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by Budi Utomo.
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ABSTRACT
An urban park is potentially a source of vector-borne disease transmission due to it being a natural and artificial mosquito breeding habitats combined with people's continuous presence. Thus, this study aims to screen the occurrence of knockdown-resistance (kdr) mutant alleles (V1016G and F1534C) in mosquito populations collected from urban parks in Surabaya, Indonesia. Cross sectional study was conducted in July 2019. A total of 28 ovitraps were installed in seven urban parks, having four ovitraps installed in each park. In total, 1,662 eggs were collected and only 187 emerged into adult mosquitoes, consisting of 97 An. stephensi and 89 An. albopictus. All female adult mosquitoes (155) were tested using allele-specific polymerase chain reaction assay (AS-PCR) to detect voltage gated sodium channel (Vgsc) gene mutations. This study found no mutations in Valine to Glycine mutation in point 1016 (V1016G) and Phenylalanine to Cysteine in point 1534 (F1534C) alleles in both species. All of mosquito samples have wild type genotype of kdr alleles (V1016V and F1534F). Data were analysed using R Studio 1.4 Version by Genetix package. Results showed that the frequency of resistant alleles (G1016 and C1534) was zero, and the frequency of susceptible allele was 1 (V1016 and F1534). Insecticide bioassay could not be established due to the limited number of adult mosquitoes, so insecticide resistance status could not be determined. However, this study can be used as preliminary monitoring for the vector control program.

Keywords: dengue, insecticide, kdr allele, mosquito, Surabaya, urban parks
INTRODUCTION

Mosquitoes can spread and carry diseases so that it can making them as one of the deadliest animals. Many mosquito-borne diseases still show an increasing number, including dengue, Zika, chikungunya, West-Nile Virus, malaria, and yellow fever. Half of the population worldwide have lived in an area where mosquitoes are present. Four genus are commonly found as the vector of mosquito-borne diseases, such as Aedes, Culex, Mansonia, and Anopheles. Genus of Aedes can transmit chikungunya virus, Zika virus, dengue virus, lymphatic filariasis, and yellow fever virus (World Health Organization 2020b). Two species of Aedes, including Aedes (Steegomyia) aegypti and Aedes (Steegomyia) albopictus, are the important vector of arboviral disease (Reinert et al. 2009). Dengue infection was a disease transmitted by both of them and became a burden for public health (Simmons et al. 2012).

Data from WHO in 2020 shows that approximately 390 million people have been estimated infected by dengue infection and distributed across 128 countries (World Health Organization 2020a). Indonesia was also endemic to dengue infection, increasing its annual incidence rate from 0.05/100,000 in 1968 to 78.8/100,000 in 2016. However, the case fatality rate was decreased from 41% in 1968 (Nathin et al. 1988) to 1.21% in 2004 (World Health Organization 2006).

Currently, there is no effective vaccine that can be a prophylaxis for all age groups. Dengvaxia (CYD-TDV) is a live recombinant tetravalent dengue vaccine by Sanofi-Pasteur that was first licensed and can be used for individuals 9 to 45 years old of age individuals in endemic areas. The efficacy of this vaccine was varied, with the highest efficacy being against dengue serotypes 3 and 4 (71.6% and 76.9%) followed by dengue serotypes 1 and 2 (54.7% and 43%) (World Health Organization 2020c). Following that, the primary method to prevent dengue infection transmission is through vector control. A meta-analysis study showed that varieties of dengue control variation significantly reduces dengue risk, such as house screening, water-container covering and community-based environmental management. Interestingly, indoor residual spraying (IRS) did not significantly reduce dengue risk, while the use of insecticide aerosol and mosquito coils was associated with increased dengue risk. In line with that, skin repellent, insecticide-treated bed nets were also had no effects (Bowman et al. 2016). Thus, the efficacy of vector control remained in question and maybe can vary in different geographical areas.

The primary method of dengue infection control in Indonesia is the combination of environmental management (eradicating larval mosquito, covering the water-container, draining the bathtub regularly) and thermal fogging. In Indonesia, Organophosphates have been used for a long decade to control adult mosquitoes. Malathion, as the derivative of Organophosphate, was used to control adult mosquitoes, while temephos was used as larvicide. Malathion was firstly introduced in Indonesia in 1969, while temephos was introduced in 1980 for dengue control (Harjianti et al. 2015). The intensive and massive use of insecticide can lead to the sensitivity decreasing of mosquito population against commonly-used insecticide. This phenomenon can be called as insecticide-resistance mechanism (World Health Organization 2012). There are four types of insecticide-resistance: metabolic resistance, target-site resistance, cuticular resistance, and behavioural resistance (Corbel & Guissain 2013).

Metabolic resistance is a mechanism in insects enzyme system to naturally detoxify from insecticides exposure. The over-expression of enzymes that can detox and amino acid substitution within the enzyme can increase the enzyme's affinity, resulting in metabolic resistance (David et al. 2007; Hemingway et al. 2004). The decreasing effectiveness of the
in mosquito to bind insecticide called target-site resistance, such as the target site of carbamates and organophosphate insecticides is acetylcholinesterase (AChE) in the nerve cell (Fournier 2005). The insect can also develop cuticular resistance as the impact of the reduced uptake of insecticide and the increasing thickness of the cuticle, but the study of this resistance is still limited in Anopheles genus (Djouaka et al. 2008). Another mechanism of insecticide resistance contributing to vector control failure is behavioural resistance, that is the mosquito’s ability to avoid insecticide exposure. The investigation of this mechanism was also limited and only found in Anopheles population (Russell et al. 2011).

Detection of insecticide resistance in mosquito populations can be conducted through various methods, such as biochemical assay and bioassays using WHO diagnosed doses, dose-response bioassays, and molecular assays. Molecular assays are the most sensitive way to predict the possibility of insecticide-resistance in the future. This method can determine the frequency of resistant allele in a population (Ranson et al. 2011). Point mutations of kdr have been revealed in some countries worldwide, such as point mutations G923V (Glisin to Valin) and I1011M (Isoleucin to Metoin) found in Brazil, Guyana, and Martinique (Brengues et al. 2003), L982W (Leusin to Tryptofan) found in Vietnam (Brengues et al. 2003), F1534C (Phenylalanin to Cystein) found in Indonesia and Thailand (Kuwada et al. 2014; Wulandari et al. 2020), and V1016G (Valin to Glisin) widely distributed in Indonesia and Thailand (Brengues et al. 2003; Rajatleka et al. 2008; Wulandari et al. 2015). The mutations are associated with the exposure of permethrin and DDT. In several regions of Indonesia, mutant allele was reported in Kuningan (Jatara), Padang, Samarinda, Pontianak, Mataram, Denpasar, Dompu, West Manggarai, and East Sumba with the frequency 0.73, 0.6, and 0.02 respectively for V1016G, S989P, and F1534C (Amelia-Yap et al. 2019). As the first place where dengue infection was found in 1968, Surabaya also used malathion as the insecticide for vector control, but the resistant alleles report in mosquito from these populations is still yet detected. This study aims to screen the occurrence of knockdown-resistance (kdr) mutant alleles in Surabaya, Indonesia. The public urban park is the chosen study site due to its importance value as the public facility.

MATERIALS AND METHODS

Materials
Mosquito eggs from the field were reared until they emerged to be adult mosquitoes, consisting of two species, namely Aedes (Stegomyia) aegypti and Aedes (Stegomyia) albopictus. Rearing processes were in accordance to the guidelines mentioned in procedure paragraph. Materials that were used in this research were QIAamp Viral RNA Extraction Kit (Qiagen, Hilden, Germany), NEXscript™ RT-PCR 2x Master Mix (NEX™ Diagnostic, USA), Nuclease Free Water (NFW), 70% ethanol, agarose gel, ethidium bromide, TBE buffer, sugar solution, cotton, and labelled paper. Instruments used were micropipette, tray, plastic pipette, board maker, microcentrifuge, freezer -80°C, vortex mixer, biosafety cabinet level-2, thermal cycler Bio Rad, Erlenmeyer, gel documentation imaging system, and stereo microscope.

Methods
Study Area
Surabaya is in Java Island and is highlighted as the most populated city in East Java, resided by two million citizens of various ethnicities. This situation can lead to urbanization and affect the transmission of mosquito-borne diseases, including dengue infection. This study was conducted in seven
Table 1. Details of parks that have successfully had mosquito hatched eggs, collection date, and their coordinates.

| Collection Site | District | Coordinates     | Collection Date |
|-----------------|---------|-----------------|-----------------|
| Apsari          | Genteng | 7°18′52.34″S 112°44′34.22″E | 15/07/2019     |
| Harmoni         | Sukolilo| 7°17′40.04″S 112°48′13.16″E | 08/07/2019     |
| Lansia          | Gubeng  | 7°16′18.85″S 112°45′17.74″E | 22/07/2019     |
| Pelangi         | Gayungan| 7°19′39.23″S 112°43′52.34″E | 15/07/2019     |
| Persahabatan    | Wonomerto| 7°16′39.93″S 112°45′07.44″E | 15/07/2019     |
| Prestasi        | Genteng | 7°15′41.30″S 112°44′34.25″E | 22/07/2019     |
| Wira Agung      | Wonomerto| 7°17′56.29″S 112°44′16.73″E | 15/07/2019     |

Figure 1. Geographical map of Surabaya City, seven urban parks were marked with circle (Ha: Harmoni Park, Pe: Prestasi Park, Lc: Lansia Park, Pd: Persahabatan Park, We: Wira Agung Park, Pl: Pelangi Park). Figure was created with QGIS 3.10.4 Version.

Urban parks in Surabaya, Indonesia. Above are the coordinate of the sampling site (Table 1) and the geographical map was shown in Figure 1.

Procedures

Mosquito Collection and Rearing

Ovitrap surveillance method was adopted from the following guidelines (Irham et al. 2014). Ovitrap were installed in some of the public urban parks distributed in Surabaya (Figure 1) and designed with black colour to attract mosquitoes to lay their eggs, but only some of the eggs from several parks were successfully attached (Table 1). A total of 28 ovitraps were installed in which 4 ovitraps were installed in each park. Observation of attached eggs in filter paper was done every seven days. All filter papers are labelled based on the date of collection and the collection site. After that, they would be
brought to Entomology Laboratory, Institute of Tropical Disease, Universitas Airlangga. Mosquito rearing was set based on the mosquito rearing guidelines (Imam et al. 2014). Adult mosquitoes were identified using an identification key from the Indonesian Ministry of Health (Indonesian Ministry of Health 1989). All of study design in present study was approved by Ethical Committee Faculty of Medicine, Universitas Airlangga with the reference number of 24-934/UN3.14/PPd/2013.

RNA Extraction
Detection of the dengue virus on the mosquito that were caught was also conducted, but the result will be reported in another study. In regards to the double purpose, RNA extraction was conducted. After mosquito identification was made, mosquitoes were extracted using QIAamp® Viral RNA by Qiagen, Germany, based on the manufacturer's instruction to extract RNA from mosquito specimens.

AS-PCR Assay for the Detection of kdr Mutant Alleles (V1016G and F1534C)
After cDNA from the procedure of 2.2.2.2 were obtained, genotyping process was conducted. Genotyping of kdr mutant alleles to detect mutant in codon 1016 and 1534 were performed using Allele-specific PCR assays following previous guidelines (Stenhouse et al. 2013; Yanola et al. 2011) AS-PCR was conducted by using forward and reverse primer below (Table 2).

Reactions were performed using PCR Thermocycler with following stage: 94°C in 2 min, 35 cycles of 30 sec in 94°C, 55°C in 30 sec, 72°C in 30 sec and final elongation 72°C in 2 min for the detection of V1016G. For the detection of F1534C, PCR was performed with the stage of 94°C in 2 min, 35 cycles of 30 sec in 94°C, 30 sec in 60°C, 30 sec in 72°C, and elongation step with 72°C in two minutes. PCR products were then loaded into 1.5% agarose gel.

Data Analysis
Data were analysed using R Application of 4.0.4 Version, using packages “HaplyWeinberg” to analyse the frequency of resistant allele.

RESULTS
A total of 55 female Aedes (Stegomyia) aegypti and Aedes (Stegomyia) albopictus were tested, consisting of 37 Aedes (Stegomyia) aegypti and 18 Aedes (Stegomyia) albopictus. The composition of mosquitoes in every park was shown in Figure 2. The electrophoresis results were shown in Figure 3. The comparison between our result and the reference was shown in Figure 4 and Figure 5.

Table 2. The sequences of oligonucleotides used to amplify fragments of the VGSC gene (Stenhouse et al. 2013; Yanola et al. 2011).

| Primer sequence | Product (bp) |
|-----------------|-------------|
| Gly1016f        | 5'-ACGGACAATAATGTTTGCCC-3' |
| Gly1016r        | 5'-GCCGGCAGGGCGGCGGGGGGGGGGGGGGGGGGG-GAGGTTAACTC-3' |
| Val1016r        | 5'-GCGGGCGACACAGGAAGCTAGAAAGGTAAAGTT-3' |
| Cys1534f        | 5'-GGGGGCGAGGCGGGGCGGGGGGGGGGCTTCTACTTT-GTGTTCATCATGAGT-3' |
| Cys1534r        | 5'-TGTTCATCATTGGTGTGAGGTTGAT-3' |
| Phe1534f        | 5'-GGGGGCTGGTGTGTTGTTGTTGATT-3' |
Figure 2. The distribution of mosquito species, dark grey indicated the occurrence of *Aedes (Stegomyia) aegypti*, meanwhile light grey indicated the occurrence of *Aedes (Stegomyia) albopictus*. Ha: Harmoni Park, Pe: Preseki Park, La: Lansia Park, Pe: Persahabatan Park, Wi: Wira Agung Park, Pe: Pelangi Park. (Figure was created with QGIS).

It was genotyped to only female mosquitoes due to the important role of female mosquitoes as the vector of mosquito-borne diseases. We estimated that random mating was occurred in this population and VGSC is not sex-linked. The results showed that no point mutation was detected in

Table 3. The distribution of species in every park, AS-PCR result (SS: Susceptible allele/V1016V/F1534F/Homozygous wildtype, SR: Susceptible resistant/V1016G/F1534C/Heterozygous mutant, RR: Resistant resistant/G1016G/C1534C/Homozygous mutant).

| Sampling Site       | *Aedes (Stegomyia) aegypti* | *Aedes (Stegomyia) albopictus* | n (pool) | Genotype | Allele Frequency |
|---------------------|-----------------------------|-------------------------------|----------|----------|------------------|
|                     |                             |                               |          | SS (%)   | SR (%) | RR | S | R |
| Apsari Park         | 5                           | 0                             | 3        | 3 (100)  | 0 (0)  | 0 (0) | 1 | 0 |
| Harmoni Park        | 12                          | 8                             | 2        | 2 (100)  | 0 (0)  | 0 (0) | 1 | 0 |
| Lansia Park         | 3                           | 0                             | 1        | 1 (100)  | 0 (0)  | 0 (0) | 1 | 0 |
| Pelangi Park        | 7                           | 1                             | 1        | 1 (100)  | 0 (0)  | 0 (0) | 1 | 0 |
| Persahabatan Park   | 5                           | 2                             | 1        | 1 (100)  | 0 (0)  | 0 (0) | 1 | 0 |
| Prestasi Park       | 1                           | 7                             | 1        | 1 (100)  | 0 (0)  | 0 (0) | 1 | 0 |
| Wira Agung Park     | 4                           | 0                             | 2        | 2 (100)  | 0 (0)  | 0 (0) | 1 | 0 |
| **Total**           | **37**                      | **18**                        | **11**   | **11**   | **0**   | **0** | **1** | **0** |

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all of the samples. In other words, all the mosquitoes collected have homozogous wildtype of V1016V and F1534F, as shown in Table 3 below. If the AS-PCR results shows the homozygous wildtype/V1016V/F1534F, it can be symbolized as genotype SS (means that this pool represents susceptible allele), heterozygous mutant/V1016G/F1534C symbolized as genotype SR (means that this pool represents susceptible and resistant allele), homozygous mutant/G1016G/C1534C symbolized as genotype RR (means that this pool represents resistant allele).

Figure 3. The electrophoresis results of the detection of kdr mutant allele (A: V1016G and B: F1534C). Amplified products could be differentiated by size (60 bp for V1016, 80 bp for G1016, 93 bp for F1534 and 113 bp for C1534).

Figure 4. The representative of the electrophoresis of V1016G. Study result compared with the reference, left figure was the study result, right figure was the figure from reference (Stenhous et al. 2013). M=marker of 25 bp; V1016V = Homozygous wildtype; V1016G = Heterozygous Mutant; G1016G = Homozygous Mutant.
**DISCUSSIONS**

This study highlighted that no $kdr$ mutant alleles (V1016G and F1534C) have been found from the study site. This study was the first report of $kdr$ mutation screening in Surabaya, East Java Province, Indonesia. Some of $kdr$ mutant alleles in Indonesia have been reported from many regions and showed various mutations on V1016G, F1534C, and S989P. A study from Amelia-Yap shows the occurrence of mutation on V1016G and S989P in mosquitoes collected from Kuningan, Padang, Denpasar, Samarinda, Mataram, and Sumba Timur (Amelia-Yap et al. 2019). Pointing out and detecting mutation of Val1016Gly/V1016G and Phe1534Cys/F1534C is important, including in national and local areas. The Allele-Specific PCR Assay (AS-PCR) can be implemented to detect $kdr$ mutant and provide a rapid result, accurate, and cost-effective genotyping (Lee et al. 2016). Rapid results and the precise target can be used to figure out the VGSC gene of mosquitoes in a population. However, this method cannot stand alone and must be combined with WHO tube test Bioassay to capture information about the susceptibility status of mosquito against insecticides (Corbel & Guissan 2013). World Health Organization (WHO) Tube Test Bioassay against insecticides require too much number of samples. Instead, AS-PCR was used for the screening of $kdr$ mutant alleles in the mosquito, using the previous method (Stenhouse et al. 2013). $kdr$ screening using Allele-Specific PCR assay is a very sensitive method that can provide early warning for future resistance detection that is suitable to use given the limited number of mosquitoes (Ranson et al. 2011). In this study, not all the mosquito eggs that were rearing successfully emerge into adult mosquitoes. Thus, AS-PCR assay screening method was adopted.

Surabaya is a dengue-endemic area and the first place where dengue cases were found in 1968. Management of dengue infection in Indonesia, including in Surabaya, was focused on vector control, which can be broken down into three methods: physical control, chemical control, and biological control. Physical control can be done by washing bathtubs regularly every seven days before the mosquito eggs become adult mosquitoes, larval
elimination through program one house one larval observer, and closing water containers. Fogging activity (hot-fogging) was applied in some Indonesian regions as chemical control, except in Surabaya where they used Ultra Low Volume spray (cold-fogging). Some chemical insecticides in ULV were from the organophosphate group (Malathion and methylparathion), Pyrethroid (Cypermethrin, Lambda-cyhalothrin, Cytfluthrin, Permethrin, S-Bioallethrin, and its derivations). In comparison, chemical control in goal for killing mosquito larvae was temephos from the organophosphate group and pirimiphos (Indonesian Ministry of Health 2017).

Since insecticides have been used for many decades, long exposure to insecticides has been a major public health problem. Continuously using the same insecticide for a long period led to mosquitoes' development resistance to insecticide exposure. In this case, every country should be responsible for their vector control program. World Health Organization (WHO) defined resistance as an insect's ability to survive against the effect of insecticides through natural selection and mutations (World Health Organization 2012). Other countries in South-East Asia were also facing the same problem. In the South-East Asia region, the number of V1016G mutations have been reported from Cambodia, Laos, Myanmar, Malaysia, Singapore, Thailand, and Vietnam. While the report from Philippine and Timor Leste are still not available (Amelia-Yap et al. 2018). Other mutations were also reported, such as mutations in Thailand that show mutations in codon 1011, where isoleucine become valin (I1011V) (Rajatileke et al. 2008). Some resistant alleles frequencies, including F1534C and V1016G have been reported in various range. A cross sectional study in India in 2015, shows the frequency of F1534C(C) and V1016G(G) were 0.51 and 0.18 respectively (Kushwah et al. 2020). In other part of India, it also has been reported the frequency of F1534C(C) was around 0.41-0.79, combined with the new point mutation T1520(H) with the frequency of 0.13 (Kushwah et al. 2015). Researches about point mutation in the voltage-gated sodium channel of dengue vector were rapidly increase, a report from Taiwan found a novel point mutation, D1794Y that occurs with the V1023G mutation (Chang et al. 2009).

Meanwhile, a study in Mexico successfully revealed the co-occurrence of point mutation V1016I and F1534C was associated with pyrethroid resistance during 16 years of observation (Savvides et al. 2015). The derivative of permethrin (λ-cyhalothrin) was also reported to be the causative of kdr point mutation D1491L in Colombia, with the frequency ranging from 0.06 to 0.46 (Granada et al. 2018).

Study of knockdown resistance (kdr) alleles in Indonesia may give another impact on vector control method. In Yogyakarta, a dengue-endemic city, the frequency of kdr mutant alleles V1016G and F1534C in the area where Wolbachia will be released as vector control method was firstly measured and shows high frequencies of V1016G mutation, but F1534C was low detected. The result of kdr screening can figure out population background, so that the vector control that may be released can be suitable (Wulandari et al. 2020). Since having the first case of dengue infection in 1968 in Jakarta and Surabaya, Indonesia has applied some vector control, but it still needs to be improved so that the dengue outbreaks can be avoided. The number of dengue incidence rate (IR) in Indonesia was increased from 0.05 to 40 per 100,000 populations in 2013. The highest epidemic was reported in 2010, with the IR value of 85.7/100,000 population (Haryanto 2018). Some risk factors of dengue infection in Indonesia were various breeding sites especially during rainy season, the mobility of citizens inside or outside the country, and the low level of awareness toward health and hygiene lifestyle (Setiati et al. 2006). Indonesia is a suitable place for some
vector’s growth and development as a tropical country, including mosquitoes. Although this study had not found kdr mutant allele in Aedes (Stegomyia) aegypti and Aedes (Stegomyia) albopictus, it does not take alleles in another district/region into account, so regular surveillance in other endemic areas are strongly suggested. kdr mutant alleles in points 989, 1534, and 1016 was a leading factor that causes mosquitoes to develop resistance phenotype against insecticide (Harris et al. 2010; Srisawat et al. 2010). A-PCR assay contributes to the early detection for future warning resistance in the mosquito that cannot be detected by bioassay methods. Thus, the presence of a single/double kdr mutant allele should be considered. In other cases, the negative result/the absence of kdr mutant allele did not lead to complacency because of the specific target that are being examined (Corbel & Guessan 2013). The absence of kdr mutant allele in one point does not reflect another absence of mutation in another point. Hence, this study might be used as complement data for the vector control program.

CONCLUSION
Result of present study confirmed that no mutations were found in Valine to Glycine in point 1016 (V1016G) and Phenylalanine to Cysteine in point 1534 (F1534C) alleles in both two species, Aedes (Stegomyia) aegypti and Aedes (Stegomyia) albopictus collected from urban parks, Surabaya, Indonesia. All of mosquito samples have homozygous wild type genotype of kdr alleles (V1016F and F1534F).

AUTHORS CONTRIBUTION
SF was responsible for conceptualization, data collection, and manuscript preparation. SS was responsible for supervision, investigation of the data, and manuscript preparation. BU was responsible for data analysis and manuscript preparation. THS was responsible for data collection and manuscript preparation. HA was responsible for manuscript preparation and grammatical check. AA was responsible for data validation and manuscript preparation. PW was responsible for data validation and manuscript preparation. SS was responsible for investigation of the data and manuscript preparation.

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CONFLICT OF INTEREST
The authors declare that there is no conflict of interest in this research.

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