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Bulan, Tahun : Januari, 2023

Author Korespondensi: Sayono Sayono

Bukti-bukti aktivitas korespondensi terlampir, secara berturut-turut:

1. Cover Letter
2. Title Page
3. Whole Manuscript
4. Submission acknowledgement
5. Editor Decision
6. Author Response (Revisions attachment)
7. Final Decision (Acceptance statement/letter)

Cover Letter

UNIMUS

UNIVERSITAS MUHAMMADIYAH SEMARANG
FACULTY OF PUBLIC HEALTH

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Email: fkm@unimus.ac.id; URL: fkm.unimus.ac.id

Semarang, November 15th 2022

The Editor-in-Chief: Biodiversitas

Dear Sir,

Attached, please find our manuscript entitled:

Morphological and molecular detection to pyrethroid and organophosphate insecticide classes in Central Java, Indonesia: indicating a cross resistance

which we would like to submit to the scientific journal that you run as an original research paper.

Information regarding mosquito vectors and their susceptibility to insecticides in Indonesia is hardly accessible to the broad scientific community and the health policy planner. As a part of our attempts to determine and map the magnitude of the Dengue vector susceptibility to insecticides in Indonesia, we would like to share our data that might be important for the establishment of a vector dengue control program in the area and also provides the scientific information for the Dengue vector in Indonesia.

We do believe that the manuscript would fill the data unavailability and is also very much relevant to your reader.

I am looking forward to hearing your favorable reply

Sincerely yours,
S. Sayono
On behalf of the authors

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Title Page

Morphological and molecular detection of insecticides resistance of the Dengue vector, *Aedes aegypti* in Central Java Province, Indonesia

Sayono Sayono[1✉], Ulfa Nurullita[1], Wahyu Handoyo[1,2], Winda Septy Tyasningrum[1], Irfanul Chakim[1], Anto Budihardjo[3]

[1]Faculty of Public Health, Universitas Muhammadiyah Semarang, Semarang, Indonesia

[2]Provincial Health Office of Central Java Province Government, Semarang, 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

Abstract word count: 191

Original article

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ACKNOWLEDGEMENTS

The authors wish to thank people who have consented to take a part in this study, to 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.

Gmail

Sayono Sayono <say.epid@gmail.com>

[biodiv] Submission Acknowledgement

1 pesan

Ahmad Dwi Setyawan <support@mail.smujo.id> 28 November 2022 pukul 17.10
Kepada: Sayono Sayono <say.epid@gmail.com>

Sayono Sayono:

Thank you for submitting the manuscript, "Morphological and molecular detection of insecticides resistance of the Dengue vector, Aedes aegypti in Central Java Province, Indonesia" to Biodiversitas Journal of Biological Diversity. With the online journal management system that we are using, you will be able to track its progress through the editorial process by logging in to the journal web site:

Submission URL: https://smujo.id/biodiv/authorDashboard/submission/13034
Username: sayono

If you have any questions, please contact me. Thank you for considering this journal as a venue for your work.

Ahmad Dwi Setyawan

Biodiversitas Journal of Biological Diversity

Gmail

Sayono Sayono <say.epid@gmail.com>

[biodiv] Editor Decision

2 pesan

Smujo Editors <support@mail.smujo.id> 22 Desember 2022 pukul 07.38
Kepada: Sayono Sayono <say.epid@gmail.com>, Ulfa Nurullita <ulfa@unimus.ac.id>, Wahyu Handoyo <wahyu_ob@yahoo.co.id>, Winda Septy Tyasningrum <septyaswinda@gmail.com>, Irfanul Chakim <irfan.unimus@gmail.com>, Anto Budiharjo <abudiharjo@yahoo.com>

Sayono Sayono, Ulfa Nurullita, Wahyu Handoyo, Winda Septy Tyasningrum, Irfanul Chakim, Anto Budiharjo:

We have reached a decision regarding your submission to Biodiversitas Journal of Biological Diversity, "Morphological and molecular detection of insecticides resistance of the Dengue vector, Aedes aegypti in Central Java Province, Indonesia".

Our decision is: Revisions Required

Reviewer Q:

The tile of the paper needs to be changed. It should be "Bioassay and molecular detection of insecticides resistance of *Aedes aegypti* , vector of dengue in Central Java Province, Indonesia.

The number of mosquitoes used for the test should be increased. Here only 150 mosquitoes were used. It will be good if the experiments can be repeated three times. this will provide more robust results. For the larvae how many larvae were tested. For the molecular testing, the number of samples should be increased. The samples are too small.

Figs 3 and 4 you have combined all the sites together? I feel they should be shown separately.

The manuscript has to be sent for editing before it can undergo proper review. I also fell that the introduction can be shortened and some of the facts can be used in the discussion

Recommendation: Revisions Required

Biodiversitas Journal of Biological Diversity

U-Morphological and molecular detection of insecticides resistance of the Dengue vector, Aedes aegypti in Central Java Provin.doc
1241K

Sayono Sayono <say.epid@gmail.com> 24 Desember 2022 pukul 16.01
Kepada: Smujo Editors <support@mail.smujo.id>

Dear Editor,
We have revised the attached article according to reviewer Q's recommendations. We highlight the parts that were changed in yellow. The essence of the change is:
1. Title (according to the reviewer's suggestion)
2. Number of samples; we describe sample counts per site and test replication and sample totals we show.
3. Request the reviewers to analyze Figures 3 and 4 separately according to the research location. This has actually been stated in more detail in Table 1 and Figure 5, so we only add explanations according to the request.

Best regard,

Sayono
Department of Epidemiology and Tropical Diseases
School of Public Health of Universitas Muhammadiyah Semarang
Jalan Kedung Mundu Raya 18, Semarang, 50273
Indonesia

[Kutipan teks disembunyikan]

U-Morphological and molecular detection of insecticides resistance of the Dengue vector, Aedes aegypti in Central Java Provin.docx
1085K

[biodiv] Editor Decision

2 pesan

Smujo Editors <support@mail.smujo.id> 1 Januari 2023 pukul 12.49
Kepada: Sayono Sayono <say.epid@gmail.com>, Ulfa Nurullita <ulfa@unimus.ac.id>, Wahyu Handoyo <wahyu_ob@yahoo.co.id>, Winda Septy Tyasningrum <septyaswinda@gmail.com>, Irfanul Chakim <irfan.unimus@gmail.com>, Anto Budiharjo <abudiharjo@yahoo.com>

Sayono Sayono, Ulfa Nurullita, Wahyu Handoyo, Winda Septy Tyasningrum, Irfanul Chakim, Anto Budiharjo:

We have reached a decision regarding your submission to Biodiversitas Journal of Biological Diversity, "Morphological and molecular detection of insecticides resistance of the Dengue vector, Aedes aegypti in Central Java Province, Indonesia".

Our decision is: Revisions Required

Reviewer A:

Dear Authors,

Thank you for submitting this manuscript that explores the insecticide resistance in Aedes mosquitoes. This is an interesting paper with some real world health consequences. The manuscript is well structured and he conclusions are clear.

There are some revisions required in order to consider this manuscript for publication. I have included specific feedback on the word document version of the manuscript, please find attached. Make sure that any changes to the manuscript are shown using highlighted text or tracked changes. Additionally, please address the following key areas when making revisions:

1. Methods. please be clear on the number of mosquitoes used per part of the experiment. The maths is unclear and it is often unclear how many locations were being sampled. please make sure this is consistent throughout the manuscript.

2. Explain the implications further. Are there any toher drugs that may be available? What should affected areas consider?

3. Number formatting and grammar. Please check through for some grammar errors that occur in the work.

Recommendation: Revisions Required

Biodiversitas Journal of Biological Diversity

A-13034-Article Text-1070663-1-4-20221231.docx
1094K

Sayono Sayono <say.epid@gmail.com> 2 Januari 2023 pukul 00.04
Kepada: Smujo Editors <support@mail.smujo.id>

Revised-Manuscript

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

Abstract. Dengue control programs in the endemic areas were hampered by the emergence of insecticide-resistant strains among *Aedes aegypti* populations. 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 which represent the different altitudes. Larval and adult stage of *Ae. aegypti* colony from each village were subjected to a bioassay test based on the WHO procedures and subsequently were sampled and subjected to the molecular analysis for identification of the 1016G kdr allele by using the allele-specific polymerase chain reaction (AS-PCR). Mortality of *Ae. aegypti* after being exposed to cypermethrin, malathion, and temephos ranged from 16–86%, 75-100%, and 6-51%, respectively. 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. 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 and Gebugan (Semarang Regency) ~~several~~and Rowosari in Semarang City ~~areas~~.

Keywords: *Aedes aegypti*, insecticide resistance, pyrethroid, organophosphate, ~~molecular~~ ~~detection~~ 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 30^0Ccausing the enhancement of the potential of the Dengue outbreak (Lee et al. 2018~~,~~; Reinhold et al. 2018). Annually, new infection in community of dengue has been estimated 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, Muso 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 ~~High intensity of the insecticide used to control the *Ae. aegypti* mosquito during the last decades impacted the emergence of resistant strains to the 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 (Putra et al. 2016, Mulyatno et al. 2012), 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). The emergence of cross/multiple resistance to some insecticide compounds was reported in some countries (Putra et al. 2016, Brengues et al. 2003, 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 (Sayono et al. 2016, Li et al. 2015, Kawada et al. 2014, Widyastuti et al. 2015, 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

Commented [REV1]: Strains of mosquito that are resistant to what?

Commented [REV2]: Include the full term on first mention in the abstract

Commented [REV3]: Why caps here?

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Commented [REV5]: Some of the key words are already included in the title. Remove any key words that are in the title and use new terms to increase paper discoverability

Commented [REV6]: Separate the references using a semi colon ; not a comma. Please adjust for all references in the text.

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Commented [REV7]: Explain this a bit further. What temperature is optimal?

Commented [REV8]: In humans?

Commented [REV9]: Estimated to be as many as 390 million cases per annum

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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 of which 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 with the highest incidence rate, namely Semarang, Pemalang, Tegal, and Kudus districts, and Semarang municipality (Figure 1) but sufficient larvae were only obtained from thirteen villages. One to two villages were selected in each district and ~~municipality~~ municipalities ~~that have~~ based on the occurrence of new dengue cases in the year 2016 ~~were selected in each district/municipality~~. 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 29.6-30^{0}C and 78 to 81 percent, respectively. ~~The~~ All of the pupae ~~emergence~~ emergences ~~of pupae~~ were ~~as~~ moved into the mosquito cage and classified based on the study cluster. The imagoes were fed with a 10% sugar solution through permeated cotton.

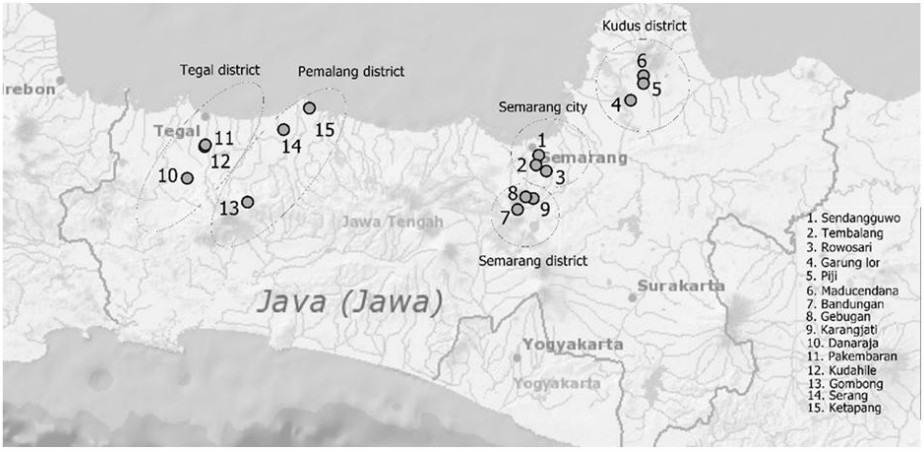

Figure 1. Map of study sites in Central Java province which included five districts or municipalities namely Kudus, Semarang, Pemalang, and Tegal districts, and Semarang city. They are indicated by the circle line surrounding each cluster of study sites.

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Commented [REV12]: Explain the selection process a bit further

Commented [REV13]: dwellings

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Figure 2. Larvae aspirator device

Bioassay

The susceptibility of *Ae. aegypti* against cypermethrin, one of the most frequently used pyrethroid class insecticides, and two organophosphate compounds, namely 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* ~~Ae. aegypti~~ by using impregnated paper containing 0.05% 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. The research subjects were filial 1 (F1) female mosquitoes that were fed sugar and healthy (3-5 days old). A total of 150 mosquitoes from each study site were subjected to a bioassay test with details of four experimental tubes (coated with impregnated paper on the inner surface) and two control tubes (without impregnated paper) where each tube contained 25 mosquitoes and was left in contact with the impregnated paper for 60 minutes. The test was carried out ~~five times~~ on three different consecutive days so that the total sample for each study site is ~~the eleven locations was 1,6~~ 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±20C 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 instar for each study site so ~~1,650~~1,950 larvae were needed for ~~eleven~~ 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 mortality of the larvae 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) using the WHO standard bioassay test based on the percentage of deaths over 98%, 90-97%, and lower than 90% respectively. (WHO 2016).

Allele-Specific Polymerase Chain Reaction

Ten resistant and susceptible mosquitoes were taken from each study site (in total 220 larvae) based on the previous test and subjected to the identification of the 1016G kdr allele of the *AaNav* gene by using the AS-PCR method. 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'-ACCGACAAATTGTTTCCC-3'), 0.125 µM Gly reverse primer (5'-GCGGGCAGGGCGGCGGGGGCGGGGCCAGCAAGGCTAAGAAAAGGTTAACTC-3') or Val (5'-GCGGGCAGCAAGGCTAAGAAAAGGTTAATTA-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 by 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.

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Commented [REV17]: Italicise here

Commented [REV18]: Why different concentrations for different chemicals?

Commented [REV19]: But five times three times 25 is 325. So there were 325 mostuiqoes used per location? This needs to be much clearer.

Commented [REV20]: But there were 15 locations stated?

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Commented [REV21]: Check sentence structure

Commented [REV22]: How did you know if they were resistant or susceptible? Was this based on your earlier tests?

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 that the 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 & 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 ~~of~~ resistant (38.36%), and resistant (38.46%), based on the mortality percentage (Table 1). ~~Malathion susceptible~~Malathion-susceptible strains were found in three villages namely Karangjati and Gebugan (Semarang district) and Rowosari (Semarang municipality).

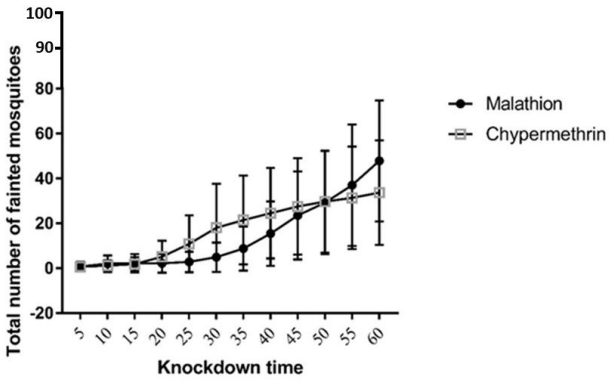

Figure 3. The trend of the knockdown mosquito number during 60 minutes exposed to two insecticide compounds

Commented [REV23]: Surely this is not a fair test if they are at different concentrations?

Dalam standar pengujian insektisida menurut WHO itu memang konsentrasi masing-masing insektisida tidak sama. Konsentrasi untuk Sipermetrin 0.05%, Malathion 5%, dan Temephos 0.02ppm.

Commented [REV24]: 0.02 ppm

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Please state how error bars were generated. Are they standard error?

No.

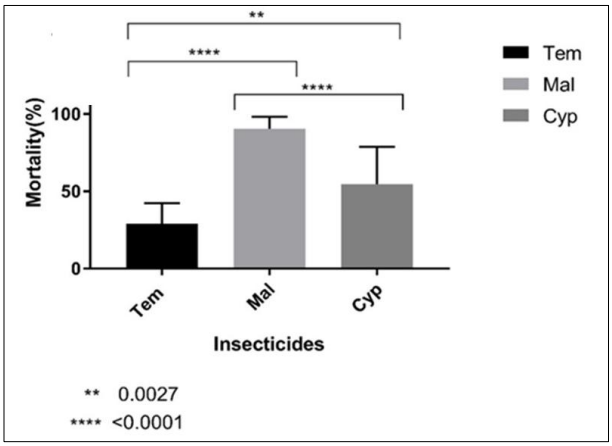

Figure 4. Comparison of mortality percentage between different types of insecticide compounds. The result of the independent samples t-test exhibited a significant distinction between insecticide compounds. There is a sequence of mortality which clearly showed by each significance level; Malathion had 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)

Commented [REV27]: Make sure the maximum on he y axis is 100%. You cannot get mortality over 100%

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

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

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 the phenotypic resistance status ($p<0.05$). Three genotype variants were detected namely the 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 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	

*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).

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The mutant genotypes of codon 1016 *AaNav* gene were detected among *Ae. aegypti* mosquito samples from all study sites although the heterozygous mutant was not detected from the high altitude of study sites (Bandungan and Gombong villages).

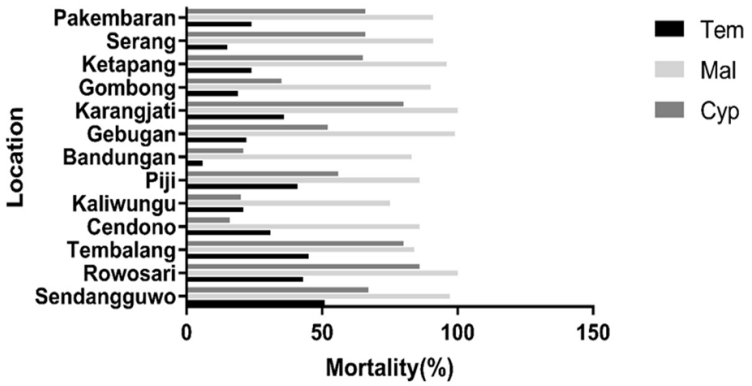

Figure 5. Contrasting different mortality levels of each insecticide compound based on study sites. The dengue vector (*Ae. aegypti*) populations were resistant to cypermethrin and temephos compounds among all of the study sites (the dengue-endemic areas) in Central Java Province, Indonesia while susceptible to Malathion compound among three villages: Karangjati and Gebugan (Semarang regency) 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 both 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 been deteriorating in the last decade (Moyes et al. 2017). This condition has been separately reported in Indonesia and this circumstance is closely correlated with the use of insecticides in the Dengue vector control program in the last two decades (Mulyatno et al. 2012, Sayono et al. 2016b, Ikawati et al. 2015, 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 covered comprehensively the wider areas in the different altitudes and geographic conditions from 12 to 1200 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 occurrence increased the community's efforts to control the disease by implementing chemical methods for dengue vector control measures, especially fogging (Krianto 2009, 46-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 dengue-endemic areas which were exposed to insecticide intensively and emerged to be resistant also participated in the migration to the other areas. This might affect the resistance status of the local population of *Ae. aegypti* (Sa 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 proof. 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). This relaxation is important to be implemented by a community experiment in the resistant populations of *Ae. aegypti*.

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 together with the migration of the human population through varying transportation lines (Sa et al. 2019). Although we only obtain 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 clearly understand the mechanism of *Ae. aegypti* resistance in this area.

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 classes of insecticides 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, but the method is considered to be expensive and unsuitable for a large number of samples (Saingamsook et al. 2017). Several numbers of PCR methods have been developed to detect kdr alleles i.e. real time-PCR and heated oligonucleotide ligation assay (HOLA), but the methods still lack efficiency (Saavedra-Rodriguez et al. 2007, Rajatileka et al. 2008). Therefore, a simpler method of genotyping i.e. AS-PCR was developed to increase the efficiency of detecting a large number of samples from the field (Stenhouse et al. 2013). Although AS-PCR is often underrated in comparison to nucleotide sequencing, several studies have shown that the method is reliable enough to be used as a detection of the mutant allele

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(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).

Resistance to insecticide will increase after 2-20 years after continuously being used for decades (Georghiou et al. 1983). Intensive use of insecticide can act as a naturally selective agent of the mosquito population which will maintain the resistant insects to survive and inherit it 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 will 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 the use 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. Surveillance of the Dengue vector susceptibility is necessary to be conducted periodically in those areas before chemical control measure is done. The actual 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.

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Dear Editor,

We have revised the manuscript attached based on the reviewer's comments. The difference in the concentration of insecticidal compounds in each type of insecticide (Cypermethrin 0.05%, Malathion 5%, and Temephos 0.02 ppm) is according to WHO diagnostic standards (WHO 2016).

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Revised-Manuscript

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

Abstract. ~~Dengue control programs in the endemic areas were hampered by the emergence of insecticide resistant strains among *Aedes aegypti* population~~The emergence of insecticide-resistant strains among Aedes aegypti populations hampered dengue control programs in the endemic areas. Moreover, t~~T~~o 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 ~~which represent the~~ different altitudes. Larval and adult stage of *Ae. aegypti* colony from each village ~~were~~ was subjected to a bioassay test based on the WHO procedures ~~and subsequent~~l. Subsequently, the~~y~~ were sampled and subjected to ~~the~~ molecular analysis ~~for identification of the 1016G kdr allele by~~to identify the 1016G kdr allele using the allele-specific polymerase chain reaction (AS-PCR). Mortality of *Ae. aegypti* after ~~being exposed~~exposure to cypermethrin, malathion, and temephos ranged from 16–86%, 75-100%, and 6-51%, respectively. These f~~F~~indings 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. 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 ~~and Gebugan (Semarang Regency),~~ Gebugan (Semarang Regency), ~~several~~and Rowosari in Semarang City ~~areas~~.

Keywords: *Aedes aegypti*, insecticide resistance, pyrethroid, organophosphate, ~~molecular detection~~ 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 30^{0}C, 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 of~~community has been estimated to have ~~dengue has been estimated~~ 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~~ dengue in areas where *Aedes* vectors are established (Fredericks et al. 2014). Zika virus has rapidly spread intercontinental (Duffy et al. 2009, Muso 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 ~~High intensity of the insecticide used to control the *Ae. aegypti* mosquito during the last decades impacted the emergence of resistant strains to the 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 (Putra et al. 2016, Mulyatno et al. 2012), 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). ~~The~~ In addition, the emergence of cross/multiple resistance to some insecticide compounds was reported in some countries (Putra et al. 2016, Brengues et al. 2003, 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 (Sayono et al. 2016, Li et al. 2015, Kawada et al. 2014, Widyastuti et al. 2015, 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.

Commented [REV1]: Strains of mosquito that are resistant to what?

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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, ~~of which~~ 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 with the highest incidence rate, ~~namely~~ Semarang, Pemalang, Tegal, and ~~and~~ Kudus districts~~, and~~ and Semarang municipality (Figure 1). ~~) but sufficient larvae were only obtained from thirteen villages. One~~ Therefore, one to two villages were selected in each district and ~~municipality~~ municipalities ~~y~~that have based on the occurrence of new dengue cases in ~~the year~~ 2016 ~~were selected in each district/municipality~~. 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'~~ 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 29.6-30^{0}C and 78 to 81 percent, respectively. ~~The All of t~~The pupae ~~emergence~~ emergences ~~of pupae~~ were ~~as~~ moved into the mosquito cage and classified based on the study cluster. The imagoes~~es were~~ were fed with a 10% sugar solution through permeated cotton.

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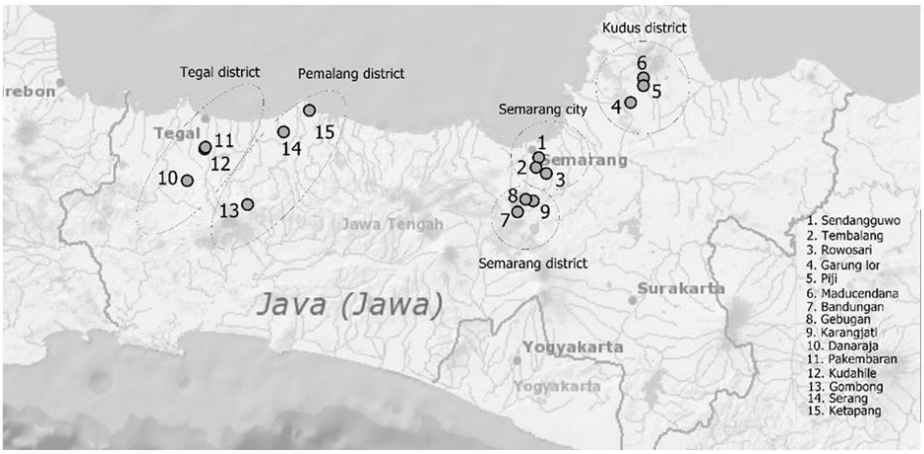

Figure 1. Map of study sites in Central Java province, which included five districts or municipalities, namely Kudus, Semarang, Pemalang, ~~and~~ Tegal districts, and Semarang city. They are indicated by the circle line surrounding each cluster of study sites.

Figure 2. Larvae aspirator device

Bioassay

The susceptibility of *Ae. aegypti* against cypermethrin, one of the most frequently used pyrethroid class insecticides, and two organophosphate compounds, ~~namely~~ 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* ~~Ae. aegypti~~ ~~by~~ using impregnated paper containing 0.05% 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. The research subjects were filial 1 (F1) female mosquitoes ~~that were~~ fed ~~s~~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, ~~of~~ four experimental tubes (coated with impregnated paper on the inner surface) and two control tubes (without impregnated paper) where each tube contained 25 mosquitoes bringing the total of 150 samples. Each sample was~~and was~~ left in contact with the impregnated paper for 60 minutes. The test was carried out ~~five times~~ on three ~~different~~ consecutive days so that the total sample for each study site ~~is was~~~~the eleven locations was 1,6~~ 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±20C 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 instar for each study site, so ~~1,650~~1,950 larvae were needed for ~~eleven~~ 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 mortality of the larvae~~, and the larvae' 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). Th~~ using th~~e 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

~~Ten resistant and susceptible mosquitoes were taken from each study site (in total 220 larvae).~~ B~~b~~ased on the previous test and subjected to the identification of the 1016G kdr allele of the *AaNav* gene ~~by~~ using the AS-PCR method, ten resistant and susceptible mosquitoes were taken from each study site (in total, 220 larvae). 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'-ACCGACAAATTGTTTCCC-~~3'~~3'), 0.125 µM Gly reverse primer (5'-GCGGGCAGGGCGGCGGGGCGGGGCCAGCAAGGCTAAGAAAAGGTTAACTC-~~3'~~3') or Val (5'-GCGGGCAGCAAGGCTAAGAAAAGGTTAATTA-~~3'~~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 ~~by~~ using a one-way comparison test was conducted to understand the difference in mortality of the pyrethroid and organophosphate-

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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 ~~that the~~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 in 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 & 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 ~~of~~ resistant (38.36%), and resistant (38.46%), based on the mortality percentage (Table 1). ~~Malathion-susceptible~~Malathion-susceptible strains were found in three villages: ~~namely~~ Karangjati and Gebugan (Semarang district) and Rowosari (Semarang municipality).

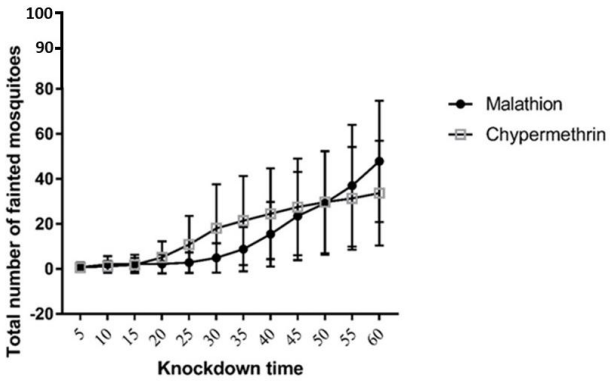

Figure 3. The trend of the knockdown mosquito number during 60 minutes exposed to two insecticide compounds

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Dalam standar pengujian insektisida menurut WHO itu memang konsentrasi masing-masing insektisida tidak sama. Konsentrasi untuk Sipermetrin 0.05%, Malathion 5%, dan Temephos 0.02ppm.

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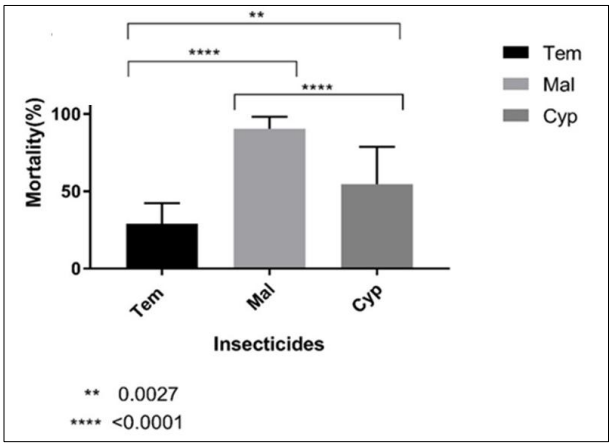

Figure 4. Comparison of mortality percentage between different types of insecticide compounds. The ~~result of the~~ independent samples t-test exhibited a significant distinction between insecticide compounds. There is a sequence of mortality which clearly showed by each significance level; Malathion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

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

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

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 ~~the~~ phenotypic resistance status ($p<0.05$). Three genotype variants were detected ~~namely the~~: 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 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	

*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).

The mutant genotypes of codon 1016 *AaNav* gene were detected among *Ae. aegypti* mosquito samples from all study sites, although the heterozygous mutant was not detected from the high altitude of study sites (Bandungan and Gombong villages).

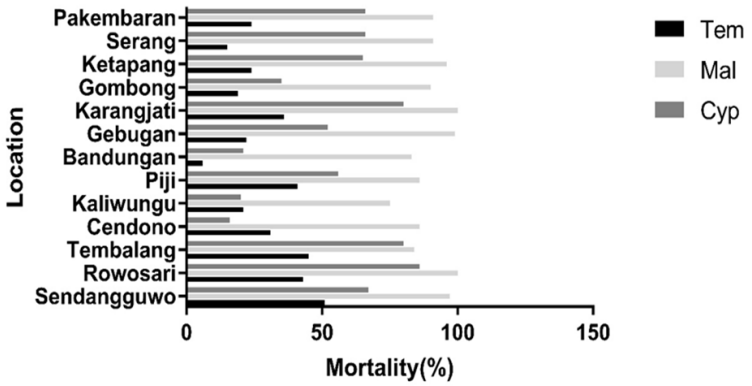

Figure 5. Contrasting different mortality levels of each insecticide compound based on study sites. The dengue vector (*Ae. aegypti*) populations were resistant to cypermethrin and temephos compounds among all of the study sites (the dengue-endemic areas) in Central Java Province, Indonesia, while susceptible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

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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 ~~been deteriorating~~deteriorated in the last decade (Moyes et al. 2017). This condition has been separately reported in Indonesia, ~~and this circumstance~~which is closely correlated with the use of insecticides in the Dengue vector control program in the last two decades (Mulyatno et al. 2012, Sayono et al. 2016b, Ikawati et al. 2015, 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 co~~vered comprehensively~~mprehensively 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'~~ 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 ~~occurrence~~ increased the ~~community's~~ community's efforts to control the disease by implementing chemical methods for dengue vector control measures, especially fogging (Krianto 2009, 46-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 ~~dengue endemic areas which were exposed to insecticide intensively and emerged to be~~intensively dengue-endemic areas exposed to insecticide and emerged resistant also participated in the migration to the other areas. Tha~~t~~i~~s~~ might affect the resistance status of the local population of *Ae. aegypti* (Sa 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). ~~This relaxation is important to be implemented by a community experiment in the resistant populations of *Ae. aegypti*~~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~~together~~ 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 ~~clearly understand the mechanism of *Ae*~~understand the mechanism of *Ae.aegypti* resistance in this area clearly. ~~*aegypti* resistance in this area~~.

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 ~~classes of~~ insecticide~~s~~ 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 standar~~standard gold method~~, but~~. Still, the method is considered to be expensive and unsuitable for a large number of samples (Saingamsook et al. 2017). ~~Several~~ Therefore, several ~~numbers of~~ PCR methods have been developed to detect kdr alleles, i.e., ~~real~~ real-

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time-PCR and heated oligonucleotide ligation assay (HOLA)~~, but~~. However, the methods still lack efficiency (Saavedra-Rodriguez et al. 2007, Rajatileka et al. 2008). Therefore, a simpler ~~method of genotyping~~genotyping method, i.e., AS-PCR, was developed to increase the efficiency of detecting ~~a large number of~~many samples from the field (Stenhouse et al. 2013). Although AS-PCR is often underrated ~~in comparison to nucleotide sequencing, several studies have shown that the method is reliable enough to be used as a detection of~~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).

~~Resistance to insecticid~~Insecticide resistance will increase ~~after~~ 2-20 years after continuously being used for decades (Georghiou et al. 1983). Intensive ~~use of insecticid~~insecticide use can act as a naturally selective agent of the mosquito population, which will maintain the resistant insects to survive and inherit ~~it~~ 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 ~~will~~ 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 the use~~ 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. ~~Surveillance~~ Therefore, surveillance of the Dengue vector susceptibility ~~is necessary to~~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.

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Kepada: Sayono Sayono <say.epid@gmail.com>, Ulfa Nurullita <ulfa@unimus.ac.id>, Wahyu Handoyo <wahyu_ob@yahoo.co.id>, Winda Septy Tyasningrum <septyaswinda@gmail.com>, Irfanul Chakim <irfan.unimus@gmail.com>, Anto Budiharjo <abudiharjo@yahoo.com>

Sayono Sayono, Ulfa Nurullita, Wahyu Handoyo, Winda Septy Tyasningrum, Irfanul Chakim, Anto Budiharjo:

We have reached a decision regarding your submission to Biodiversitas Journal of Biological Diversity, "Morphological and molecular detection of insecticides resistance of the Dengue vector, Aedes aegypti in Central Java Province, Indonesia".

Our decision is: Revisions Required

Reviewer A:

Dear Authors,

Many thanks for submitting this revised version of the manuscript for review. You have taken into account the feedback provided on the initial review of the paper. You have also shown clearly where changes have been made to the work, as shown with the highlighted sections of text. The developments to the manuscript have resulted in a more robust paper overall. In light of the revisions, the paper is now in a much better position for consideration.

Recommendation: Accept Submission

Reviewer B:
Recommendation: Accept Submission

Acceptance Statement

Biodiversitas Journal of Biological Diversity

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Sayono Sayono <say.epid@gmail.com> 6 Januari 2023 pukul 07.07
Kepada: Smujo Editors <support@mail.smujo.id>

Dear Editors,
Thank you very much for updating the progress of our manuscript. There is a decision: "Revision required" in the email above, but after I read deeper there are no recent corrections from reviewers that we should follow up on. Please indicate the parts we should revise. Thank you.

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

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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.

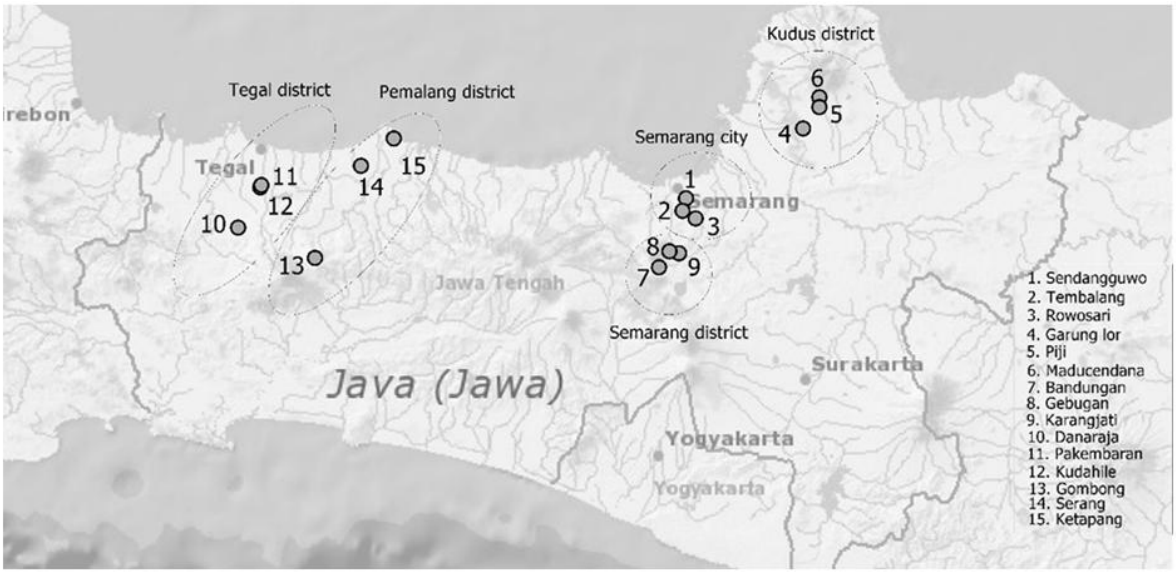

Figure 1. Map of study sites in Central Java province, which included five districts or municipalities, namely Kudus, Semarang, Pemalang, Tegal districts, and Semarang city. They are indicated by the circle line surrounding each cluster of study sites

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±2ºC 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'-ACCGACAAATTGTTTCCC-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)

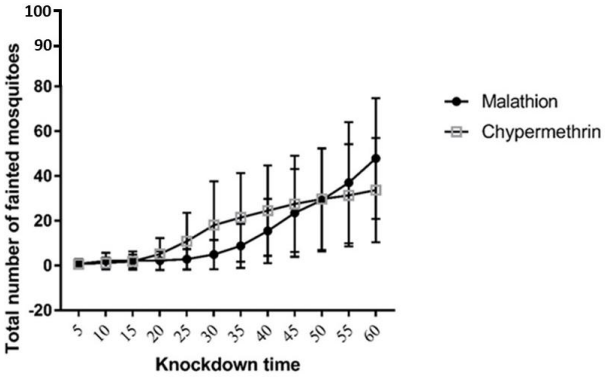

Figure 3. The trend of the knockdown mosquito number during 60 minutes exposed to two insecticide compounds

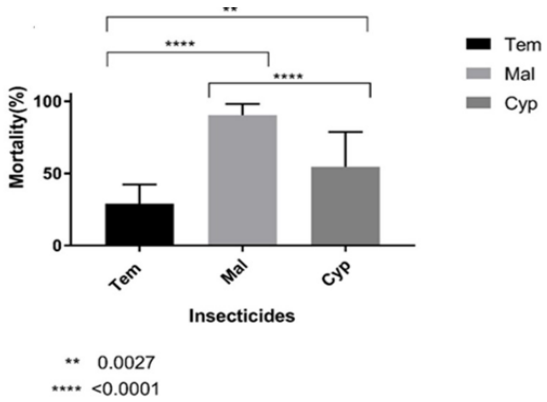

Figure 4. Comparison of mortality percentage between different types of insecticide compounds. The independent samples t-test exhibited a significant distinction between insecticide compounds. There is a sequence of mortality which clearly showed by each significance level; Malathion had the highest 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)

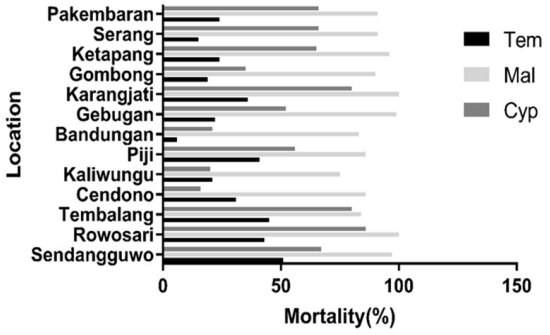

Figure 5. Contrasting different mortality levels of each insecticide compound based on study sites. The Dengue vector (*Ae. aegypti*) populations were resistant to cypermethrin and temephos compounds among all of the study sites (the Dengue-endemic areas) in Central Java Province, Indonesia, while susceptible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

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[Kutipan teks disembunyikan]

Sayono Sayono <say.epid@gmail.com> 6 Januari 2023 pukul 08.28
Kepada: Smujo Editors <support@mail.smujo.id>

Dear Editors,
We have revised all reviewer notes one by one. We highlight all corrected parts in yellow, including additional sentences or statements. Hopefully, our efforts have met the expectations of the reviewers. Thank you very much.

Best regards
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Acceptance Letter

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[biodiv] Editor Decision

2 pesan

Agustina Putri <support@mail.smujo.id> 16 Januari 2023 pukul 22.10
Kepada: SAYONO <say.epid@gmail.com>, ULFA NURULLITA <ulfa@unimus.ac.id>, WAHYU HANDOYO <wahyu_ob@yahoo.co.id>, WINDA SEPTY TYASNINGRUM <septyaswinda@gmail.com>, IRFANUL CHAKIM <irfan.unimus@gmail.com>, ANTO BUDIHARJO <abudiharjo@yahoo.com>

SAYONO, ULFA NURULLITA, WAHYU HANDOYO, WINDA SEPTY TYASNINGRUM, IRFANUL CHAKIM, ANTO BUDIHARJO:

We have reached a decision regarding your submission to Biodiversitas Journal of Biological Diversity, "Bioassay and molecular detection of insecticides resistance of Aedes aegypti, vector of dengue in Central Java Province, Indonesia".

Our decision is to: Accept Submission

Biodiversitas Journal of Biological Diversity

Sayono Sayono <say.epid@gmail.com> 17 Januari 2023 pukul 09.21
Kepada: Agustina Putri <support@mail.smujo.id>

Dear Editor,
We have a few corrections for author names and author affiliations. I put the correction in the copyediting file, and I highlighted it in yellow.

Best regard,
Sayono
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3 pesan

DEWI NUR PRATIWI <support@mail.smujo.id> 8 Januari 2023 pukul 08.33
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Ahmad Dwi Setyawan

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Sayono Sayono <say.epid@gmail.com> 8 Januari 2023 pukul 15.47
Kepada: Ahmad Dwi Setyawan <editors@smujo.id>

Dear Editors,
We sent the article corrected-proof and proof of payment of the article processing charge worth 4.5 million according to the invoice

Best Regards
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