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Detection of *Aedes aegypti* Resistance towards Cypermethrin using Polymerase Chain Reaction (PCR) Techniques in Ambarawa Semarang Regency

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Abstract

Ambarawa Sub-district is one of the endemic sub-districts since 2017 which always accounts for the highest DHF cases in Semarang Regency. The purpose of this study was to detect molecular resistance of Aedes aegypti towards cypermethrin using Polymerase Chain Reaction (PCR) technique. This study was conducted to look at the mutation in the gene VGSC Aedes aegypti. This research is a pure experimental research. The results showed that in Tambakboyo Village, 2 samples were susceptible (V / V), 7 samples were detected as homozygous (G/G) resistant, 1 sample was detected as heterozygous (V/G) resistant; in Kupang Village 5 samples were detected as being homozygous resistant (G/G) and 5 samples were detected as heterozygous resistant (V/G); and in Panjang Village 1 susceptible sample (V/V), 8 samples were detected homozygous resistant (G/G), 1 sample detected heterozygous resistant (V/G). Resistance is detected by mutations. Mutations have been found in the VGSC gene in codon V1016G.

INTRODUCTION

Dengue Hemorrhagic Fever (DHF) is a disease caused by Dengue virus which is classified as Arthropod-Borne Virus, genus Flavivirus, and family Flaviviridae. DHF is transmitted by mosquito bites from the *Aedes* genus, especially *Aedes aegypti* and *Aedes albopictus*. The number of cases in America, Southeast Asia and the Western Pacific has surpassed 1.2 million in 2008 and more than 2.3 million in 2010. WHO data in 2014 recorded 198 million cases of dengue occurred globally and caused 584,000 deaths in 2013 (WHO, 2014).

DHF is reported to be a health problem for the people of Indonesia. DHF is spread throughout Indonesia, in some areas having a high level of endemicity. In 2017 there were 68,407 DHF cases, with 493 deaths (Indonesian Ministry of Health, 2017). DHF is still a serious problem in Central Java Province, out of 35 districts / cities all have been infected with DHF. The DHF Incidence Rate (IR) in Central Java Province in 2017 was 21.68 per 100,000 populations. This means that the IR of DHF in Central Java is lower than the national target (<51 / 100,000 population) and the Renstra target (<48 / 100,000) (Central Java Province Health Office, 2017).

DHF cases according to public health cen-

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ter in Semarang Regency in 2015-2017 show there are three public health centers (*Puskesmas*) with the highest number of cases, namely Ambarawa Public Health Center 267 cases and 2 cases of death, Pringapus Public Health Center 235 cases and 1 case of death, and Bergas Public Health Center is 216 and 3 cases of death (Semarang Regency Health Office, 2017). Data from the Ambarawa Public Health Center in 2019 showed that in the work area of the Ambarawa Public Health Center during 2016-2019, 47 fogging had been carried out in 8 villages out of 10. Fogging was carried out by the Semarang Regency Health Office including 9 times in Kupang Village, 8 times in Panjang Village, and 5 times in Tambakboyo Village using 15 UL zeta trademark insecticide which contains the main active ingredient zeta- cypermethrin 15 g/l (*Puskesmas* Ambarawa, 2019). Ambarawa Sub-district is one of the endemic sub-districts since 2017 which always contributes the highest DHF cases (Health Office in Semarang Regency, 2017).

One of the efforts to control DHF is chemical control for DHF vectors such as the application of insecticides with fogging. Prolonged and repeated application of insecticides in an ecosystem will lead to vector resistance towards the insecticides used (Mulyani et al., 2017; Cahyati et al., 2019). Many countries have reported that some mosquito vectors are resistant to various insecticides. Research in Pakistan on the susceptibility status of *Aedes aegypti* taken from the population in Lahore, Pakistan reports that the *Aedes aegypti* tested has been resistant to cypermethrin (Jahan & Shahid, 2013). Resistance of mosquito vectors has been reported in Indonesia in a study of the use of insecticides to control DHF vectors, *Aedes aegypti* mosquitoes captured in Bandung, Surabaya, Jakarta, and Palu are resistant to permethrin and deltamethrin. While mosquitoes that are caught in Palembang are resistant to *malathion* (Ahmad et al., 2009). *Aedes aegypti* mosquitoes captured in Semarang are also reported resistant to cypermethrin (Sayono, et al., 2012). Other study reported *Aedes aegypti* resistance for Pyrethroid in Palu (Purwaningsih et al., 2019).

The low mortality of *Aedes aegypti* due to cypermethrin belongs to the group of pyrethroid insecticides which is the most effective insecticide of all types of insecticides when it is in direct contact with insects because it causes death only in a short time. Therefore, fogging conducted in Central Java uses the insecticide malathion and cynof (Central Java Province Health Office, 2011). The longest (over 10 years) pyrethroid insecticide and is often used in Central Java is the type of cypermethrin (Sayono et al., 2012). Cypermethrin has been used as an active ingredient in vector control in various countries, causing resistance to cypermethrin (Susanti & Boesri, 2012). The resistance of *Aedes aegypti* to cypermethrin in Brazil has been reported since 2004, 2005 and 2011 with the same result that *Aedes aegypti* has been resistant to cypermethrin (Lima et al., 2011). Reports of vector resistance to cytmetrin in Indonesia have been confirmed, one of which is a study in Yogyakarta that reported that *Aedes aegypti* has been resistant to cipmetrin 0.05% with an average mortality rate of 4.03% (Mulyani et al., 2017).

Detection of vector resistance to insecticides can be useful as program information for the selection of appropriate insecticides in vector control. The basic principle of resistance detection in molecular vectors is to identify mutations in genes that are targeted conventionally by insecticide groups, one of which is gen voltage gated sodium channel (VGSC). In insects that have been resistant to pyrethroid group insecticide and DDT an important resistance mechanism is the occurrence of changes or mutations in the VGSC gene (Widiarti et al., 2012). One of the mutations that has been found is that the *Aedes aegypti* mosquito undergoes heterozygous mutations in the second domain of the VGSC gene (V1016G) (Widiastuti et al., 2015).

The Polymerase Chain Reaction (PCR) method is one of the methods used to multiply DNA. PCR is important as a diagnostic tool to detect and determine virus serotypes (Ghiffari et al., 2013). This study uses the PCR method by detecting mutations of DNA gen VGSC. VGSC gene mutations indicate resistance. The PCR method in resistance testing can be used to determine the mechanism of resistance in a molecular way. The purpose of this study was to detect molecular resistance of *Aedes aegypti* towards cypermethrin using Polymerase Chain Reaction (PCR) technique.

METHOD

This research is pure experimental research. The *Aedes aegypti* samples examined were 10 each of the villages taken from 3 endemic DHF villages with high fogging intensity in the Sub-District of Ambarawa, namely Tambakboyo Village, Kupang Village and Panjang Village. Sampling using a random sampling technique taken with ovitrap which was first installed during the month of August 2019. Based on population data taken from each village in 2019, the number of fogged houses in each village were Tam-

Table 1. Examination results of ***Aedes aegypti*** larvae samples from Tambakboyo Village

No	Sample Number	Sample Code	Result
1	468	Tambakboyo 1	Homozygous resistant
2	469	Tambakboyo 2	Homozygous resistant
3	470	Tambakboyo 3	Homozygous resistant
4	471	Tambakboyo 4	Homozygous resistant
5	472	Tambakboyo 5	Homozygous resistant
6	473	Tambakboyo 6	Susceptible
7	474	Tambakboyo 7	Homozygous resistant
8	475	Tambakboyo 8	Susceptible
9	476	Tambakboyo 9	Homozygous resistant
10	477	Tambakboyo 10	Homozygous resistant
11	448	Kupang 1	Homozygous resistant
12	449	Kupang 2	Homozygous resistant
13	450	Kupang 3	Homozygous resistant
14	451	Kupang 4	Homozygous resistant
15	452	Kupang 5	Homozygous resistant
16	453	Kupang 6	Homozygous resistant
17	454	Kupang 7	Homozygous resistant
18	455	Kupang 8	Homozygous resistant
19	456	Kupang 9	Homozygous resistant
20	457	Kupang 10	Homozygous resistant
21	458	Panjang 1	Homozygous resistant
22	459	Panjang 2	Homozygous resistant
23	460	Panjang 3	Homozygous resistant
24	461	Panjang 4	Homozygous resistant
25	462	Panjang 5	Homozygous resistant
26	463	Panjang 6	Homozygous resistant
27	464	Panjang 7	Homozygous resistant
28	465	Panjang 8	Susceptible
29	466	Panjang 9	Homozygous resistant
30	467	Panjang 10	Homozygous resistant

bakboyo Village 171 houses, Kupang Village 533 houses, and Panjang Village 236 houses. Based on the preliminary survey, HI (House Index) was found to be more than 5% in the 3 villages. From the calculation results it is concluded that when examining a population of 200 houses must examine 51 houses in order to detect HI >5%. When examining a population of 500 houses, they must examine 56 houses in order to detect HI >5%. In total, 51 houses should be larvae-surveyed in the Tambakboyo Village, 56 in Kupang, and 51 in Panjang. Sampling of *Aedes aegypti* larvae uses ovitrap that is installed in every house, inside or outside the house. After that, *Aedes aegypti* larvae is taken and carried out to the laboratory for DNA samples.

Resistance detection tests using the PCR method were conducted at the Research and Development Center of Banjarnegara on September. The independent variable in this study is the application of the cypermethrin insecticide with the dependent variable is the mutation of the gen VGSC F1 *Aedes aegypti* on the codon 1016. Secondary data were obtained from the relevant agencies in this study, namely the Health Office of Semarang Regency and the Ambarawa Public Health Center which were used as a reference in conducting research. Primary data in this study were obtained from the results of the *Aedes aegypti* mosquito resistance detection test using the PCR

Table 2. Examination results of ***Aedes aegypti*** larvae samples according to village

No	Type of Resistance	Total	Percentage
1	Susceptible	3	10%
2	Homozygous resistant	20	66.7%
3	Homozygous resistant	7	23.3%
	Total Samples	30	

method during the study. The data is then analyzed descriptively to illustrate the results of the study.

RESULTS AND DISCUSSION

The examination results of 10 larvae samples of *Aedes aegypti* from Tambakboyo Village showed 7 samples detected homozygous resistance, 1 sample detected heterozygous resistance, and 2 susceptible samples. The examination results of 10 *Aedes aegypti* larvae samples from Kupang Village showed that all examined samples had detected resistance, i.e. 5 samples were detected homozygous resistance and 5 samples were detected heterozygous resistant. The results of the examination of 10 larvae of *Aedes aegypti* from Panjang Village showed 8 samples detected homozygous resistance, 1 sample detected heterozygous resistance, and 1 susceptible sample.

The results of resistance detection of *Ae. aegypti* larvae samples using the Polymerase Chain Reaction (PCR) technique that was carried out in three villages in Ambarawa Sub-District, Semarang Regency, showed 27 of the 30 examined samples had detected resistant.

Mutations in the gen VGSC caused by pyrethroid insecticide also occur in the world and in Indonesia. Resistance detection of by looking at mutations in the gen VGSC in the world occurs in populations of *Aedes aegypti* in Panama with the discovery of mutations for the first time in America in codons V1016G and I1011M (Murcia et al., 2019), and in Taiwan which detected mutations in the V1016G codon (28.03%)), S989P (17.83%), F1534C (21.97%), and D1763Y (66.69%) (Chung, 2019). Similar mutations were also found in Indonesia in samples of *Aedes aegypti* originating from Palembang in studies showing the results of the gen V1016G with a DNA target of 82 bp (Ghiffari et al., 2013). In addition, mutations in the VGSC codon V1016G and S989P *Aedes aegypti* also occurred in Padang with a DNA target of 579 bp (Hasmiwati et al., 2016), in West Sumatra the identification of mutation points in the gen VGSC showed positive results on the S989P and V1016G codons (Hasmiwati & Supargiyono, 2018), in West Sumatra. and in Palu mutations in VGSC were detected in samples examined from Balaroa codon of V1016G and S989P with DNA targets 619 bp (Umniyati, 2019).

This can indicate that the examined larvae of *Aedes aegypti* have been subjected to selective suppression of pyrethroid groups in this case cypermethrin. As it is known that pyrethroid insecticides are widely used by the community / household so *Aedes aegypti* is often exposed to these insecticides and supplemented with insecticides from the health sector. Health Office at Central Java Province informed that cynoff insecticide has been used in several cities in Central Java besides malathion to control *Ae. egypti* by fogging (Widiarti et al., 2012). In the V1016G mutation is a change in the valine coding codon to glycine, where there is a transition of thymine base with guanine in the GTA to GGA (Ghiffari et al., 2013). The results of this study showed that most mosquitoes of *Ae. aegypti* has undergone a V1016G mutation in the gen VGSC which is the target of synthetic pyrethroid insecticides (Widiastuti et al., 2015).

The mechanism of resistance to pyrethroid insecticides can be detected molecularly. Target mutations in the gen VGSC regarding resistance to pyrethroid indicate that there is an ongoing resistance mechanism. Detection of the gen VGSC mutation can directly assess the transformation of target cells that become insecticide targets. Gene mutations cause conformational changes in sodium channels because they cannot be opened by insecticide molecules. Mutations like this can only be detected by molecular methods. The basic principle of detection of molecular resistance in vectors is to identify genes (Umniyati, 2019). Pyrethroid insecticide works by attaching to the VGSC part located in the insect neuron vector (Ghiffari et al., 2013).

Pyrethroid insecticide binds to the VGSC protein that regulates nerve impulses. Initially, the pyrethroid insecticide molecule will attach to open the sodium channel and bind it up to keep it in an open condition. This will trigger repetitive nerve firing which will cause movements or activities out of control. The target insect will experience convulsion and cannot control its flying behavior. However, if there is a mutation in the gen

VSGC, the amino acids produced will change, thereby reducing the sensitivity of the pyrethroid insecticide molecule to form bonds in that part. (Widiastuti et al., 2015). Detection of pyrethroid insecticide resistance by molecular testing is known in two ways: changes in detoxification enzymes and changes in the target site VGSC. Detoxification enzyme detection is the detection of gene point mutations that cause increased levels of enzymes that detoxify insecticides (metabolic resistance) (Ghiffari et al., 2013).

In this study molecular tests were only carried out by looking at changes or mutations in gen VGSC. Changes in the mosquito gene of *Ae. aegypti* as the major vector of dengue virus is thought to be the cause of difficulty in controlling DHF. Resistance to insecticides also has the potential for high vector mosquito cycles of DHF cannot be completely overcome. Specific gene expression and autosomes are indicated to affect loci in the gene even more so if there is a resistance effect from insecticide exposure (Yudhana et al., 2017). VGSC can be an important indication of the extent to which the development of mosquitoes of *Ae. Aegypti* is resistant to certain class of insecticides so that their use can be evaluated and improved to enhance preventive action. The results of the study are related to the species of *Ae. aegypti* which has undergone selective suppression of insecticides from the pyrethroid group (Sinkins, 2010).

It is known that pyrethroid insecticides have been widely used by the community or households so that the mosquito of Ae. aegypti is often exposed to these insecticides and supplemented with insecticides from other groups. Based on the results of the susceptibility test of the mosquito of *Aedes aegypti* from the Regency of Semarang, it has been resistant to cytmetrin insecticide. Thus, the possibility of other resistance mechanisms in this case the molecular mechanism can take place in the mosquito species (Ilham et al., 2017). The use of pyrethroid insecticide, which is carried out continuously for a long time, does not kill 100% of mosquitoes of *Aedes aegypti* that are exposed and there are always insects that remain alive. In the beginning the number was only small, but in a certain period there will be an increase in the population of mosquitoes that live because of the breeding process while leaving the ability to be resistant to the same insecticide to subsequent offspring (Yudhana et al., 2017).

In this study, mutations were found in 2 types of mutations, namely mutations of homozygotes (G/G) and heterozygous mutations (V/G). The results showed that in of Tambakboyo Village 2 mosquitoes had not mutated (V/V), 7 mosquitoes had a mutation of homozygous (G / G), and 1 mosquito had a heterozygous mutation (V/G). All examined mosquitoes in Kupang Village had mutations, 5 mosquitoes had homozygous mutations (G/G) and 5 mosquitoes had heterozygous mutations (V/G). The results of the Panjang Village showed that 1 mosquito has not mutated (V/V), 8 mosquitoes have a homozygous mutation (G/G), and 1 mosquito had a heterozygous mutation (V/G). This can be interpreted that from total examined samples, there were 3 mosquitoes that had not undergone a mutation (V/V), 20 mosquitoes had a homozygous mutation (G/G) in the allele of 1016G, and 7 mosquitoes undergo heterozygous mutations in the second domain of the gen V1016G. There are 3 from 30 samples that have not been mutated, in line with the results of research in Thailand which showed that 74 out of 170 samples of mosquitoes of *Aedes aegypti* is still susceptible or not yet mutated (V/V) (Stenhouse et al., 2013). *Aedes aegypti* susceptible was also found in a study conducted in West Bengal, India, examining 5 samples showed that the results of the study found no mutations in the 1016G allele associated with observed resistance, both homozygous and heterozygous mutations, all still susceptible. In areas with individual mosquitoes of *Ae. aegypti* susceptible frequency of use of pyrethroids used and polymorphisms in target genes must be monitored regularly to detect the appearance of pyrethroid resistance in populations *Ae. aegypti* (Saha et al., 2019).

The results of this study showed 20 of 30 samples of Ae. the aegypti examined was detected as having homozygous mutations (V/V). This is in line with research in Yogyakarta, the mosquito of *Aedes aegypti* was found to have developed resistance to pyrethroids. Homozygous mosquitoes are almost as genetically mutated as heterozygous levels of individual resistance. No generation of pure homozygous susceptible strains has been found. Resistance is genetically inherited and the rate of development of resistance depends on the frequency of the resistant gene, the frequency of use of the insecticide, and the length of application. Resistance studies will also provide important information to recognize resistance mechanisms. The results of molecular screening found the target-site mutation and the high mutation kdr of V10161 (87%). Formation of susceptible homozygous strains requires time over five generations (Isfanda et al., 2017). A study showed that not all individual mosquitoes that had a V1016G mutation of homozygous were

able to survive or be resistant to permethrin insecticide exposure (0.75%) and 23.4% of the group that had heterozygous mutations were able to survive (Harris et al., 2010). This indicates that the possible mechanism of resistance to pyrethroid insecticides in mosquitoes of *Aedes aegypti* is influenced by several factors that do not stand alone. Apart from the V1016G mutation, *Aedes aegypti* resistance to synthetic pyrethroid insecticides can also be caused by several mutations in the VGSC gene in other positions including mutations V1016G, F1534C, and S989P (Kawada et al., 2014).

Heterozygous mutations were found in 7 of the 30 samples of *Aedes aegypti* examined in this study. The 1016G allele is recessive; this indicates that mosquitoes that have heterozygous mutations still have a great chance to remain sensitive to pyrethroid insecticides (Stenhouse et al., 2013). The results showed that most mutations occurred heterozygous, but this needs to be a concern in determining the type of insecticide that will be used in the mosquito control program of *Aedes aegypti*. The existence of this mutation will cause resistance also to other types of insecticides that come from the synthetic pyrethroid group (Widiastuti et al., 2015). There are differences in the level of resistance in each strain caused by differences in the level of use and type of insecticide in each region, in addition to genetic background and ecological variations. The application of stopped insecticides will provide an opportunity for susceptible genotypes to survive. Vulnerable genotypes originate from the inheritance of heterozygous resistant recessive gene traits generated by crossbreeding between susceptible and resistant individuals (Mantolu et al., 2016). Higher frequencies of heterozygous mutations compared to homozygous mutations accelerate the occurrence of resistance in the future because there is no protective mechanism in mosquitoes. The mechanism of protection depends on genetic factors; single or recessive, semi dominant or dominant, in the process of heredity. Heterozygous resistance is uncommon in a population, but if heterozygous mutations occur and survive until mating with other heterozygotes produces homozygous mutants with stronger resistance to insecticides. If homozygous mutants become dominant, resistance spreads rapidly in a population due to the ease of *Aedes aegypti* to adapt to its environment (Hasmiwati & Supargiyono, 2018).

CONCLUSION

DHF control with fogging method using pyrethroid pyrethrin insecticide has been proven to cause resistance by supporting molecular test results. The molecular test in this study was carried out on samples from three endemic DHF villages and high fogging frequency in the work area of Ambarawa Public Health Center, Semarang Regency. The results showed a mutation in the gen VGSC codon 1016 of the examined *Ae. aegypti*. The mutation of the VGSC gene marked the resistance of *Ae. aegypti*.

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5	Sébastien Marcombe, Bénédicte Fustec, Julien Cattel, Somesanith Chonephetsarath et al. "Distribution of insecticide resistance and	<1%

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6 Pabitra Saha, Moytrey Chatterjee, Sudeep Ballav, Akash Chowdhury, Nandita Basu, Ardhendu Kumar Maji. "Prevalence of kdr mutations and insecticide susceptibility among natural population of Aedes aegypti in West Bengal", PLOS ONE, 2019 <1%
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7 Koichi Hirata, Osamu Komagata, Kentaro Itokawa, Atsushi Yamamoto, Takashi Tomita, Shinji Kasai. "A Single Crossing-Over Event in Voltage-Sensitive Na+ Channel Genes May Cause Critical Failure of Dengue Mosquito Control by Insecticides", PLoS Neglected Tropical Diseases, 2014 <1%
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