethroid exposure, while mosquitoes from Pakembaran showed the shortest after organophosphate exposure. The mortality of *Ae. aegypti* mosquitoes after exposed to cypermethrin 0.05%, malathion 5%, and temephos 0.02 ppm ranged from 16-86%, 75-100%, and 6-45%, respectively, indicating the different susceptibility statuses. All of the *Ae. aegypti* populations from the thirteen study sites were resistant to cypermethrin and temephos. Of the thirteen studies sites were classified into susceptible (23.08%), suggestive of existing resistant (38.36%), and resistant (38.46%), based on the mortality percentage (Table 1). Malathion-susceptible strains were found in three villages: Karangjati and Gebugan (Semarang district) and Rowosari (Semarang municipality).

Molecular analysis

In molecular analysis of pyrethroid resistance using AS-PCR, only 11 live (resistant) and 6 dead (susceptible) mosquito specimens were identified clearly, where the 1016G kdr alleles of the *AaNav* gene were detected in the homozygous and heterozygous. Statistical analysis (Table 2) showed that there was a significant difference between allele frequencies and phenotypic resistance status ($p<0.05$). Three genotype variants were detected: homozygous wild type 1016V/V, homozygous mutant 1016G/G, and heterozygous mutant 1016V/G. Allele frequencies for wild type and mutant are 45% and 55%, while the genotype frequencies for V/V, V/G, and G/G are 36%, 18%, and 45%, respectively. The 1016G kdr allele was detected from the resistance *Ae. aegypti* of all altitudinal study sites, but the kdr allele was not detected in the susceptible one. A high frequency of the homozygous 1016G kdr allele was detected in the low altitudinal locality.

Table 1. Susceptibility status of *Ae. aegypti* mosquito towards pyrethroid and organophosphate insecticides

Study area		Pyrethroid (Cypermethrin 0.05%)		Organophosphate (Malathion 5%)		Organophosphate (Temephos 0.02%)	
District/city	Location	Mortality (%)	Resistance status	Mortality (%)	Resistance status	Mortality (%)	Resistance status
Semarang District	Karangjati	80	R	100	S	36	R
	Gebugan	52	R	99	S	22	R
	Bandungan	21	R	83	R	6	R
Pemalang District	Ketapang	65	R	96	SER	24	R
	Serang	66	R	91	SER	15	R
	Gombong	35	R	90	SER	19	R
Tegal District	Pakembaran	66	R	91	SER	24	R
Kudus District	Piji	56	R	86	R	41	R
	Maducendana	16	R	86	R	31	R
	Kaliwungu	20	R	75	R	21	R
Semarang City	Sendangguwo	67	R	97	SER	51	R
	Rowosari	86	R	100	S	43	R
	Tembalang	80	R	80	R	45	R

Note: WHO criteria: mortality rate <90% is resistance (R), a mortality rate of 90%-97% is suggestive of the existence of resistance (SER), and a mortality rate >98% is fully susceptible (S)

Table 2. Altitudinal distribution of genotype and allele frequencies of codon 1016 *Ae. aegypti AaNav* gene in Central Java Province, Indonesia

Habitat origin (village)	Altitude (m asl)*	Resistance status#	Number of mosquitoes	Genotype+			G Allele frequency	p
				V/V	V/G	G/G		
Gombong	1,112	R	2	1	0	1	0.50	0.035
		S	1	1	0	0	0.00	
Bandungan	910	R	2	1	0	1	0.50	
		S	2	2	0	0	0.00	
Gebugan	524	R	3	1	1	1	0.50	
		S	1	1	0	0	0.00	
Karangjati	486	R	2	1	0	1	0.50	
		S	0	0	0	0	0.00	
Tembalang	225	R	2	0	1	1	0.75	
		S	2	2	0	0	0.00	
Total		R	11	4	2	5	0.55	
		S	6	6	0	0	0.00	

Note: *m asl: meter above sea level, #Bioassay test result of *Ae. aegypti* to cypermethrin 0.05%: resistant (R), susceptible (S). +Detected genotypes: V/V (wild-type), V/G (heterozygous mutant), G/G (homozygous mutant)

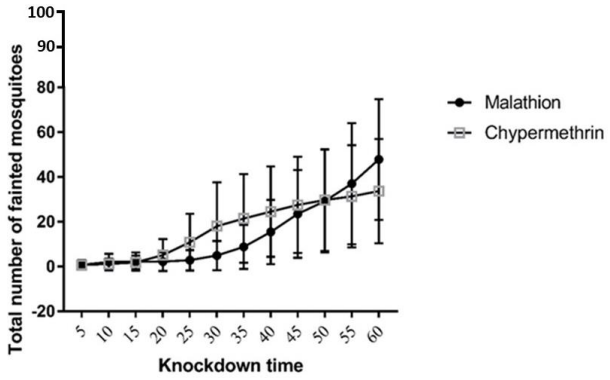

Figure 3. The trend of the knockdown mosquito number during 60 minutes exposed to two insecticide compounds

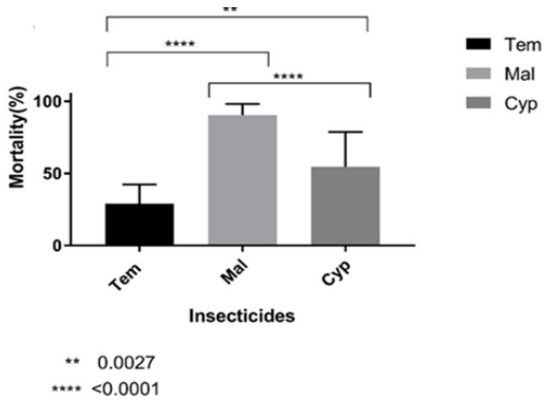

Figure 4. Comparison of mortality percentage between different types of insecticide compounds. The independent samples t-test exhibited a significant distinction between insecticide compounds. There is a sequence of mortality which clearly showed by each significance level; Malathion had the highest mortality rate ($p<0.0001$), while *Ae. aegypti* had the most resistance to temephos ($p<0.0001$ compared to malathion and $p=0.0027$ to cypermethrin)

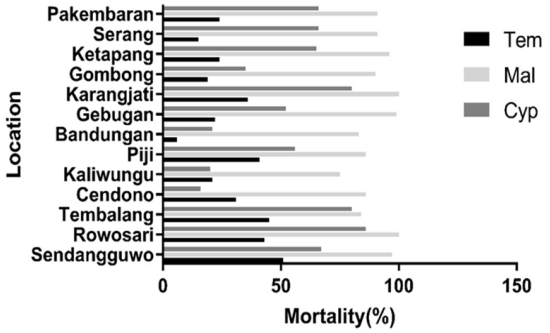

Figure 5. Contrasting different mortality levels of each insecticide compound based on study sites. The Dengue vector (*Ae. aegypti*) populations were resistant to cypermethrin and temephos compounds among all of the study sites (the Dengue-endemic areas) in Central Java Province, Indonesia, while susceptible to Malathion compound among three villages: Karangjati and Gebugan (Semarang district) and Rowosari (Semarang city)

Discussion

Monitoring the susceptibility of Dengue vectors to pyrethroid and organophosphate insecticide classes is an important method for understanding and mapping the distribution of the susceptible populations of the vectors. This situation needs to be understood before chemical control measure is done to accompany the selective insecticide-use policy in Indonesia. This study completed information on the previous studies by covering the wider Dengue endemic areas that have not been studied before (Sayono et al. 2016b). The result of this study showed two different susceptibility situations of the *Ae. aegypti* populations to three insecticide compounds. The susceptible strain to malathion 5% emerged in several study sites, although the species was resistant to cypermethrin-0.05% and temephos 0.02 ppm in all study sites. This study also found that *Ae. aegypti* populations in several study sites were resistant to three different classes of insecticide compounds simultaneously. This phenomenon indicated a multiple resistance of the species to pyrethroid and organophosphate insecticide classes (Nkya et al. 2014). Further investigations are needed to understand the emergence of the resistance genes conferring the knockdown and metabolic resistance among the populations when the bioassay test resulted in 90-97% of mortality (WHO 2016).

The susceptibility of *Ae. aegypti* to organophosphate and pyrethroid compounds has deteriorated in the last decade (Moyes et al. 2017). This condition has been separately reported in Indonesia, which is closely correlated with the use of insecticides in the Dengue vector control program in the last two decades (Mulyatno et al. 2012; Ikawati et al. 2015; Sayono et al. 2016b; Rahayu et al. 2017). A similar phenomenon has also been reported worldwide in other countries *Ae. aegypti* was resistant to pyrethroid and organophosphate compounds (Moyes et al. 2017).

This recent study comprehensively covered the wider areas in the different altitudes and geographic conditions from 12 to 1,200 meters above sea level (m asl) and from coastal to mountainous areas. The resistance status of *Ae. aegypti* to cypermethrin 0.05% and temephos 0.02 ppm were distributed throughout the localities. This condition is similar to the altitudinal distribution of this species' density in previous research (Sayono et al. 2017). The findings showed that the resistance of *Ae. aegypti* mosquitoes toward pyrethroid and organophosphate are not only focusing on urban areas but also on the high-altitude areas which possess more than 1,000 m asl where very limited studies have reported. This phenomenon is influenced by complex factors, including vector control measures, human movement, and agricultural pesticide use (Kamgang et al. 2011; Marcombe et al. 2012).

The expansion of the multiple resistance status of *Ae. aegypti* to the wider areas is in line with the expansion of Dengue cases from the epicenter in the Dengue-endemic cities to the neighboring areas. Dengue cases increased the community's efforts to control the disease by implementing chemical methods for Dengue vector control measures, especially fogging (Krianto 2009; Zahir et al. 2016). The

growth of transportation line intercity and from the city to villages is the main factor of Dengue expansion (Ren et al. 2019). This phenomenon also affects the Dengue vector displacement from the endemic to other areas. *Ae. aegypti* mosquitoes in the intensively Dengue-endemic areas exposed to insecticide and emerged resistant also participated in the migration to the other areas. That might affect the resistance status of the local population of *Ae. aegypti* (Sá et al. 2019). Further research on the genetic diversity of *Ae. aegypti* mosquitoes in the areas are needed to prove the displacement flow and mechanisms.

The low level of *Ae. aegypti* susceptibility to pyrethroid and organophosphate insecticide classes is predicted to be related to the use of those insecticide classes for decades to control the mosquito vector in adult and larval stages (Macoris et al. 2007). Another causal factor of the lower susceptibility of *Ae. aegypti* to pyrethroid insecticide is related to the intense use of commercial insecticides in the community (Gray et al. 2018). Most commercial insecticides contain pyrethroid compounds. This finding also proved that the mosquito susceptibility to insecticide is not affected by the altitudes of population habitats but indicated by the number of Dengue cases and endemicity of areas. The high occurrence of Dengue cases is usually followed by the vector control efforts of the community, mainly by applying chemical methods (Zahir et al. 2016).

This study indicated a reemerging of susceptible strains of *Ae. aegypti* to malathion compound in several parts of Central Java Province, Indonesia, after ten years of delay of the compound, although further studies are needed to extend the scientific proofing. The relaxation of insecticide exposure for a certain period will recover the genetic structure and increase the susceptibility of mosquitoes to an insecticide compound (Son-un et al. 2018). A community experiment in the resistant populations of *Ae. aegypti* must implement this relaxation.

Also, this finding presents the altitudinal distribution of 1016G kdr allele from 225 to 1,112 m asl study sites that have not been reported before in the Dengue endemic areas of Central Java Province, Indonesia. This phenomenon indicated the resistance of *Ae. aegypti* to cypermethrin 0.05% compound has spread widely across the elevation localities. The distribution of the kdr allele may occur in line with the *Ae. aegypti* mosquitoes spreading from Dengue endemic areas at the lower to the higher elevation influenced by some conditions, including the warming temperature, migration of population, the growth of transportation lines, the existence of breeding sites, and agricultural pesticide use (Marcombe et al. 2012). The resistant strains of *Ae. aegypti* in the foci of Dengue endemic areas may spread to other places along with the migration of the human population through varying transportation lines (Sa et al. 2019). Although we only obtained very limited molecular samples, this study founds 1016G kdr alleles scattered at various altitudes. With all the limitations, this preliminary data can be used as a starting point to develop further research on genetic diversity and the distribution of resistant genes to understand the mechanism of *Ae. aegypti* resistance in this area clearly.

Based on the susceptibility status of *Ae. aegypti* from this research, the allele frequency of 1016G is more dominant in the resistant group than the susceptible one. The absence of a mutant allele in the susceptible group of *Ae. aegypti* is hypothetically affected because allele 1016G is recessive as part of the kdr gene (Harris et al. 2010; Yanola et al. 2011). Thus, the mutational site of V1016G is not the only correlated point mutation of knockdown resistance in the *AaNav* gene of *Ae. aegypti* mosquitoes in the sampled location. Exposure to other insecticide classes and environmental factors could affect the resistance mechanism of mosquitoes.

DNA sequencing is the most precise method for detecting mutational location in a gene as it is the gold standard method. Still, the method is considered to be expensive and unsuitable for a large number of samples (Saingamsook et al. 2017). Therefore, several PCR methods have been developed to detect kdr alleles, i.e., real-time-PCR and heated oligonucleotide ligation assay (HOLA). However, the methods still lack efficiency (Saavedra-Rodriguez et al. 2007; Rajatileka et al. 2008). Therefore, a simpler genotyping method, i.e., AS-PCR, was developed to increase the efficiency of detecting many samples from the field (Stenhouse et al. 2013). Although AS-PCR is often underrated compared to nucleotide sequencing, several studies have shown that the method is reliable enough to detect the mutant allele (Yanola et al. 2011; Saingamsook et al. 2017). Additionally, the assay was validated to be comparable and in complete agreement with the DNA sequencing method (Saingamsook et al. 2017).

Insecticide resistance will increase 2-20 years after continuously being used for decades (Georghiou et al. 1983). Intensive insecticide use can act as a naturally selective agent of the mosquito population, which will maintain the resistant insects to survive and inherit them to the next generation (Srisawat et al. 2010). As an impact, the percentage of resistant insects will increase, and the susceptible strain will be eliminated due to insecticide utilization. Eventually, there will be an ineffective use of insecticide due to the imbalance between the number of resistant and susceptible strains. Son-un et al. (2018) identified that the recovery rate of mortality level would be reverted after 12 generations, which is estimated to be 6 months in time. Therefore, it is plausible hypothetically for the mosquito to revert to a vulnerable state after 5 years based on a previous explanation of the resistance spread rate. However, the previous findings did not account for natural circumstances such as random mating, migration, and other population genetic measures. The impact of household spray or other commercial insecticides was not covered by any research in which the application will cause a more complex strategy to control the resistance (Gray et al. 2018). Further research needs to be carried out to understand comprehensively the recovery rate of resistant individuals phenotypically and genotypically.

In conclusion, the resistant population of *Ae. aegypti* to cypermethrin 0.05% and temephos 0.02 ppm compounds spread widely throughout the Dengue endemic areas in Central Java Province along with the Dengue occurrence,

while the Malathion 5% susceptible strains are reemerging in several parts. Therefore, surveillance of the Dengue vector susceptibility must be conducted periodically in those areas before chemical control measure is done. The factual information is important to determine the suitable methods and strategies for controlling the Dengue, Chikungunya, and Zika vectors. This study showed that the genotypic change from valine to glycine of codon 1016 of the *AaNav* gene was present in all sampled areas following the phenotypic status.

ACKNOWLEDGMENTS

The authors wish to thank people who have consented to take part in this study, to the Directorate of Research and Development, Ministry of Research and Technology and Higher Education of Indonesia; Health Office of Central Java Province; Health Office of Semarang City, Kudus District, Semarang District, Pemalang District, and Tegal District, and those who have helped.

REFERENCES

Ahmad I, Astari S, Rahayu R, Hariani N. 2009. Status kerentanan *Aedes aegypti* (Diptera: Culicidae) pada Tahun 2006-2007 terhadap Malation di Bandung, Jakarta, Surabaya, Palembang dan Palu. Biosfera 26 (2): 85-89. DOI: 10.20884/1.mib.2009.26.2.119. [Indonesian]

Amelia-Yap ZH, Chen CD, Sofian-Azirun M, Lau KW, Suana IW, Harmonis. 2018a. Efficacy of mosquito coils: Cross resistance to pyrethroid in *Aedes aegyypti* (Diptera: culicidae) from Indonesia. J Econ Entomol 20 (10): 1-7. DOI: 10.1093/jee/toy296.

Amelia-Yap ZH, Chen CD, Sofian-Azirun M, Low VL. 2018b. Pyrethroid resistance in the dengue vector *Aedes aegypti* in Southeast Asia: Presence situation and prospects for management. Parasit Vector 11 (1): 332. DOI: 10.1186/s13071-018-2899-0.

Astuti EP, Ipa M, Pradani FY. 2012. Resistance detection of *Aedes aegypti* larvae to cypermethrin from endemic area in Cimahi city West Java. Aspirator 6: 7-12. DOI: 10.22435/aspirator.v6i1.3517.7-12.

Bharati M, Saha D. 2018. Multiple insecticide resistance mechanisms in primary dengue vector, *Aedes aegypti* (Linn.) from dengue endemic districts of sub-Himalayan West Bengal, India. PLoS ONE 13 (9): e0203207. DOI: 10.1371/journal.pone.0203207.

Brady OJ, Gething PW, Bhatt S, Messina JP, Brownstein JS, Hoen AG, Moyes CL, Farlow AW, Scott TW, Hay SI. 2012. Refining the global spatial limits of Dengue virus transmission by evidence-based consensus. PLoS Negl Trop Dis 6 (8): e1760. DOI: 10.1371/journal.pntd.0001760.

Brengues C, Hawkes NJ, Chandre F, McCarroll L, Duchon S, Guillet P, Manguin S, Morgan JC, Hemingway J. 2003. Pyrethroid and DDT cross-resistance in *Aedes aegypti* is correlated with novel mutations in the voltage-gated sodium channel gene. Med Vet Entomol 17 (1): 87-94. DOI: 10.1046/j.1365-2915.2003.00412.x.

Duffy MR, Chen T-H, Hancock WT, Powers AM, Kool JL, Lanciotti RS, Patick M, Marfel M, Holzbauer, Dubray C, Guillaumot L, Griggs A, Bel M, Lambert AJ, Laven JJ, Kosoy OL, Panella AJ, Biggerstaff BJ, Fischer M, Hayes EB. 2009. Zika virus outbreak on Yap Island, Federated States of Micronesia. N Engl J Med 360 (24): 2536-2543. DOI: 10.1056/NEJMoa0805715.

Elsinga J, Lizarazo EF, Vincenti MF, Schmidt M, Velasco-Salas ZI, Arias L, Bailey A, Tami A. 2015. Health seeking behaviour and treatment intentions of Dengue and fever: A household survey of children and adults in Venezuela. PLoS Negl Trop Dis 9 (12): e0004237. DOI: 10.1371/journal.pntd.0004237.

Fredericks AC, Fernandez-Sesma A. 2014. The burden of Dengue and Chikungunya worldwide: Implications for the Southern United States and California. Ann Glob Health 80: 466-475. DOI: 10.1016/j.aogh.2015.02.006.

Georghiou GP, Mellon RB. 1983. In: Georghiou GP, Sito T. (eds). Pest Resistance to Pesticides. Plenum Press, New York.

Gray L, Florez SD, Barreiro AM, Vadillo-Sánchez J, González-Olvera G, Lenhart A, Manrique-Saide P, Vazquez-Prokopec GM. 2018. Experimental evaluation of the impact of household aerosolized insecticides on pyrethroid-resistant *Aedes aegypti*. Sci Rep 8: 12535. DOI: 10.1038/s41598-018-30968-8.

Hamid PH, Prastowo J, Widyasari A, Taubert A, Hermosilla C. 2017. Knockdown resistance (kdr) of the voltage-gated sodium channel gene of *Aedes aegypti* population in Denpasar, Bali, Indonesia. Parasit Vectors 10: 283. DOI: 10.1186/s13071-017-2215-4.

Hardjanti A, Indrawati I, Donanti E, Wibowo H, Zulhasril. 2015. Detection of insecticide resistance in *Aedes aegypti* to organophosphate in Pulogadung, East Jakarta. Makara J Health Res 19 (3): 117-120. DOI: 10.7454/mjhr.v19i3.5563.

Harris AF, Rajatileka S, Ranson H. 2010. Pyrethroid resistance in *Aedes aegypti* from Grand Cayman. Am J Trop Med Hyg 83: 277-284. DOI: 10.4269/ajtmh.2010.09-0623.

Ikawati B, Sunaryo S, Widiastuti D. 2015. Peta status kerentanan *Aedes aegypti* (Linn.) terhadap insektisida cypermethrin dan malathion di Jawa Tengah. Aspirator 7: 23-28. DOI: 10.22435/aspirator.v7i1.3722.23-28. [Indonesian]

Kamgang B, Marcombe S, Chandre F, Nchoutpouen E, Nwane P, Etang J, Corbel V, Paupy C. 2011. Insecticide susceptibility of *Aedes aegypti* and *Aedes albopictus* in Central Africa. Parasit Vectors 4: 79. DOI: 10.1186/1756-3305-4-79.

Kawada H, Oo SZM, Thaung S, Kawashima E, Maung YNM, Thu HM, Thant KZ, Minakawa T. 2014. Co-occurrence of point mutations in the voltage-gated sodium channel of pyrethroid-resistant *Aedes aegypti* Populations in Myanmar. PLoS Negl Trop Dis 8 (7): e3032. DOI: 10.1371/journal.pntd.0003032.

Krianto T. 2009. Masyarakat Depok memilih fogging yang tidak dimengerti. KESMAS 4 (1): 29-35. [Indonesian]

Kwong JC, Druce JD, Leder K. 2013. Case report: Zika virus infection acquired during brief travel to Indonesia. Am J Trop Med Hyg 89 (5): 516-517. DOI: 10.4269/ajtmh.13-0029.

Lee H, Kim JE, Lee S, Lee CH. 2018. Potential effects of climate change on Dengue transmission dynamics in Korea. PLoS ONE 13 (6): e0199205 DOI: 10.1371/journal.pone.0199205.

Leung GH, Baird RW, Druce J, Anstey NM. 2015. Zika virus infection in Australia following a monkey bite in Indonesia. Se Asian J Trop Med 46 (3): 460-464.

Li CX, Kaufman PE, Xue RD, Zhao MH, Wang G, Yan T, Guo XX, Zhang YM, Dong YD, Zhang HD, Zao TY. 2015. Relationship between insecticide resistance and *Kdr* mutations in the Dengue vector *Aedes aegypti* in Southern China. Parasit Vectors 8: 325. DOI: 10.1186/s13071-015-0933-z.

Linss JGB, Brito LP, Garcia GA, Araki AS, Bruno RV, Lima JBP, Danies V, Martins AJ. 2014. Distribution and dissemination of the Val1016Ile and Phe1534Cys *Kdr* mutations in *Aedes aegypti* Brazilian natural populations. Parasit Vectors 7: 25-35. DOI: 10.1186/1756-3305-7-25.

Lozano-Fuentes S, Hayden MH, Welsh-Rodriguez C, Ochoa-Martinez C, Tapia-Santos B, Kobylinski KC, Uejio CK, Zielinski-Gutierrez E, Monache LD, Monaghan AJ, Steinhoff DF, Eisen L. 2012. The Dengue virus mosquito vector *Aedes aegypti* at high elevation in México. Am J Trop Med Hyg 85 (7): 902-909. DOI: 10.4269/ajtmh.2012.12-0244.

Macoris MLG, Andrighetti MTM, Otrera VCG, Carvalho LR, Junior ALC, Brogdon WG. 2007. Association of insecticide use and alteration on *Aedes aegypti* susceptibility status. Mem Inst Oswaldo Cruz 102 (8): 895-900. DOI: 10.1590/S0074-02762007000800001.

Marcombe S, Mathieu RB, Pocquet N, Riaz M-A, Poupardin R, ´lior SS, Darriet F, Reynaud S, Ye´bakima A, Corbel V, David JP, Chandre F. 2012. Insecticide resistance in the Dengue vector *Aedes aegypti* from Martinique: Distribution, mechanisms and relations with environmental factors. PLoS ONE 7 (2): e30989. DOI: 10.1371/journal.pone.0030989.

Martins AJ, Brito LP, Linss JGB, Rivas GBDS, Machado R, Bruno RV, Lima JBP, Valle D, Piexoto AA. 2013. Evidence for gene duplication in the voltage-gated sodium channel gene of *Aedes aegypti*. Evol Med Public Health 1: 148-160. DOI: 10.1093/emph/eot012.

Ministry of Health of the Republic of Indonesia. 2017. Profil Kesehatan Indonesia Tahun 2016. http://www.depkes.go.id/resources/down-

load/pusdatin/profil-kesehatan-indonesia/Profil-Kesehatan-Indonesia-2016.pdf. [Indonesian]

Moyes CL, Vontas J, Martins AJ, Ng LC, Koou SY, Dusfour I, Raghavendra K, Pinto J, David J-P, Weetman D. 2017. Contemporary status of insecticide resistance in the major *Aedes* vectors of arboviruses infecting humans. PloS Negl Trop Dis 11 (7): e0005625. DOI: 10.1371/journal.pntd.0005625.

Mulyatno KC, Yamanaka A, Ngadino, Konishi E. 2012. Resistance of *Aedes aegypti* (L.) larvae to temephos in Surabaya, Indonesia. Se Asian J Trop Med 43 (1): 29-33.

Musso D, Nilles EJ, Cao-Lormeau V-M. 2014. Rapid spread of emerging Zika virus in the Pacific area. Clin Microbiol Infec 20 (10): 595-596. DOI: 10.1111/1469-0691.12707.

Nkya TE, Poupardin R, Laporte F, Akhouayri I, Mosha F, Magesa S, Kisinza W, David JP. 2014. Impact of agriculture on the selection of insecticide resistance in the malaria vector *Anopheles gambiae*: A multigenerational study in controlled conditions. Parasit Vectors 7: 480. DOI: 10.1186/s13071-014-0480-z.

Olson JG, Ksiazek TG, Suhandiman, Triwibowo. 1981. Zika virus, a cause of fever in Central Java, Indonesia. Trans R Soc Trop Med Hyg 75 (3): 389-395. DOI: 10.1016/0035-9203(81)90100-0.

Perkasa A, Yudhaputri F, Haryanto S, Hayati RF, Ma'roef CN, Antonjaya U, Yohan B, Myint KS, Ledermann JP, Rosenberg R, Powers AM, Sasmoto RT. 2016. Isolation of Zika virus from febrile patient, Indonesia. Emerg Infect Dis 22 (5): 924-925. DOI: 10.3201/eid2205.151915.

Peterson J, Sammon M, Garg M. 2016. Dengue, Zika, and Chikungunya: Emerging Arboviruses in the new world. West J Emerg Med 17 (6): 671-679. DOI: 10.5811/westjem.2016.9.30904.

Putra RE, Ahmad I, Prasetyo DB, Susanti S, Rahayu R, Hariani N. 2016. Detection of insecticide resistance in the larvae of some *Aedes aegypti* (Diptera: Culicidae) strains from Java, Indonesia to temephos, malathion and permethrin. Intl J Mosq Res 3 (3): 23-28.

Rahayu N, Sulasmi S, Suryatinah Y. 2017. Status kerentanan *Aedes aegypti* terhadap beberapa golongan insektisida di Provinsi Kalimantan Selatan. J Health Epidemiol Commun Dis 3 (2): 56-62. DOI: 10.22435/jhecds.v3i2.1792. [Indonesian]

Rajatileka S, Black WC, Saavedra-Rodriguez K, Trongtokit Y, Apiwathnasorn C, McCall PJ, Ranson H. 2008. Development and application of a simple colorimetric assay reveals widespread distribution of sodium channel mutations in Thai populations of *Aedes aegypti*. Acta Trop 108: 54-57. DOI: 10.1016/j.actatropica.2008.08.004.

Ren H, Wu W, Li T, Yang Z. 2019. Urban villages as transfer stations for dengue fever epidemic: A case study in the Guangzhou, China. PLoS Negl Trop Dis 13 (4): e0007350. DOI: 10.1371/journal.pntd.0007350.

Reinhold JM, Lazzari CR, Lahondère. 2018. Effects of the environmental temperature on *Aedes aegypti* and *Aedes albopictus* mosquitoes: A review. Insects 9: 158. DOI: 10.3390/insects9040158.

Sá ELR, Rodovalho CM, Sousa NPR, Sá ILR, Bellinato DF, Dias LS, Silva LC, Martins AJ, Lima JBP. 2019. Evaluation of insecticide resistance in *Aedes aegypti* populations connected by roads and rivers: The case of Tocantins state in Brazil. Mem Inst Oswaldo Cruz 114: e180318 DOI: 10.1590/0074-02760180318.

Saavedra-Rodriguez K, Urdaneta-Marquez L, Rajatileka S, Moulton M, Flores AE, Fernandez-Salas I. 2007. A mutation in the voltage-gated sodium channel gene associated with pyrethroid resistance in Latin American *Aedes aegypti*. Insect Mol Biol 16: 785-798. DOI: 10.1111/j.1365-2583.2007.00774.x.

Saingamsook J, Saeung A, Yanola J, Lumjuan N, Walton C, Sombon P. 2017. A multiplex PCR for detection of knockdown resistance mutations, V1016G and F1534C, in pyrethroid-resistant *Aedes aegypti*. Parasit Vector 10: 465. DOI: 10.1186/s13071-017-2416-x.

Sayono S, Hidayati APN, Fahri S, Sumanto D, Dharmana E, Hadisaputro S, Asih PBS, Syafruddin D. 2016a. Distribution of voltage-gated sodium channel (Nav) alleles among the *Aedes aegypti* populations in Central Java Province and its association with resistance to pyrethroid insecticides. PLoS ONE 11 (3): e0150577. DOI: 10.1371/journal.pone.0150577.

Sayono, Nurullita U. 2016b. Situasi terkini vektor Dengue (*Aedes Aegypti*) di Jawa Tengah, Indonesia. KEMAS 11 (2): 285-294. [Indonesian]

Sayono S, Nurullita U, Sumanto D, Handoyo W. 2017. Altitudinal distribution of *Aedes* indices during the dry season in the dengue-endemic area of Central Java, Indonesia. Ann Parasitol 63 (3): 213-221. DOI: 10.17420/ap6303.108.

Son-un P, Choovattanapakorn N, Saingamsook J, Yanola J, Lumjuan N, Walton C, Somboon P. 2018. Effect of relaxation of deltamethrin pressure on metabolic resistance in a pyrethroid-resistant *Aedes aegypti* (Diptera: Culicidae) strain harboring fixed P989P and G1016G kdr Alleles. J Med Entomol 55 (4): 975-981. DOI: 10.1093/jme/tjy037.

Srisawat R, Komalamisra N, Eshita Y, Zheng M, Ono K, Itoh TQ, Matsumoto A, Petmitr S, Rongsriyam Y. 2010. Point mutations in domain II of the voltage-gated sodium channel gene in deltamethrin-resistant *Aedes aegypti* (Diptera: Culicidae). Appl Entomol Zool 45 (2): 275-282. DOI: 10.1303/aez.2010.275.

Stenhouse SA, Plernsub S, Yanola J, Lumjuan N, Dantrakool A, Choochote W, Somboon P. 2013. Detection of the V1016G mutation in the voltage-gated sodium channel gene of *Aedes aegypti* (Diptera: Culicidae) by allele-specific PCR assay, and its distribution and effect on deltamethrin resistance in Thailand. Parasit Vectors 6 (1): 253. DOI: 10.1186/1756-3305-6-253.

Widiastuti D, Sunaryo, Pramestuti N, Sari TF, Wijayanti N. 2015. Deteksi mutasi V1016G pada gen *Voltage-Gated Sodium Channel* pada populasi *Aedes aegypti* (Diptera: Culicidae) di Kabupaten Klaten, Jawa Tengah dengan metode *Allele-Specific* PCR. Vektora 7: 65-70. DOI: 10.22435/vk.v7i2.4505.65-70. [Indonesian]

Widjanarko B, Martini M, Hestiningsih R. 2017. Resistance status of *Aedes* sp. strain from high land in Central Java, Indonesia, as an indicator of increasing vector's capacity of dengue hemorrhagic fever. Ann Trop Med Public Health 10 (1): 71-75. DOI: 10.4103/ATMPH.ATMPH_78_17.

World Health Organization. 2016. Test procedures for insecticide resistance monitoring in malaria vector mosquitoes. 2nd Ed. Geneva: WHO Press. https://apps.who.int/iris/bitstream/handle/10665/250677/9789241511575-eng.pdf.

Wuliandari JR, Lee SF, White VL, Tantowijoyo W, Hoffmann AA, Endersby-Harshman NM. 2015. Association between three mutations, F1565C, V1023G and S996P, in the voltage-sensitive sodium channel gene and knockdown resistance *in Aedes aegypti* from Yogyakarta, Indonesia. Insects 6: 658-685. DOI: 10.3390/insects6030658.

Yanola J, Somboon P, Walton C, Nachaiwieng W, Somwang P, Prapanthadara L. 2011. High-throughput assays for detection of the F1534C mutation in the voltage-gated sodium channel gene in permethrin-resistant *Aedes aegypti* and the distribution of this mutation throughout Thailand. Trop Med Intl Health 16: 501-509. DOI: 10.1111/j.1365-3156.2011.02725.x.

Zahir A, Ullah A, Shah M, Mussawar A. 2016. Community participation, Dengue fever prevention and control practices in Swat, Pakistan. Intl J MCH AIDS 5 (1): 39-45. DOI: 10.21106/ijma.68.