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MOLECULAR DETECTION OF GENE RESISTANT TO VARIOUS INSECTICIDES IN *Aedes aegypti* AT BANYUWANGI EAST JAVA USING POLYMERASE CHAIN REACTION

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ABSTRACT

The purpose of this study was to determine the type of insecticides which *Aedes aegypti* mosquitoes are resistant to, so that other susceptible insecticides still can be used. The study was a cross sectional epidemiological study with cluster sampling in Sub-district Wongsorejo, Banyuwangi, Muncar, Tegaldlimo, Kalibaru which is considered sufficient to represent Banyuwangi Regency. Mosquito samples appropriate to the characteristics were isolated and tested for insecticide-resistance primer namely voltage gated sodium channel (VGSC) to determine the specific resistance expressed in mosquitoes. The result of resistance test using WHO standard method showed that *Aedes aegypti* mosquito from Banyuwangi Regency were resistant to cypermethrin 0.25% and malathion 0.8%. There was a VGSC coding gene with 250 bp band detected using polymerase chain reaction (PCR) technique which was associated with the resistance of *Aedes aegypti* mosquitoes in Banyuwangi Regency to organophosphate insecticides (malathione) and pyrethroid insecticides (cypermethrin).

Key words: *Aedes aegypti*, insecticide, polymerase chain reaction, resistance

ABSTRAK

Tujuan penelitian ini adalah mengetahui jenis insektisida yang sudah resisten terhadap nyamuk Aedes aegypti sehingga terdapat kebijakan alternatif penggunaan insektisida yang belum resisten. Metode penelitian yang digunakan adalah studi epidemiologi cross sectional dengan sampel kluster di Kecamatan Wongsorejo, Banyuwangi, Muncar, Tegaldlimo, Kalibaru yang dianggap mewakili Kabupaten Banyuwangi. Sampel nyamuk sesuai dengan karakteristik diisolasi dan diuji dengan primer spesifik resisten insektisida yaitu voltage gated sodium channel (VGSC) untuk mengetahui ekspresi spesifik dari DNA yang sudah resisten terhadap nyamuk. Hasil uji resistensi sampel nyamuk menggunakan metode standar WHO menunjukkan bahwa nyamuk Aedes aegypti dari Kabupaten Banyuwangi tahan terhadap cypermethrin 0,25% dan malathion 0,8%. Terdapat gen penyandi VGSC dengan pita 250 bp yang dideteksi menggunakan teknik polymerase chain reaction (PCR) yang terkait dengan resistensi terhadap insektisida organofosfat (malathion) dan insektisida piretroid (cypermethrin) pada nyamuk Aedes aegypti di Kabupaten Banyuwangi.

Kata kunci: Aedes aegypti, insektisida, polymerase chain reaction, resistensi

INTRODUCTION

Banyuwangi Regency is still listed as an area that is not completely free from dengue hemorrhagic fever (DHF) outbreak. Healthcare report in 2014 found that total DHF cases in Banyuwangi contributed 14% of total incidents in East Java Province. All efforts have been made to prevent and cope with DHF outbreaks including chemical control such as periodic abatization throughout the season in order to reduce the incidence of DHF (Banyuwangi Health Office, 2015).

Community active participation in controlling DHF outbreaks could be done through improvement and modification of environmental hygiene, physical control with electric rackets and insect webs, biological controls such as mosquito larvae fish farming, and chemical control such as fogging and insecticides. Another method to control DHF using counseling and surveillance has been carried out regularly, however the case is still prevalent (Komalamisra *et al*., 2011).

Aedes aegypti mosquito has been reported to be resistant to cypermethrin 0.05% insecticide in some areas in Central Java i.e. Jepara, Blora, Semarang City, Salatiga City, Surakarta City, Tegal City, Magelang City, and Purwokerto City (Widiarti *et al.*, 2011). Similar results are reported by Sayono *et al*. (2012) in Semarang, that cypermethrin insecticide resistance occurred in *Aedes aegypti* also affects the resistance status of *Culex quinquefasciatus* mosquitoes due to large numbers and the occurance in the same target genes. The results of a recent study in 2015 also showed that *Aedes aegypti* in Semarang City had 100% resistance to organophosphate insecticide group namely malathione 0.8% and permethrin 0.25%.

Factors causing resistance include genetic, biology, and operational factors (insecticide use). Genetic factors consist of frequency, number, and dominance of resistant alleles while biological-ecological factors consist of insect behavior, number of generations per year, mobility, and migration. Operational factors include the type and characteristic of the insecticide used, types of insecticide used before, number of applications, target stage, dose, frequency, application model, and formulation. Vector mosquito resistance can be detected in two ways, conventionally based on WHO standard sensitivity test for mosquitoes using impregnated paper and biochemical or enzymatic tests or plate tests on larvae. Biochemical test is a technique to detect mosquito resistance against highly essential insecticide based on quantity of enzyme responsible for resistance process (Komalamisra *et al*., 2011).

The change *of Aedes aegypti* mosquito genome as the main vector of dengue virus predicted to be the reason why DHF is difficult to be controlled.

Insecticide resistance is probably the cause of dengue fever vectors cannot be controlled completely. The expression of specific genes and autosomes is indicated to affect loci in the genome even when exposed to insecticide occur. Voltage gated sodium channel (VGSC) can also be an important indicator of the development of *Aedes aegypti* resistance to certain insecticides so that its use can be evaluated and improved to determine the appropriate disease control measures (Sinkins, 2010).

MATERIALS AND METHODS

This quantitative study used a combination of epidemiological studies with a cross sectional study approach. Determination of sample area was done based on specific criteria of cluster sampling which represent sample needed as research data of sampling, by dividing Banyuwangi Regency into North (Sub-district Wongsorejo), East (Sub-district Muncar), South (Sub-district Tegaldlimo), West (Sub-district Kalibaru), and Middle (District Banyuwangi). The determination of the area also shows the potential distribution and affinity of mosquitoes because Banyuwangi Regency is surrounded by Baluran National Park, Alas Purwo, and Meru Betiri which called Diamond Triangle Area *(Kawasan Segitiga Berlian)*.

Research samples from each region were 25 larvae of *Aedes aegypti* mosquitoes. The mosquito larvae obtained was then isolated in a buffer solution which contain nutrients for the larvae and maintained until become adult mosquitoes. Samples were obtained using ovitrap that had been randomly installed in the area. Samples were then maintained for insecticide susceptibility tests at Banyuwangi PSDKU Instrument Laboratory and the detection of insecticide resistance genes was carried out at the Institute of Tropical Diseases (ITD) Airlangga University Surabaya. The dengue vector susceptibility test against insecticide was done based on impregnated paper WHO standard method followed by detection of VGSC-coding gene using polymerase chain reaction (PCR) technique. Visualization of VGSC PCR result was done by electrophoresis method on 2% agarose gel.

Resistance Test

The captured mosquito larvae were kept until they become adult mosquitoes in the laboratory. Every day, the larvae are fed with powdered mixture of rice bran and meat in a ratio of 10:4, as much as 75 to 200 mg adjusted for the instar size. After the larvae become adult mosquito (F1), susceptibility test or WHO standard resistance test was conducted using impregnated paper or conventional test. The standard WHO method with impregnated paper insecticides was conducted for organophosphates (malathion 0.8%), carbamate (bendiokarb 0.1%), pyrethroid (deltamethrin 0.05%), permethrin 0.75%, and cypermethrin 0.25%.

As many as 4-5 tubes were prepared for susceptibility test according to WHO standard and an insecticide paper was stick in each tube circularly. Subsequently, 20-25 mosquitoes were put into each test tube (marked with red). As control group, 2 tubes with green mark and insecticides free paper were used. Mosquitoes tested were exposed to insecticide paper for 1 hour then transferred into a green holding tube (storage). The deaths mosquitoes were observed and calculated after 24 hours of storage. During storage, moisture was maintained and the holding tube was equipped with a wet towel. The vulnerability criteria was determined by Herath (2012), sensitive (deaths rate of 99-100%), required verification/tolerance (death rate of 80-98%), and resistance (death rate of <80%).

Mosquito DNA Isolation

DNA isolation was performed using Chelex-100 Ion-Exchanger method. Each mosquito was inserted into a 1.5 mL microcentrifuge tube. A total of 50 µL Phosphate buffered saline (PBS) was added to the tube and the mosquito sample was mechanically destroyed using teflon pestle. 1 µL 0.5% cold saponin was then added into the homogenate solution and stirred slowly. After completion, the tube was incubated in 4° C for 24 hours. The homogenates were then centrifuged at 3000 rpm for 5 minutes; the supernatant was discharged to remove saponins content. After the supernatant was removed into a tube, 1 mL of PBS was added and then further centrifuged for 5 minutes at a rate of 3000 rpm. Afterwards, 150 µL of distilled water and 50 µL of 20% suspense chelex were added. The mixture was then heated at >90° C for 10 minutes, followed by vortex a few seconds. To separate the chelex, the tube was centrifuged for 10 minutes at 12,000 rpm. The supernatant (the upper liquid) was removed and separated from the pellet. The supernatant was then used for PCR. The supernatant was transferred into a new 1.5 mL tube which directly used (Wooden *et al*., 1993).

Amplification of VGSC Genes with Semi-Nested Method PCR

Prior to PCR, the component reagents, i.e 10x buffer PCR; $MgCl_2$ 50 mM; dNTP 10 mM; *kdr*F primer; *kdr*R primer, Taq DNA polymerase enzyme; ddH_2O; and the DNA samples were mixed in a 0.2 mL micro centrifuge tube. Afterwards, the tube was inserted into thermal cycler machine. The primers used were forward primer AgF_kdr; reverse primerAn_ kdr_R2; and reverse primer AgR_kdr. The primer sequences used in the VGSC gene amplification strategy in Anopheles mosquitoes by Kazanidou *et al*. (2009) were as follows AgF_kdr (5' GACCATGAT CTGCCAAGATGGAAT 3'), An_kdr_R2 (5' GAGG ATGAACCGAAATTGGACA 3'), and AgR_kdr (5' GCAAGGCTAAGAAAAGGTTAAGCA 3').

RESULTS AND DISCUSSION

The results (Table 1) showed that *Aedes aegypti* species were resistant to insecticides from organophosphate group (malathione 0.8%) and pyrethroid (0.25% cypermethrin) which Malathione 0,8% is the most resistant and Cypermethrin 0.25%

respectively if compared with the others insecticides. Based on Table 1, *Aedes aegypti* species were also resistant to others insecticides such as Bendiocarb 0.1%, Deltamethrin 0.05%, and Permethrin 0.75% with different mortality rate. It is well known that insecticides from organophosphate and pyrethroid groups have been widely used so that *Aedes aegypti* mosquitoes are often exposed to these insecticides and insecticides from other classes.

The VGSC coding gene was also detected with PCR method from all insecticides which used in this study respectively (Figure 1). The VGSC coding gene have 250 bp length and succesfully detected from all samples (Figure 1) and it is also describe that *Aedes aegypti* mosquito from Banyuwangi Regency are resistant to various insecticides. Based on the resistance results from Figure 1, *Aedes aegypti* mosquito from Banyuwangi Regency are resistant to malathione and cypermethrin, and the possibility of resistance might be transferred to other mosquito species.

The Banyuwangi District Health Office confirmed that malathione insecticides have been used in some Sub-districts in Banyuwangi for vector control by fogging, while cypermethrin was often used as mosquito repellent at household scale. The organophosphate group causes metabolic resistant, which means there are enzymes that decrease insecticide concentration before reaching the target location (Brogdon and McAllister, 1998). All of the mosquito samples were resistant to malathione (organophosphate group); proven by the occurrence of kdr-e when DNA sequencing is performed. The kdr-e type is the second type of mutation resulted in a change of the amino acid leucine into serine (TTA becomes TCA) (Soderlund, 2008; Kazanidou *et al*., 2009).

This suggests that the used of organophosphate group insecticides should be very selective since some farmers are already using this insecticide group as agricultural pest control. This is supported by Najera and Zaim (2001) that *Aedes aegypti* tends to have cross-resistance from the organochlorine insecticide group and the pyrethroid insecticide group. This caused by *Aedes aegypti* has undergone selective suppression of both agricultural insecticides during larval stages in rice fields and indoor residual spraying. In addition, it might be as the result of cross resistance from dichlorodiphenyltrichloroethane (DDT) (class of organochlorine) and pyrethroid group. It has been reported that *Aedes aegypti* is resistant to DDT and have cross-resistance tendency to pyrethroid insecticide group (Hemingway *et al*., 1997). Insecticide sensitivity test of WHO standard is used to detect and to monitor insecticide resistance. If there is any indication of resistance in vectors, further confirmation of the potential for cross-resistance is required. Identifying the resistance mechanisms involved using biochemical methods will predict the potential for cross-resistance at an early stage and provide resistance management guidance. Biochemical endurance tests in combination with WHO standard susceptibility tests are very effective to detect cross-resistance of several disease vectors including dengue vectors. The development of insecticide resistance vector is influenced by various factors such as genetic (specific gene frequency), operational (type and application of insecticides), and

Table 1. Insecticide resistance test result on DHF vector in Banyuwangi

Sample location	Vector species	Vector mortality rate (%)				
		Bendiocarb 0.1%	Deltamethrin 0.05%	Permethrin 0.75%	Cypermethrin 0.25%	Malathione 0.8%
Kalibaru	*Aedes aegypti*	55	60	45	40	0
Muncar	*Aedes aegypti*	75	90	30	10	0
Wongsorejo	*Aedes aegypti*	60	85	90	5	0
Tegaldlimo	*Aedes aegypti*	85	75	65	35	0
Banyuwangi	*Aedes aegypti*	45	10	20	15	0

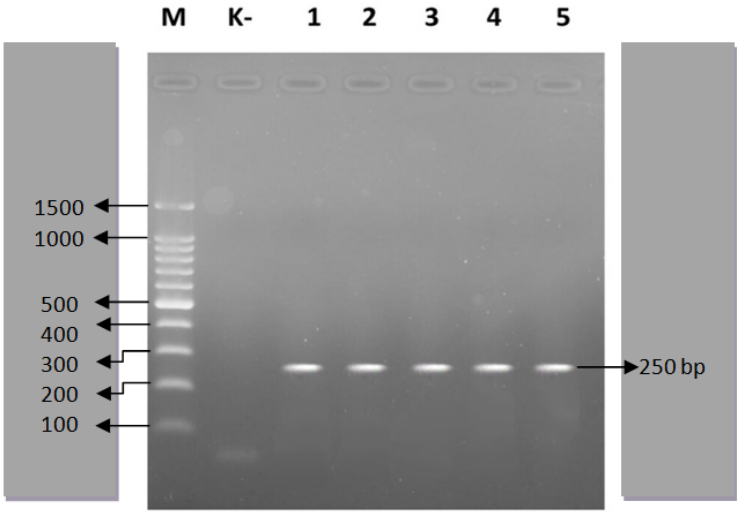

Figure 1. *Aedes aegypti* VGSC-coding gene

biology (size and characteristics of vector population) (David and Gilles, 2002). The occurrence of vector resistance does not go through a gradual adaptation process to toxic chemical compounds, but over an acceleration process based on natural selection. Selection occurs when a small number of insects undergone the individual genetic mutations.

CONCLUSION

This study has proved that *Aedes aegypti* mosquitoes from five Sub-districts in Banyuwangi Regency (Kalibaru, Muncar, Tegaldlimo, Wongsorejo, and Banyuwangi) were resistant to malathione insecticide (organophosphate) and cypermethrin insecticide.

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