Indonesian Journal of
Tropical and Infectious Disease
Vol. 7 No. 5 May-August 2019

Research Report

COMBINED TARGET SITE VGSC MUTATIONS PLAY A PRIMARY ROLE IN PYRETHROID RESISTANT PHENOTYPES OF *AEDES AEGYPTI* AS DENGUE VECTOR FROM PALU CITY, CENTRAL SULAWESI

Purwaningsih[1], Sitti Rahmah Umniyati[2a], Budi Mulyaningsih[2]
[1] Post Graduate Student of Tropical Medicine Program, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta.
[2] Department of Parasitology, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta.
[a] Coresponding author: sitti-rahmah@ugm.ac.id

ABSTRACT

It has been reported that Aedes aegypti mosquitoes in Palu City had been resistant to cypermethrin insecticide but the resistance mechanism is not well known. This study aimed to determine the resistance status of Ae. aegypti to cypermethrin and whether the mutation of voltage-gated sodium channel (VGSC) was associated with pyretroid resistance in high and low dengue endemic areas in Palu City. Aedes aegypti collected from each village was reared to adult and assayed for susceptibility test to cypermethrin using the CDC bottle bioassay method. PCR primers of AaSCF1 and AaSCR4 were used for screening of IIS6 VGSC gene mutation. PCR primers of AaSCF7 and AaSCR7 were used for screening of IIIS6 VGSC gene mutation. For an identification of mutation sites were sequenced and aligned to Gene bank (access No. AB914689 and AB914690) for IIS6 VGSC and Gene bank (access No. AB914687 and AB914688) for IIIS6 VGSC gene mutation. The susceptibility status of Ae. aegypti to cypermethrin was resistant in high dengue endemic areas and moderately resistant in low dengue endemic areas. It was found double point mutation at S989P and V1016G in Ae. aegypti from high and low dengue endemic areas in Palu City and there was a single point mutation only in high dengue endemic area at target site V1016G. Aedes aegypti from both high and low dengue endemic areas were resistant to cyperpethrinn and the two alleles had a major role in the occurrence of cypermethrin resistance in Palu City.

Keywords: *Aedes aegypti, resistance, pyrethroid, VGSC gene mutation*

ABSTRAK

Telah dilaporkan bahwa nyamuk Aedes aegypti di Kota Palu telah resisten terhadap insektisida sipermetrin, tetapi mekanisme resistensinya belum diketahui dengan baik. Penelitian ini bertujuan untuk mengetahui status resistensi Ae. aegypti terhadap sipermetrin dan untuk menentukan apakah mutasi voltage gated sodium channel (VGSC) dikaitkan dengan resistensi piretroid di daerah endemis dengue yang tinggi dan rendah di Kota Palu. Aedes aegypti yang dikumpulkan dari masing-masing desa dipelihara sampai dewasa dan diuji untuk uji kerentanan terhadap sipermetrin menggunakan metode CDC botol bioassay. Primer PCR AaSCF1 dan AaSCR4 digunakan untuk skrining mutasi gen IIS6 VGSC. Primer PCR AaSCF7 dan AaSCR7 digunakan untuk skrining gen IIIS6 VGSC. Untuk identifikasi lokasi mutasi disekuensing dan disejajarkan dengan bank Gene (akses No. AB914689 dan AB914690) untuk IIS6 VGSC dan Gene bank (akses No. AB914687 dan AB914688) untuk mutasi gen IIIS6 VGSC. Status kerentanan Ae. aegypti terhadap sipermetrin resisten di daerah endemik dengue tinggi dan resisten sedang di daerah endemik dengue rendah. Ditemukan titik ganda mutasi pada S989P dan V1016G Ae. aegypti dari daerah endemik dengue tinggi dan rendah di Kota Palu, dan ada satu titik mutasi hanya di daerah endemik dengue tinggi pada target site V1016G. Aedes aegypti dari daerah endemik dengue tinggi dan rendah resisten terhadap sipermetrin, dan kedua alel memiliki peran besar dalam terjadinya resistensi sipermetrin di Kota Palu.

Kata Kunci: *Aedes aegypti, resisttensi, pyrethroid, mutasi gen VGSC*

INTRODUCTION

Aedes aegypti is the most efficient vector for arboviruses because it is highly anthropophilic, frequently bites, and thrives in close proximity to humans.[1] Indonesia is a hyperendemic area with the spread of cases in both urban and rural areas.[2,3] DHF often causes outbreaks in several districts/cities in Indonesia. The number of dengue cases always increase every year. In 2016, Palu City had the highest number of DHF cases in Central Sulawesi Province, 2 people were died from Balaroa Village. Balaroa Village is categorized as high dengue endemic area while Siranindi Village is categorized as low dengue endemic area of DHF. Almost all Primary Health Facilities in Palu City had problems with DHF every year, and there are reported cases of death.[4]

Various DHF prevention policies and strategies have been programmed. The Government of Palu City issued Regional Regulation No. 2, 2016 about concerning the control of DHF as proof of the seriousness in DHF control efforts, but the results are still not optimal until now.[4] *Aedes aegypti* resistance to insecticides is a global phenomenon. Resistance is inherited and a single obstacle to the success of chemical vector control.[5] The using of insecticides from the community is a factor that triggers resistance.[6] The Health Office of Palu City used malathion insecticides for vector control since its establishment in 1997, but the using of malathion was stopped in 2013. The using of cypermethrin insecticide began in 2014.[4]

The latest information from several areas in Palu City is that adult mosquitoes of *Ae. aegypti* have been resistant to cypermethrin 0.05% based on the conventional method of impragnated paper.[7] Resistance mechanism of *Ae. aegypti* to cypermethrin insecticide has not been identified using this method. There are several methods to determine the resistance mechanism, including biochemical testing to detect the mechanism of metabolic resistance and molecular tests to determine the target site resistance mechanism to insecticides. An example of the target site resistance mechanism to pyrethroids is known as knockdown resistant (kdr)/voltage gated sodium channel (VGSC) gene. The molecular method can determine mutations in the VGSC gene due to selection pressure of insecticides in the organochlorine and pyrethroid groups.[8]

This study was aimed to determine the resistance status of *Ae. aegypti* to cypermethrin and to determine whether the mutation of the voltage-gated sodium channel (VGSC) gene was associated with pyrethroid resistance in high and low dengue endemic areas in Palu City.

MATERIALS AND METHODS

The observational study with cross-sectional analytical design[9] was approved by the Medical and Health Research Ethics Committee of Faculty of Medicine of Universitas Gadjah Mada number KE /FK/262/EC/2017.

One hundred and twenty ovitraps were installed in high and low dengue endemic area (Balaroa and Siranindi Villages). The coordinates of sampling locations are recorded using GPS (Global Positioning System). The ovitraps were installed for 3-4 days and the ovistrips were carefully dried, labeled and inserted in clear plastics and labeled to be stored.

The mosquito eggs were colonized in the insectarium of Department of Parasitology of the Faculty Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta. The eggs hatch into larvae were kept until they became pupae. They were taken with a pipette and kept in a cage. Pupae developed into adult mosquitoes after 2 days. Adult mosquitoes were fed 10% sugar solution which was absorbed in cotton. The temperature of test room was 27 ± 2°C, humidity was 75 ± 10% and photoperiod consisted of 12 hours of light: 12 hours of darkness.[10] The Adult mosquitoes were identified to determine *Ae. aegypti* mosquito and were colonized to the F1 Generation mosquito

CDC Bottle Bioassay

Each testing was involved 125 female adults of *Ae. aegypti* F1 generation, aged 3-5 days. Female mosquitoes were fed only with 10% sugar solution the day before testing. The test used 1 control bottle, and 4 test bottles. Each bottle was labeled (4 test bottles, 1 control bottle). The test bottle was filled with 1ml of cypermethrin 10µg/ml solution and the control bottle was filled with 1ml of acetone, then the bottle was tightly closed and the solution was coated on the wall, bottom and bottle cap. Bottles were dried at room temperature for 24 hours. Using an aspirator 25 mosquitoes were introduced into each test bottle and control bottle. A number of knockdown and or alive mosquitoes were recorded every 5 minutes during the diagnostic time of 30 minutes of exposure. Observation was continued until all are dead or up to 2 hours. Mortality was corrected with Abbott's Formula if the mortality at 2 hours in the control bottle is between 3% and 10%. The result should be discarded if the mortality in the control bottle > 10%. Mosquitoes were moved to an insecticide-free recovery cage and were administered with 10% sugar for 24 hours and recorded the number of dead mosquitoes.

$$\text{Abbott's Formula} = \frac{\%\ \text{mortality in test} - \%\ \text{mortality in control}}{100 - \%\ \text{mortality in control}} \times 100\%$$

The resistance status was classified in three categories according to WHO as follows: 98–100% mortality at the recommended diagnostic time indicates susceptibility; 80–97% mortality at the recommended diagnostic time suggests the possibility of resistance that needs to be confirmed; <80% mortality at the recommended diagnostic time suggests resistance.[11]

The Molecular Test

The isolation of genomic DNA was done individually using Genomic DNA mini Kit Geneaid™ Cat No. GB100. Lot No.JM02202 according to the manufacturer's

instructions.[12] PCR primers AaSCF1 (AGA CAA TGT GGA TCG CTT CC) and AaSCR4 (GGA CGC AAT CTG GCT TGTTA) were used for screening of IIS6 VGSC gene mutation. PCR primers of AaSCF7 (GAG AAC TCG CCG ATG AAC TT) and AaSCR7 (GAC GAC GAA ATC GAA CAG GT) were used for screening of IIIS6 VGSC gene mutations.[13] The PCR mixtures contained 15 µl of Mix PCR (Go Taq® Green Master mix. 2x), 11 µl of ddH2O (nuclease-free water lot. 0000123190. Promega), 2 µl of R & F primers (20 µM), 2 µl of the DNA template in a total volume of 30µl. PCR was performed under the following conditions: initial denaturation at 94°C for 3 min; 35 cycles each of 94°C for 15s, 55°C for 30s, and 72°C for 30s; and a final elongation step at 72°C for 10 min.[13]

The DNA amplification results were separated according to the size of the base pair by electrophoresis technique. Electrophoresis technique used 2% agarose gel which added 1 µl gel red. The Gel was inserted in chamber electrophoresisis which already contained solution buffer 50X TAE to cover surface gel. Product PCR was taken up 7 µl and inserted on gel well. Standard molecules were used 100 kb ladder marker. Power supply was run with potential difference of 100 volts for approximately 45 minutes. Observation of the DNA bands was done below UV light on Gel doc. The electrophoresis results were read on the target band and documented. The samples which showed bands of target DNA were sent to PT. Genetica Science Indonesia. Samples would be sent to 1st Base Laboratories Singapore for sequencing.

For an identification of mutation sites were sequenced and aligned to Gene bank (access No. AB914689 and AB914690) for IIS6 VGSC gene mutation (S989P, I1011M/V, V1016G/I) and aligned to Gene bank (access No. AB914687 and AB914688) for IIIS6 VGSC gene mutation (T1520I and F1534C)[13] using mega version 7.0.18 and bio edit version 7.2.6.

RESULTS AND DISCUSSION

Result of CDC Bottle Bioassay

It was shown the susceptibility status of *Ae. aegypti* to cypermethrin based on CDC bottle bioassays in high and low dengue endemic areas. The result of statistical analysis through bivariate test using independent T-test, obtained that the result the susceptibility status based on mortality rate between high and low dengue endemic area were significant differences with p value = 0.000 (p <0,05).

It was indicated that there was knockdown resistance (kdr) of *Ae. aegypti* mosquitoes from high and low dengue endemic areas, because there were reduction of mortality, about 26% dan 19,5% after 24 hours in recovery cages (Table 1).

Result of Molecular Assays and Sequencing Analysis

The amplification results of IIS6 and IIIS6 VGSC gene were visualized with 2% agarose gel electrophoresis and read under UV obtained specific band, 619 bp and 748 bp respectively (Figure 1 and Figure 3).

The PCR product was sequenced to determine the mutation of IIS6 VGSC gene (Figure 2). The point mutation of S989P at IIS6 site occurred because one of nucleotide base changed from thymine to citocin at codon TCC → CCC caused the amino acid changed from serine to proline.

Figure 2 showed, there were double point mutation in target site S898P and V1016G and there was single point mutation in target site V1016G.

The PCR product was sequenced to determine the mutation of IIIS6 VGSC gene (Figure 4).

Table 1. The result of CDC bottle bioassay of *Ae. aegypti* to cypermethrin 10 µg/bottle (diagnostic dose)

Location (villages)	Generation	Mortality (%)			Category
		30 minutes	2 hours	Holding 24 hours 24 jam	
High endemic dengue area (Balaroa)					
Test bottle-1	F1	65	98	66	resistant
Test bottle-2	F1	59	100	82	resistant
Test bottle-3	F1	66	99	71	resistant
Average high endemic		63,33	99	73	resistant
Control bottle	F1	0	0	0	
Low endemic dengue area (Siranindi)					
Test bottle-1	F1	91,2	95,2	75,2	moderate resistant
Test bottle-2	F1	89,6	95,2	80,8	moderate resistant
Test bottle-3	F1	90,4	97,6	73,6	moderate resistant
Average low endemic		90,4	96	76,5	moderate resistant
Control bottle	F1	0	0	0	
Laboratory Strain	F1057	99	100	100	susceptible

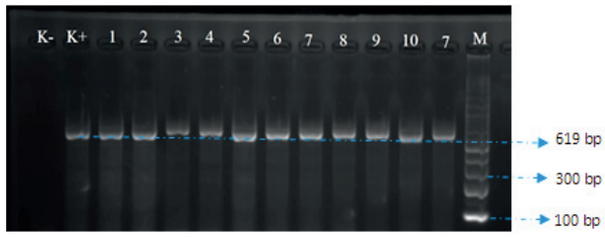

Figure 1. Visualization of IIS6 VGSC gen amplification of *Ae. aegypti* from Balaroa (1-6) and Siranindi (7-10), M (100 lader DNA marker), K- (negative control, without VGSC gene DNA), K+ (positive control of VGSC gene)

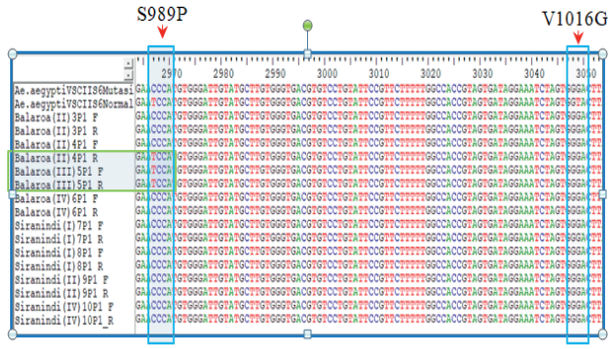

Figure 2. Result sequensing aligned with gen bank access AB914689 and AB914690 indicated mutation of IIS6 VGSC gene of *Ae. aegypti* target site Serin (TCC) 989 Prolin (CCC) and Valin (GTA) 1016 Glycin (GGA) (Mega version of 7.0.18 and Bio edit version of 7.2.6)

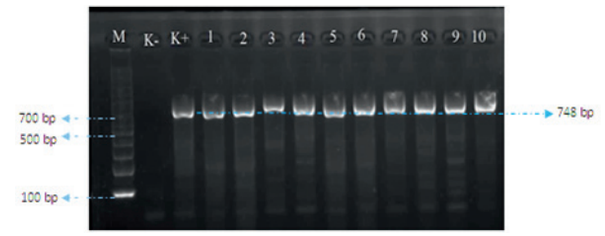

Figure 3. Visualization of IIIS6 VGSC gene amplification of *Ae. aegypti* from Balaroa (1-6) and Siranindi (7-10), M (100 lader DNA marker), K- (negative control, without VGSC gene DNA), K + (positive control of VGSC gene)

DISCUSSION

It was shown that the susceptibility status of *Ae. aegypti* to cypermethrin insecticide was resistant in high endemic dengue area and moderate resistant in low endemic dengue area.

Differences in susceptibility status between regions can occur because they are influenced by differences in knowledge, education, control efforts and frequency of insecticides used for health and agricultural purposes.[14] Cypermethrin insecticide began to be used for the benefit

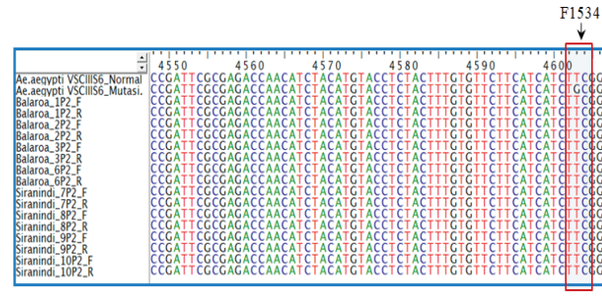

Figure 4. Results of sequencing aligned with Gene bank access AB914687 and AB914688. There weren't change TTC (phenilalanine) to Cystein (TGC) (Mega version of 7.0.18 and Bio edit version of 7.2.6)

of the program in Palu City, in 2014. Insect resistance to insecticides generally occurs after 2-20 years of use. The use of insecticides on a large scale, continuously for a long period of time and high frequency can cause a decrease in susceptibility to mosquitoes targeted.[15]

The process of the occurrence of vector resistance to certain insecticides is influenced by multiple factors, namely genetic (presence of specific gene frequencies), operational (insecticide type and application) and biological (size and characteristics of vector populations).[16] The different of susceptibility status in these two regions due to operational factors, namely vector control through fogging from the program until now is still being used. Fogging is carried out when there is a DHF case report. Dengue endemic areas have a higher fogging frequency compared to low dengue endemic areas.

Another factor that triggers a decrease in the susceptibility status of *Ae. aegypti* mosquitoes from these two villages were the use of household insecticides by the local community. Some household insecticides such as aerosol formulations and other formulations were used in Balaroa and Siranindi Villages, 28.2% and 36.95% respectively. The active ingredients used were malation and other active ingredients such as propoxur (bendiocarb) which can cause multiple resistance. *Aedes aegypti* were still undergoing selection pressure on organophosphate insecticides in Palu City, but selection pressure was higher for cypermethrin insecticides because of the use of the program and the effect of exposure from household insecticides. The data of research through structured interviews showed that household insecticides in Balaroa and Siranindi Villages were equal to 76.67%. Most household insecticides were pyrethroids. The percentage of household insecticides which was quite high affects the susceptibility status to cypermethrin insecticides in both regions.

Another factor that influences the increase in resistance status is the ability of mosquitoes to adapt and evolve well. Mosquitoes have a high reproductive speed and a short generation period so that mosquitoes are susceptible to genetic mutations.[17] This is evidenced by research in Malaysia which shows an increase in *Ae. aegypti* resistance to permethrin is 5-18 times after five generations.[18,19]

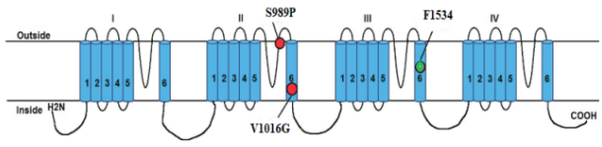

Figure 5. IIS6 VGSC double point mutation target site S989P and V1016G associated to resistance to pyrethroid from *Ae. aegypti* were mostly detected in this study

Aedes aegypti resistance against cypermethrin insecticide occurs in West Venezuela,[20] and several regions in Central Java (Semarang, Grobogan, Purbalingga and Kendal).[21] The resistance of *Ae. aegypti* against deltamethrin is also reported in Central Java (Semarang, Jepara, Blora, Salatiga, Surakarta, Tegal, Magelang and Purwokerto).[22]

The mechanism of resistance to pyrethroid insecticides can be detected molecularly. This study indicated that there was a target site mutation in the VGSC gene. The target site mutation in VGSC gene regarding resistance to pyrethroids suggests that there is an ongoing resistance mechanism. Detection of VGSC gene mutations can directly assess the transformation of target cells which are the target of insecticides. Gene mutation causes conformational changes in the sodium channel because it can not be opened by insecticide molecules.

Mutations like this can only be detected by molecular methods. The basic principle of molecular detection of resistance in vectors is identify genes.

The results showed that most of the samples of *Ae. aegypti* from Balaroa and Siranindi villages, Palu City experienced double-point mutations (two-point mutations simultaneously) at S989P, and V1016G in IIS5-S6 and IIS6 VGSC genes respectively (Figure 5). The results of susceptibility test gave a very specific description of phenotypic resistance events and were supported by mutations found in *Ae. aegypti* mosquito in VGSC gene (genotypic resistance). A valine to glycine transversion in domain II of the VGSC (V1016G) is associated with resistance to type I and II pyrethroids, such as permethrin and deltamethrin, respectively.[23]The V1016G mutation is often found with a serine to proline mutation (S989P) in domain II. They have also been found in several other regions of Asia. Mutations were reported at these points in Thailand, Myanmar, Vietnam, Taiwan and Indonesia.[24]

In this study, the only one sample experienced a single point mutation at the V1016G target site in Balaroa Village. It was also reported in Klaten, Central Java.[23] The results of study by Rajatileka[25] and Srisawat[26] found a point mutation at target site V1016G in VGSC gene of *Ae. aegypti* that was associated with pyrethroid synthetic resistance in Thailand.

The V1016G allele seems limited in Southeast Asia, but recently the V1016G allele was found in Mecca.[27] Different substitutions of V1016I are found in *Ae. aegypti* populations from Brazil V1016I.[28,29] The V1016I allele was distributed in South and North America (Alvarez *et al*., 2013)[20], but the V1016I allele was also found in Palembang-Indonesia (Ghiffari *et al*., 2013).[30]

Transformation in valine to glycine IIS6 VGSC (V1016G) were associated with resistance to type I and II pyrethroids, such as permethrin and deltamethrin.[23] Pyrethroids mainly affect the peripheral and central nervous system in insects by binding to the VGSC target site in the nerve membrane. Some of the advantages of insecticides from this group include low levels of toxicity to humans and mammals in general and easily decompose in the soil (Martins *et al*., 2009).[28]

Pyrethroid is divided into 2 types based on chemical structure & the effects, such as pyrethroid Type I and Type II. Pyrethroid type II contains parts of α-cyano-3-phenoxybenzyl alcohol such as cypermethrin, sifulthrin, deltamethrin, fenvalerate, esfenvalerate and lamellalhalrin (Ishak *et al*, 2015).[31] It was reported by Al Nazawi[27] that the *Ae. aegypti* sample was resistant to deltamethrin. The strain from Mecca experienced a point mutation simultaneously S989P and V1016G. Point mutations found simultaneously were also reported in *Ae. aegypti* populations from Latin America in different substitutions and alleles, I1011V and V1016I (Plernsub *et al*., 2016).[32]

Mutation is a marker for monitoring resistance (Ishak et al., 2015).[31] According to Widyastuti *et al.* (2015)[33] that VGSC gene mutations in several positions can occur simultaneously in one individual mosquito and the possible effect will be greater on mosquito resistance properties. *Aedes aegypti* V1016G strains (occurring with and without S989P) and F1534C mutations are common and widespread throughout Asia. The G1016 allele was known to be associated with resistance to type I and II pyrethroids. The C1534 allele was mainly associated with resistance to pyrethroid type I and known as recessive alleles (Ghiffari *et al*., 2013.[30] F1534 allele of this study are similar to those conducted by Stenhouse.[24]

CONCLUSION

Aedes aegypti from high and low dengue endemic areas were resistant to cyperpethrinn, and the two alleles (V1016G and S989P) had a major role in the occurrence of cypermethrin resistance in Palu City.

ACKNOWLEDGEMENT

The author would like to express sincerely gratitude the health office of Central Sulawesi Province for funding this research and Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada Yogyakarta for providing an opportunity to do this study in laboratory of department of parasitology and integrated research laboratory.

REFERENCES

1. WHO. *Dengue Guidelines for Diagnosis, Treatment, Prevention and Control.* Geneva, Switzerland: WHO; 2009.
2. World Health Organization. Comprehensive guidelines for prevention and control of dengue and dengue haemorrhagic fever. South-East Asia: WHO, 2011.
3. World Health Organization. National guidelines for clinical management of dengue fever. India: WHO, 2015.
4. Dinas Kesehatan Kota Palu. 2017. Pengendalian Penyakit Demam Berdarah Dengue. Peraturan Daerah (PERDA) Kota Palu No. 2 Tahun 2016. Available from: https://peraturan.bpk.go.id/Home/Details/20800.
5. Kementrian Kesehatan Republik Indonesia. Pedoman penggunaan insektisida (pestisida) dalam pengendalian vektor. Jakarta: Direktorat Jendral Pengendalian dan Penyehatan Lingkungan, 2012. Available from:http://perpus.b2p2vrp.litbang.depkes.go.id/opac/index.php?p=show_detail&id=1107&keywords=
6. Yuliani TS, Triwidodo H, Mudikdjo K, Panjaitan, NK, Sjafrida Manuwoto S. Pestisida rumah tangga untuk pengendalian hama permukiman pada rumah tangga. JPSL, 2011;2 (1): 73-83.
7. Jastal. Pemetaan status resistensi insektisida di Indonesia tahun 2015. Laporan penelitian. Balai Litbangkes Donggala, 2015. Available from: https://docplayer.info/44333679-Laporan-tahunan-tahun-balai-penelitian-dan-pengembangan-pengendalian-penyakit-bersumber-binatang-donggala.html
8. Smith LB, Kasai S, Scott JG. Pyrethroid resistance in *Aedes aegypti* and *Aedes albopictus*: Important mosquito vectors of human diseases. Pestic. Biochem. Physiol. 2016. 133: 1-12.
9. Sastroasmoro S dan Ismail S. Dasar-dasar metodologi penelitian klinis. 5th Ed. Jakarta: CV. Sagung Seto. 2014. 104-110.
10. World Health Organization. Monitoring and managing insecticide resistance in Aedes mosquito populations. Genewa: WHO, 2016. Available from:https://www.who.int/csr/resources/publications/zika/insecticide-resistance/en/
11. Center for Desease Control and Prevention Gudline for evaluating insecticide resistance in vector using the CDC bottle bioassay. USA: CDC, 2010. 1-83.
12. Anonymus. Genomic DNA Mini Kit (Blood/Cultured Cell). Geneaid www.geneaid.com Available from: Geneaid www.geneaid.com. http://www.geneaid.com/products/genomic-dna-purification/dna-extraction-kit-blood-cultured-cell-miniprep.
13. Kawada H, Oo SZM, Thaung S, Kawashima E, Maung YNM, Thu HM, et al. (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. https://doi.org/10.1371/journal.pntd.0003032.
14. Ponlawat A, Scott JG, Harington LC. Insecticide susceptibility of *Aedes aegypti* an *Aedes albopictus* across Thailand. J Med Entomol. 2015; 42: 821-825.
15. Chareonviriyaphap T, Bangs MJ, Suwonkerd W, Kongmee M, Corbel V, Ngoen-Klan R. Review of insecticide resistance and behavioral avoidance of vectors of human diseases in Thailand. Parasit Vectors. 2013. 6: 280. doi: 10.1186/1756-3305-6-280
16. Seixas G, Grikoraki L, Weetman D, Vicente JL, Silva AC, Pinto J, Vontas J, Sousa JA. Insecticide resistance is mediated by multiple mechanisms in recently introduced *Aedes aegypti* from Madeira Island (Portugal). PLoS Negl Trop Dis. 2017. https://doi.org/10.1371/journal.pntd.0005799
17. Ahmad I. Adapatsi serangga dan dampaknya terhadap kehidupan Manusia. 2011.Pidato ilmiah Guru Besar Institut Teknologi Bandung (ITB). Available from: http://rektorkita.itb.ac.id/files/balon/IA_CV.pdf
18. Mantolu Y, Kustiati, Ambarningrum, TB, Yusmalinar S, Ahmad I. Status dan perkembangan resistensi *Ae. aegypti* (Linnaeus) (Diptera: Culicidae) Strain Bandung, Bogor, Makassar, Palu dan VCRU terhadap tnsektisida permetrin dengan seleksi lima generasi. J Entomol Indonesi. 2018. 13(1):1-8.
19. Amelia-Yap ZH, Ahee DC, Azirun MS, Van LL, Pyrethroid resistance in the dengue vector *Aedes aegypti* in Southeast Asia: present situation and prospects for management. Parasite Vectors.2018. 11.332:1-17. doi: 10.1186/s13071-018-2899-0
20. Alvarez LC, Ponce G, Oviedo M, Lopez B, Flores AE. Resistance to malation and deltamethrin in *Aedes aegypti* (Diptera : Culicidae) From Western Venezuela. Entomol Soc Am. 2013. 50(5):1031–9.
21. Widiarti, Heriyanto B, dan Boewono, DT. Peta resistensi vektor demam berdarah dengue *Aedes aegypti* terhadap insektisida kelompok organophosfat, karbamat dan piretroid di Provinsi Jawa Tengah dan Yogyakarta. Buletin Penelitian Kesehatan. 2011. (4): 39.
22. Sunaryo, Ikawati B, Rahmawati., Widiastuti D. Status resistensi vektor demam berdarah dengue (*Aedes Aegypti*) terhadap malation 0,8% dan permethrin 0,25% di Provinsi Jawa Tengah. Ekologi Kesehatan. 2014. Vol. 13 No. 2.
23. Ikawati B, Sunaryo,Widiastuti D. Peta status kerentanan *Aedes aegypti* (Linn.) terhadap insektisida cypermethrin dan malation di Jawa Tengah. Aspirator. 2015. 7(1): 23-8.
24. Stenhouse SA, Plernsub S, Yanola J, Lumjuan N, Dantrakool A, Choochote W, Somboon P. 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. Parasites & Vectors. 2013.6: 253.
25. Rajatileka S, William CB, Saavedra KR, Yuwadee T, Chamnarn A, McCalla PJ, and Hilary R. Development and application of a simple colorimetric assay reveals widespread distribution of sodium shannel mutations in thai populations of *Aedes aegypti*. Acta Tropica. 2008. 108: 54–7.
26. Srisawat R, Komalamisra N, Eshita Y, Zheng M, Ono K, Itoh TQ, Matsumoto A, Petmitr S, and Rongsriyam Y. Point mutations in domain II of the voltage-gated sodium channel gene in deltamethrin-resistant *Aedes aegypti* (diptera: Culicidae). Appl. Entomol. Zool. 2010; 45. 275–282.
27. Al Nazawi AM, Aqili J, Alzahrani M, McCall PJ, and Weetman D. Combined target site (kdr) mutations play a primary role in highly pyrethroid resistant phenotypes of Aedes aegypti from Saudi Arabia. Parasites & Vectors. 2017;10:161.
28. Martins AJ, Lima JB, Peixoto AA, and Valle D. Frequency of Val1016Ile mutation in the voltage-gated sodium channel gene of Aedes aegypti Brazilian populations. Trop Med Int Health. 2009.14(11): 1351-5, ISSN 1365-3156.
29. Lima EP, Paiva MHS, De Araújo AP, Da Silva ÉVG, Da Silva UM., De Oliveira A.E.G. Santana, C.N.Barbosa, C.C.P. Neto, M.O.F Goulart, C.S. Wilding, Ayres LN, and Santos MAVM. Insecticide resistance in Aedes aegypti populations from Ceará, Brazil. Parasites & Vectors. 2011. 4(5):1-12.
30. Ghiffari A, Fatimi H, dan Anwar C. Deteksi resistensi insektisida sintetik piretroid pada Aedes aegypti (l.) strain Palembang menggunakan teknik polymerase chain reaction. Aspirator. 2013. 5 (2): 37-44.
31. Ishak IH, Jaal Z, Ranson H, and Wondji CS. contrasting patterns of insecticide resistance and knockdown resistance (kdr) in the dengue vectors Aedes aegypti and Aedes albopictus from Malaysia. Parasites & Vectors. 2015. 8:181.
32. Plernsub S, Saingamsook J, Yanola J, Lumjuan N, Tippawangkoso P,Sukontason K, Walton C, and Somboon P. Additive effect of knockdown resistance mutations, S989P, V1016G and F1534C,in a heterozygous genotype conferring pyrethroid resistance in *Aedes aegypti* in Thailand. Parasites & Vectors, 2016; 9:417.
33. Widyastuti D, Sunaryo, Pramestuti N, Sari TF, Wijayanti N. 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. 2015. 7 (2): 65-70.