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RESEARCH ARTICLE

Characterization of *Aedes* sp. larvae: Detection of dengue, Japanese encephalitis and West Nile virus with knockdown resistance mutations in Mulyorejo, Surabaya, Indonesia

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Abstract

Indonesia recorded the highest dengue mortality rate in Asia in 2022, with 45,387 cases and 432 deaths. This study aimed to investigate the coexistence of arboviruses and insecticide resistance mechanisms in *Aedes* vectors mosquitos' populations in Mulyorejo District, Surabaya, during the early monsoon season of 2021. A cross-sectional survey was conducted in January 2021, and *Aedes* larvae were collected and reared to adulthood under laboratory conditions. A total of 309 *Aedes* larvae (comprising 65% *Aedes aegypti* and 35% *Aedes albopictus*) were collected from 36 breeding sites, identified, and georeferenced using QGIS software. RNA extraction and RT-PCR assays were performed to detect Dengue virus (DENV), Japanese encephalitis virus (JEV), West Nile virus (WNV), and knockdown resistance (KDR) mutations. Results showed seven samples positive for DENV-2 and seven for JEV, with two samples (codes 9 and 12) exhibiting co-infection with both viruses, while no WNV was detected. The detection of JEV in *Aedes* larvae is a notable finding, suggesting a potential secondary vector role for these species in the study area, as Culex mosquitoes are the primary vectors of JEV. Mutations at codons 1016 (V1016G) and 1534 (F1534C), associated with pyrethroid and DDT resistance, were detected in eight and seven samples, respectively. These findings confirm the circulation of DENV-2 and JEV in local *Aedes* populations, highlighting the presence of KDR mutations that confer insecticide resistance. The results underscore the urgent need for integrated vector management in Surabaya, combining arbovirus surveillance, insecticide resistance monitoring, and community-based source reduction to mitigate arboviral disease transmission.

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1. INTRODUCTION

Indonesia had the highest mortality rate from Dengue virus (DENV) in Asia in 2022 (1). First reported in Indonesia (1968), with 58 cases and 24 deaths (case fatality rate (CFR) = 41.32). DENV was highly prevalent in 2019, with 277 cases and 3 deaths (IR=9.56 and CFR=1.08) and 6 cases from Mulyorejo. This number experienced a decline in 2020; there were 73 cases and no deaths (IR=2.51 and CFR=0.00), and 1 case was from Mulyorejo. However, according to the latest data from the Ministry of Health reported before the 39th week of 2022, dengue hemorrhagic fever' (DHF) cases exploded again, with an incidence rate (IR DHF) of 34.3% and a case fatality rate (CFR of DHF) of 0.90%, with 45,387 cases and 432 deaths, and for the end of 2022, the number of dengue cases reached 187 cases and 11 deaths in Surabaya (2).

Despite ongoing vector control efforts through community-based *Pemberantasan Sarang Nyamuk* (PSN) programs, several districts continue to report dengue cases, indicating the persistence of *Aedes* mosquito populations capable of sustaining transmission. One such area is Mulyorejo District, which recorded nine dengue cases in 2019, no reported cases in 2020, and two cases in 2021, according to data from the Surabaya City Health Office (3-5). The occurrence of sporadic cases despite intensive control measures suggests that local *Aedes* populations may exhibit adaptive or genetic variations that contribute to their survival and potential vector competence. Therefore, Mulyorejo was selected as the sampling site in this study to characterize the *Aedes* species and explore possible genetic features related to their persistence in an urban setting with ongoing vector management interventions (6-10).

DHF disease management is complex, and current disease prevention plans is symptomatic supportive care are ineffective (11). The primary method for preventing the spread of Dengue viruses is the elimination of the vector using insecticides (12,13) . This approach is detrimental because of long-term use development of knockdown resistance (kdr) in *Aedes* sp. (14). The resistance of *Aedes* sp. mosquitoes to pyrethroid pesticides can be characterized by mutations in codon 1016, where valine is substituted by glycine, which causes mutations that cause immunity or kdr. However, the dichloro-diphenyl-trichloroethane (DDT) pesticide is mutated at codon 1534, where phenylalanine is replaced by cysteine, serine, or leucine (14,15). Previous research has conducted a cross-sectional survey in urban parks in Surabaya, they used allele-specific polymerase chain reaction (AS-PCR) to kdr mutations V1016G and F1534C but found no mutations (14). In this process, the viral RNA that may contain mutations associated with knockdown resistance (kdr), such as V1016G or F1534C, is transcribed into cDNA using reverse transcriptase. Once converted into cDNA, standard PCR techniques are applied to amplify specific gene regions that contain these resistance mutations. This allows for the detection and analysis of genetic variations contributing to resistance against insecticides, even when only RNA samples are available.

Virus detection can help to monitor and prevent the spread of the virus, enhancing the surveillance system to respond to cases, reducing the number of DENV cases, and promoting an integrated vector management approach that involves community participation, environmental management, biological and chemical control, and intersectoral collaboration (3). The number of DHF patients has increased due to misdiagnosis or inadequate treatment for the Dengue virus during the COVID- 19 pandemic. This study also helps to validate and identify the serotypes found to guide the appropriate treatment and prevention measures. Recent study revealed that in addition to carrying the Dengue virus, *Aedes* sp. could also be a vector for Japanese encephalitis virus (JEV) and West Nile virus (WNV) (4). JEV cases in Indonesia were first found in Bali Province, with the highest rate of JEV virus transmission, mainly due to the presence of pigs as primer vectors periodically (5,9). WNV cases were first found in Bandung, Indonesia in 2005, where two patients showed hemorrhagic manifestations, renal insufficiency, liver dysfunction, non-cardiogenic pulmonary edema, and platelet count < 1000,000/mm^3. WNV is transmitted between avian hosts and *Aedes* sp. are involved as important vectors in the enzootic cycle (10,11). Another research in Surabaya isolated DENV-2 from 2008 to 2014, but this study didn't include other DENV serotypes that may co-circulate in the area. Detection of JEV has been conducted in Bali Island, Indonesia from *Culex* sp. and found that the JEV genotype IV isolates in 2017, but no studies have investigated the detection of JEV potential in *Aedes* sp. and its implications for Surabaya (16).

Reverse transcriptase polymerase chain reaction (RT-PCR) methods used to amplify and identify the RNA of viruses with specific primers (17-19). In the first step of the RT-PCR reaction, the extracted RNA is converted into complementary Deoxyribonucleic Acid (cDNA) using the Superscript III Reverse Transcriptase enzyme (Invitrogen) and the D2 primer, which targets conserved genomic sequences of the virus for the serotype being tested (8,14,20,21). In the second step, the formed cDNA serves as a template for the PCR reaction to detect the Dengue virus, using Taq Polymerase enzyme and its primers. A positive reaction is indicated by the presence of a DNA band on agarose gel electrophoresis. The determination of the Dengue virus serotype is based on the size of the DNA bands formed after visualization on agarose gel electrophoresis. For both detection and serotype determination, positive controls are always included for each serotype, consisting of RNA extracted from the supernatant of reference virus culture. The presence of *Aedes* sp. larvae in an area can indicate the presence of *Aedes* sp. populations, and this study will enhance the understanding of the epidemiology and evolution of the Dengue virus and other arboviruses in Indonesia (22-25).

2. MATERIALS AND METHODS

2.1. Study Area

This study was carried out in Mulyorejo District, Surabaya, East Java, Indonesia (longitude 112°47.0243'E-7°16.3381'S Longitude). The spatial distribution of the mosquito samples was analyzed using QGIS Desktop 3.28.6 software (https://qgis.org/download/), and a district, Mulyorejo, was preferred as the sampling site for this study.

2.2. Larva Collection

All sample collection were used a code of ethics that was authorized by the Institute for Research and Community Service Universitas Airlangga with approval number 24-934/UN3.14/PPd/2013. The classification of natural breeding habitats was adopted in Kitching in 1971 with modification. Larval collection was conducted in January 2021, which was the early monsoon season. Because of population of these mosquitoes increases dramatically during the rainy season when many puddle (23,26,27). After being collected, the mosquito larvae were raised via artificial breeding sites, which are artificial places in the laboratory for breeding mosquito larvae to become adult mosquitoes. Due to the limitations of this study, only *Aedes* larvae were collected. A simple method for differentiating between *Aedes* larvae and other larvae involves conforming the siphon and movement pattern of the larvae. All potential breeding habitats were examined using a flashlight to ensure the occurrence of larvae. Larvae were collected using a plastic pipette from 36 potential mosquito habitats.

2.3. Artificial Breeding and Mosquito Identification

All the larvae collected were reared under laboratory conditions following the mosquito rearing guidelines (14). After all of the larvae emerged into adult mosquitoes, they were frozen at -80°C until death. Mosquito identification was performed under a stereomicroscope. Mosquitoes were separated based on the guidelines for Indonesian *Aedes* mosquitoes published by the Indonesian Ministry of Health. The mosquitoes were identified as *Aedes aegypti* and *Aedes albopictus* based on their morphological characteristics. *A. aegypti* is characterized by a lyre-shaped pattern on its head, consisting of two curved and two straight white lines, while *A. albopictus* has a single white line on its mesonotum (26-28). A digital microscope (model and manufacturer) was used to examine the larvae. In terms of size, *A. albopictus* is slightly smaller, measuring approximately 8 mm, than *A. aegypti*, which measures approximately 10 mm (26,29,30).

2.4. RNA Extraction and Purity Measuring Using Nanodrop Spectrophotometer

RNA was isolated from frozen adult mosquitoes using a Zymo Kit™ (USA) according to the manufacturer's instructions. To each mosquito sample was added 300 µL of DNA/RNA Shield™, 30 µL of PK digestion buffer, and 15 µL of proteinase K, after which the mixture was vortexed and incubated at 55°C for up to 5 hours for maximum extraction. The tissue sample that had dissolved in the liquid was then centrifuged at 15,000 × g for 1 minute. The supernatant (300 µL) was transferred to an RNase-free tube, an equal volume of RNA lysis buffer was added, and the mixture was vortexed.

For RNA purification, the sample was put into a yellow Spin-Away™ filter attached to the collection tube and then centrifuged. The filtrate was transferred to a new collection tube, ethanol was added at a 1:1 ratio to the sample volume, and the mixture was vortexed. The mixture was then transferred to a green Zymo-Spin™ III CG column attached to the collection tube and centrifuged. The filtered sample was removed from the collection tube, and the collection tube was reattached. Subsequently, 400 µL of RNA prep buffer was added, the sample was centrifuged, the filtered sample was removed, the solution was removed from the collection tube, and the collection tube was reattached. The filtrate was discarded, and the column was washed twice with 700 µL and 400 µL of RNA wash buffer. The column was then transferred to an RNase-free tube and centrifuged at maximum speed for 2 minutes to ensure the cleanliness of the RNA sample.The RNA sample was eluted with 100 µL of DNase/RNase-free water and centrifuged.

The sample in the spectrophotometer was wiped with ethanol and dried with a KimWipe before each measurement. Two microliters of dispensing water (blank) were loaded, and the system was initialized according to the instructions on the screen. The computer setting was changed to RNA, and the blank button was clicked. Then, for each sample, 2 µL was loaded, and the "measurer" button was clicked. The results, including the A260/A280 and A260/A230 ratios and the amount of RNA obtained (in ng/µL), were recorded. The samples were wiped with a clean, dry KimWipe between samples.

2.5. RT-PCR Assay for Detecting DENV

An RT-PCR assay was performed to detect and serotype Dengue virus in the RNA samples (20,31) using specific primers, probes, and targets for each serotype (DEN-1, DEN-2, DEN-3, and DEN-4) by using primers from the journal Lanciotti (17). Three microliters of each RNA sample were added to 5 µL of PCR mix one step, 1 µL of nuclease water, and

0.2 µL of the targeted primers forward (D1) or reverse (TS1, TS2, TS3, and TS4) for each primer. The next stage is the RT-PCR protocol consisting of reverse-transfer stage at 55°C for 45 minutes, following by pre-denaturation at 94°C for 2 minutes, then the primary stage of denaturation, annealing, and extension is cycled 35 times with following temperature are 94°C for one minute, 60°C for 1 minute, and 72°C for 1 minute, after cycling at 35 times the final extension is 72°C and after that takes 16°C constant for incubation until the sample taken (32).

2.6. RT-PCR Assay for Detecting the V1016G and F1543C Mutations

At this stage, *Aedes* resistance was detected via RT-PCR with V1016G and F1543C Primer (14). Three microliters of extracted RNA were mixed with 5 µL of PCR mix, 1 µL of nuclease-free water, and specific primers. For the VG locus, two reverse primers (5′-GCGGGCAGGGCGGCGGGGGCGGGGCCAGCAAGGCTAAGAAAAGGTTAACTC-3′ and 5′-GCGGGCAGCAAGGC TAAGAAAAGGT TAATTA-3′) and one forward primer (5′-ACCGACAAATTGTTTCCC-3′) were used, while for the FC locus, one reverse primer (5′-TCTGCTCGTTGAAGTTGTCGAT-3′) and two forward primers (5′-GCGGGCAGGGCGGCGGG GGCGGGGCCTCTACTTTGTGTTCTTCATCATGTG-3′ and 5′-GCGGGCTCTACTTTGTGTTCTTCATCATATT-3′) were used. Each primer required 0.5 µL. After vortex mixing to achieve optimal homogeneity, the reactions were run on a SimpliAmp Thermal Cycler to amplify the RNA. The start temperature running is started at 55°C for the reverse-transfer stage for 45 minutes, next stage is pre-denaturation at 94°C for 2 minutes, then the primary stage of denaturation, annealing, and extension is cycled 35 times with the following temperature are 94°C for one minute, 55°C for 1 minute, and 72°C for 1 minute, after cycling at 35 times the final extension is 72°C for one minute (33-35).

In this study, the RT-PCR assay for detecting the V1016G and F1534C mutations, which typically requires DNA, was conducted using RNA samples instead, as only RNA was available. Despite this deviation, the assay was successfully adapted to amplify the target mutations from RNA, demonstrating the flexibility of the method in cases where DNA samples are not accessible.

2.7. RT-PCR for Detecting JEV and WNV

The detection of JEV and WNV in mosquitoes was performed via RT-PCR with JEV and WNV primers (21,36). In the first step of the detection of JEV and WNV, RNA was extracted from stage 3, and as much as 3 µL of RNA was mixed with 5 µL of PCR Mix (one step), 1 µL of nuclease-free water, and the JV forward primer and JR reverse primer. However, for the detection of WNV viruses, forward WF primers and reverse WR primers were used. The volume of forward and reverse primer required was 0.5 µL. Next stage, one-step RT-PCR assay using a thermal cycler performed with the following thermal: 55°C for reverse-transfer stage for 45 minutes, next stage is pre-denaturation with 94°C for 15 minutes, then the primary stage of denaturation, annealing, and extension is cycled for 35 times with following temperature are 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute, after cycling at 35 times the final extension is 72°C for one minute.

3. RESULTS AND DISCUSSION

3.1. Area of Study

The results of location visualization with QGIS are shown in Figure 1, where the location in red indicates the scope of the Surabaya area and in blue indicates the scope of the Mulyorejo City Park area, which is the location where the *Aedes* larvae samples were collected. *Aedes* larvae are found in places with standing water as well as damp water. Table 1 presents data on the breeding sites of *Aedes* sp., highlighting the variability in habitats utilized by these mosquito species. It identifies diverse breeding environments, including artificial containers like scrap tires, trays, jars, and bottles, as well as natural habitats such as tree holes, coconut shells, and village pots. *A. aegypti* appears to be predominantly associated with artificial containers, reflecting its adaptation to urban settings, while *A. albopictus* shows a broader range, utilizing both artificial and natural sites. A total of 36 samples were classified by habitat; 309 mosquitoes were collected. Every sample was analyzed according to the following sample collection procedure in Figure 2.

Figure 2 demonstrates various breeding sites of *Aedes* sp. larvae sampled from the Mulyorejo area, showcasing a range of both artificial and natural habitats. These include man-made containers such as trays, bottles, scrap tires, aviaries, jars, and villager pots, as well as natural settings like tree holes and coconut shells. The diversity of these sites highlights the adaptability of *Aedes* mosquitoes to different environments, particularly in urban and semi-urban areas.

3.2. RNA Extraction and Purity Level

The samples were grouped by sampling location and calculated the RNA purity, which affects the accuracy of the analysis results and the risk of contamination in the RT-PCR process. The A260/A280 ratio was used to assess the purity of the RNA. The ideal purity for RNA is approximately 1.7-2.5 A ratio lower than 1.7 indicates possible DNA or protein contamination, while a ratio higher than 2.5 indicates possible contamination by polysaccharides, washing solutions, chaotropic salts, phenols or proteins (37,38). The total of 36 samples showed that the extraction results using the Zymo kit™ met the RNA purity criteria (Table 2).

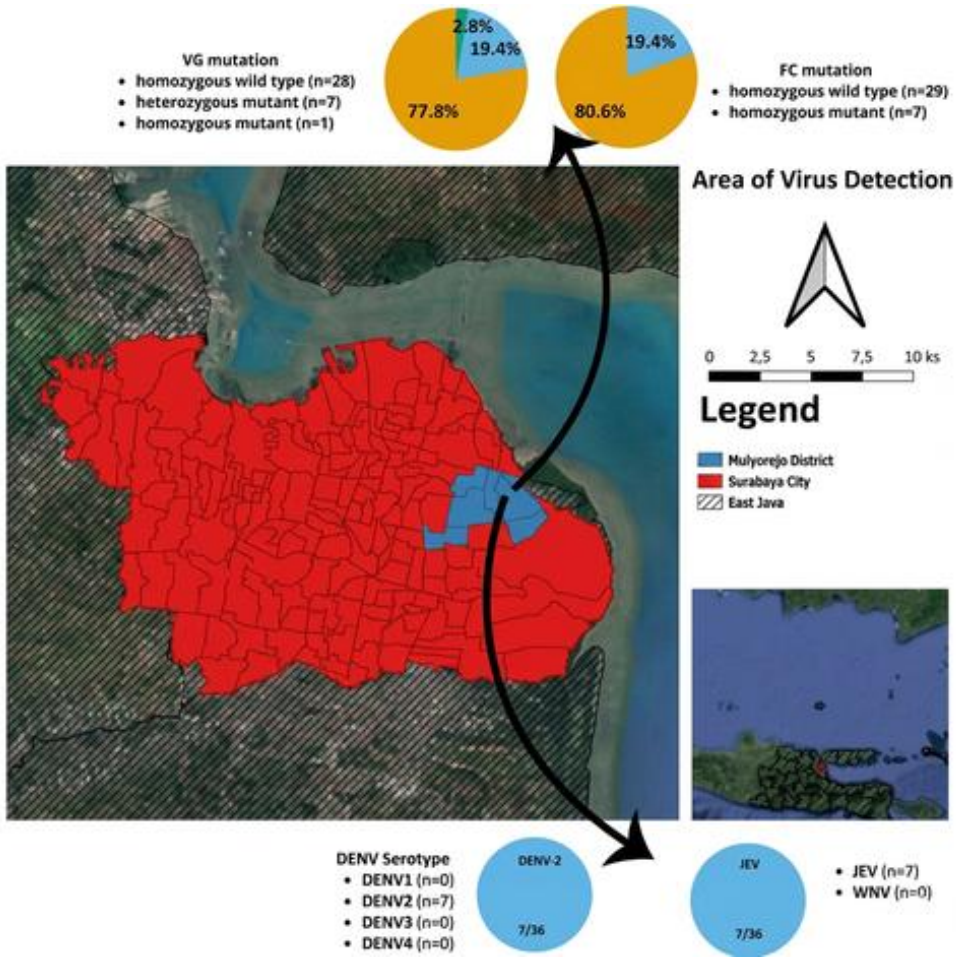

Figure 1. The geographic distribution of virus detection in *Aedes* larvae samples collected from the Mulyorejo City Park area in Surabaya, visualized using GIS (Geographic Information System) applications. The red zones represent the Mulyorejo District, highlighting areas of potential virus activity, while the blue area specifically marks the Surabaya City Park, where the larvae samples were obtained

Table 1. Breeding sites of *Aedes* sp. collection

Sample Code	Breeding	*Aedes aegypti*		*Aedes albopictus*	
		♂	♀	♂	♂
R1	Tray		6		
R2	Scrap Tires 2	20			
R3	Scrap Tires 2		20		
R4	Villager Pot 3			4	
R5	Trees Hole 1			20	
R6	Trees Hole 1				20
R7	Trees Hole 1		1		
R8	Bottle 2				5
R9	Villager Pot 6		4		
R10	Villager Pot 6				21
R11	Coconut Shell 3		12		
R12	Aviary 2		5		
R13	Scrap Tires 1		6		
R14	Jar 3			4	
R15	Jar 3				1
R16	Coconut Shell 1		20		
R17	Bottle 1			3	
R18	Tray			10	

Sample Code	Breeding	*Aedes aegypti*		*Aedes albopictus*	
		♂	♀	♂	♂
R19	Tray				21
R20	Scrap Tires 2		8		
R21	Scrap Tires 2	9			
R22	Coconut Shell 1		11		
R23	Coconut Shell 1				1
R24	Villager Pot 6			2	
R25	Trees Hole 1			8	
R26	Trees Hole 1				20
R27	Jar 1	6			
R28	Jar 1		4		
R29	Jar 1				8
R30	Aviary 2	14			
R31	Aviary 2				1
R32	Bottle 1	1			
R33	Villager Pot 3				4
R34	Villager Pot 3			3	
R35	Tray				3
R36	Villager Pot 6				3

Figure 2. Locations of the larvae sampled in the Mulyorejo area: (A) tray, (B) coconut shell, (C) tree hole, (D) bottle, (E) scrap tire, (F) aviary 1, (G) aviary 2, (H) villager pot 1-6, and (I) jar

Table 2. Purity level of RNA extracted from sample

Sample Code	Yield (ng/µL)	A260 (10 mm) nm	A260/A280 nm	Sample Code	Yield (ng/µL)	A260 (10 mm) nm	A260/A280 nm
R01	13.6	0.340	2.06	R19	74.5	1.853	1.99
R02	74.0	1.851	1.91	R20	29.9	0.747	2.04
R03	52.2	1.304	1.93	R21	15.3	0.383	2.05
R04	4.9	0.122	2.44	R22	14.2	0.354	1.94
R05	47.7	1.193	1.95	R23	0.8	0.021	1.94
R06	21.6	0.540	2.01	R24	3.3	0.082	1.73
R07	1.3	0.035	2.19	R25	24.0	0.599	1.95
R08	8.8	0.219	2.17	R26	44.2	1.106	1.92
R09	19.2	0.481	2.06	R27	16.4	0.410	2.04
R10	62.0	1.549	1.72	R28	5.1	0.126	2.52
R11	36.1	0.902	2.11	R29	27.2	0.680	1.75
R12	18.9	0.479	2.07	R30	39.5	0.987	1.87
R13	9.9	0.249	2.19	R31	3.1	0.076	2.92
R14	9.5	0.238	2.09	R32	2.3	0.057	2.24
R15	3.7	0.093	2.43	R33	31.5	0.788	1.95
R16	81.4	2.035	1.96	R34	5.7	0.142	2.44
R17	7.7	0.193	2.13	R35	8.6	0.214	2.21
R18	12.0	0.315	2.02	R36	6.0	0.150	2.21

Table 2 provides data on the yield and purity of RNA extracted from various samples, assessed by spectrophotometric measurements (A260 and A260/A280 ratios). The RNA yield varies significantly across samples, ranging from 0.8 ng/µL to 81.4 ng/µL, reflecting differences in RNA concentration. The A260/A280 ratio, which indicates RNA purity, generally falls within the acceptable range of 1.8–2.1, suggesting that most samples are of sufficient quality for downstream applications such as molecular analyses. However, some samples, such as R07 and R24, have very low A260 values and might be of poor quality or contaminated. Variability in RNA yield and purity could result from differences in sample handling, extraction protocols, or the inherent nature of the samples.

3.3. RT-PCR Findings

Figure 3 presents the results of Dengue virus serotyping, based on agarose gel documentation under UV light. Among the samples analyzed, 7 samples, specifically those coded 7, 8, 9, 10, 11, 12, and 13 in Figure 2, exhibited a base pair (BP) band at 119, indicating the presence of Dengue virus with type 2. However, the remaining 29 samples (Shown on Figure 1, 3, 4) did not produce BP bands in the region corresponding to Dengue virus-infected, confirming they were negative for Dengue virus (20). The presence of BP band on the gel documentation indicates that the sample shares the same BP size as the target viral BP; serving as the basis for determining positivity for the associated virus. These findings align with data from the Surabaya City Health Profile, which report that dengue cases in the Mulyorejo area are remain relatively low (8).

The detection of mutations in the target sites of insecticide active ingredients indicates reduced insecticide effectiveness, as these mutations (V1016G and F1534C) can confer resistance detectable through RT-PCR. V1016G is used to detect mutase at codon 1016, while F1543C is used to detect mutation at codon 1543 (14). Figure 4 shows the detection of VG mutations, including the detection of mutations in pyrethroid-type insecticides. UV light analysis revealed 25 samples of the homozygous wild type (60 bp) with sample codes 3, 4, 7, 8, 9, 10, 11, 12, 13, 14, 15, 17, 18, 19, 23, 24, 25, 26, 27, 28, 31, 33, 34, 35, and 36. Furthermore, 7 samples were detected as heterozygous mutants (60 bp or 80 bp), namely, samples 2, 5, 6, 16, 20, 22, and 32. One sample was detected as a homozygous mutant (80 bp), namely, sample code 21 (14). For samples 1, 29, and 30, no bands appear on the agarose gel. Generally, the presence or absence of mutations in mosquitoes still causes bands on the agarose gel, so all three samples were analyzed in duplicate.

Figure 5 presents the results of the repetition, exhibited that samples with codes 1, 29, and 30 were all homozygous for the wild type (60 bp). Because a band appeared on the agarose gel in the second PCR test, it is possible that, in the first PCR result, there was contamination or that the primer did not stick to the target so that the RNA was not amplified. VG resistance detection revealed that 8 out of 36 samples were mutated.

For the detection of FC mutations, namely, mutations in DDT-type insecticides, Figure 6 shows the results of gel documentation with UV light showed that 29 samples were homozygous for the wild type (93 bp), namely, sample codes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, and 36 bp. A homozygous mutant (113 bp) was detected in 7 samples, namely, with 14, 15, 16, 17, 18, 19, and 20. In the detection of mutase in FC, 7 samples were mutated (1). These findings show that most mosquitoes in the Mulyorejo area are resistant to pyrethroids and DDT, making these insecticides ineffective for eradicating mosquitoes in the area.

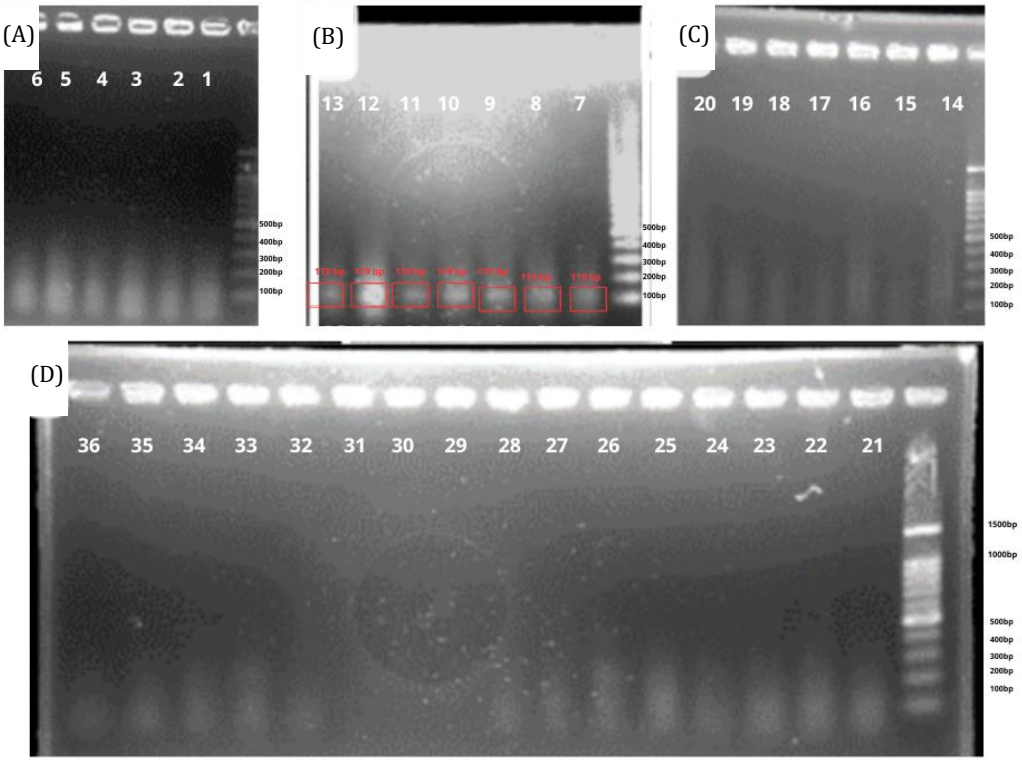

Figure 3. (A) Sample code: 1-6 [negative]. (B) Sample code: 7-13 [positive DENV-2]. (C) sample code: 14-20 [negative]. (D) Sample code: 21-36 [negative], compared with the 100 bp marker

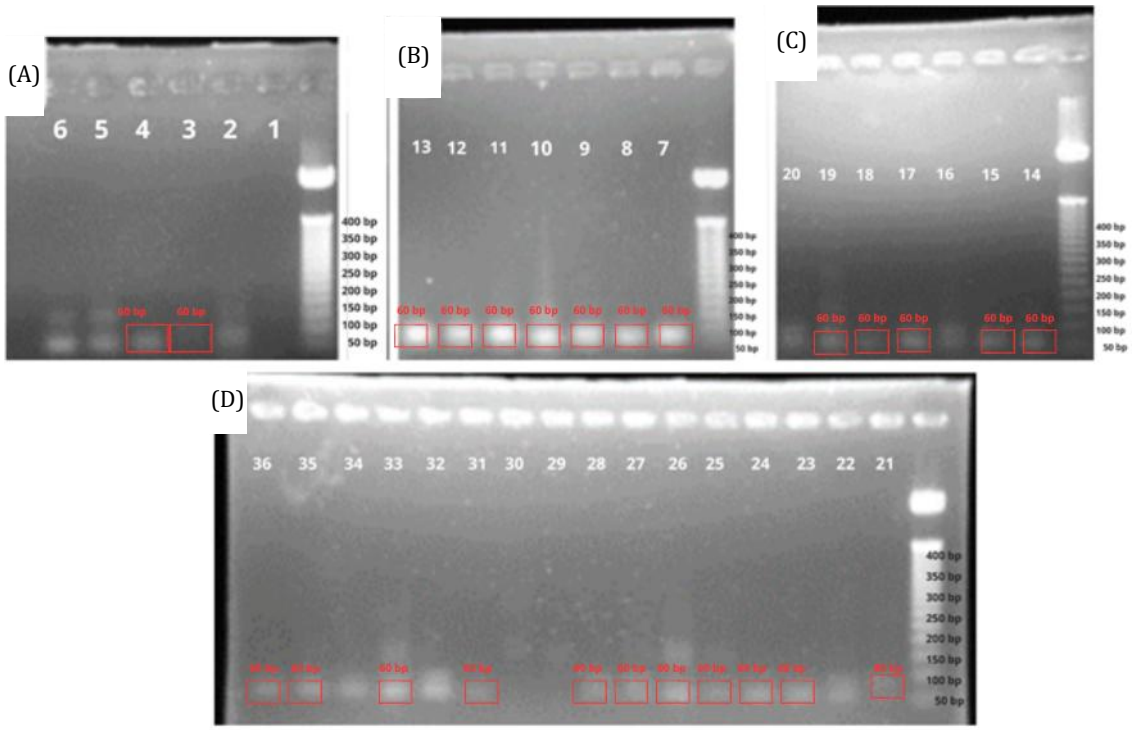

Figure 4. (A) Sample code:1-6 [1 doesn't appear ribbon, 3.4 homozygote wildtype, 2.5.6 heterozygote mutant]. (B) Sample code:7-13 [all wildtype homozygotes (60 bp)]. (C) Sample code:14-20 [16.20 heterozygote mutant, 14.15.17.18.19 homozygote wildtype]. (D) Sample code: 21-36 [21 homozygote mutant, 22.32 heterozygote mutant, 29.30 doesn't appear ribbon, 23.24.25.26.27.28.31.33.34.35.36 homozygote wildtype], compared with marker 50 bp

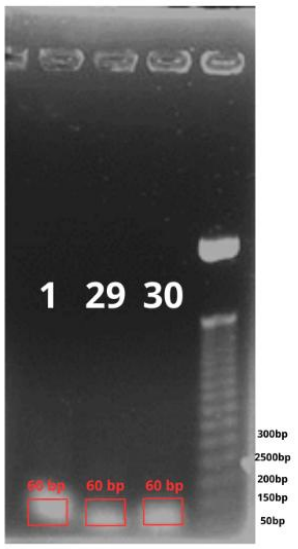

Figure 5. Repetition for sample codes: 1, 29, and 30 [homozygote wild-type] compared with the marker 50 bp

Furthermore, detecting whether *Aedes* mosquitoes in the Mulyorejo area also harbor JEV using RT-PCR. Considering that, at the beginning of 2020, the world was shocked by the increase in COVID-19 cases, which caused a high mortality rate. All forms of the virus can hopefully be detected early to avoid widespread spread and mutation into a deadly virus. In addition, collecting data on the spread and potential of the virus can serve as a reference for the urgency of developing a vaccine or cure as soon as possible. This is also based on the findings of (16), which described cases of JEV in patients in Bali, Indonesia As a results from the Figure 7, gel documentation for the detection of the JEV in mosquito samples in the Surabaya area, 7 samples were found to be positive (shown ribbon at 241 bp) also carrying this virus, namely, in sample codes 3, 9, 12, 16, 17, 21, and 22 which were characterized by the appearance of bands in the area of 119 bp 2 of 7 samples carrying the JEV were also positive for carrying the Dengue virus, namely, sample codes 9 and 12 so that it can be concluded even though the mosquitoes are negative for carrying the Dengue virus However, these mosquitoes still have the potential to carry other viruses such as the JEV.

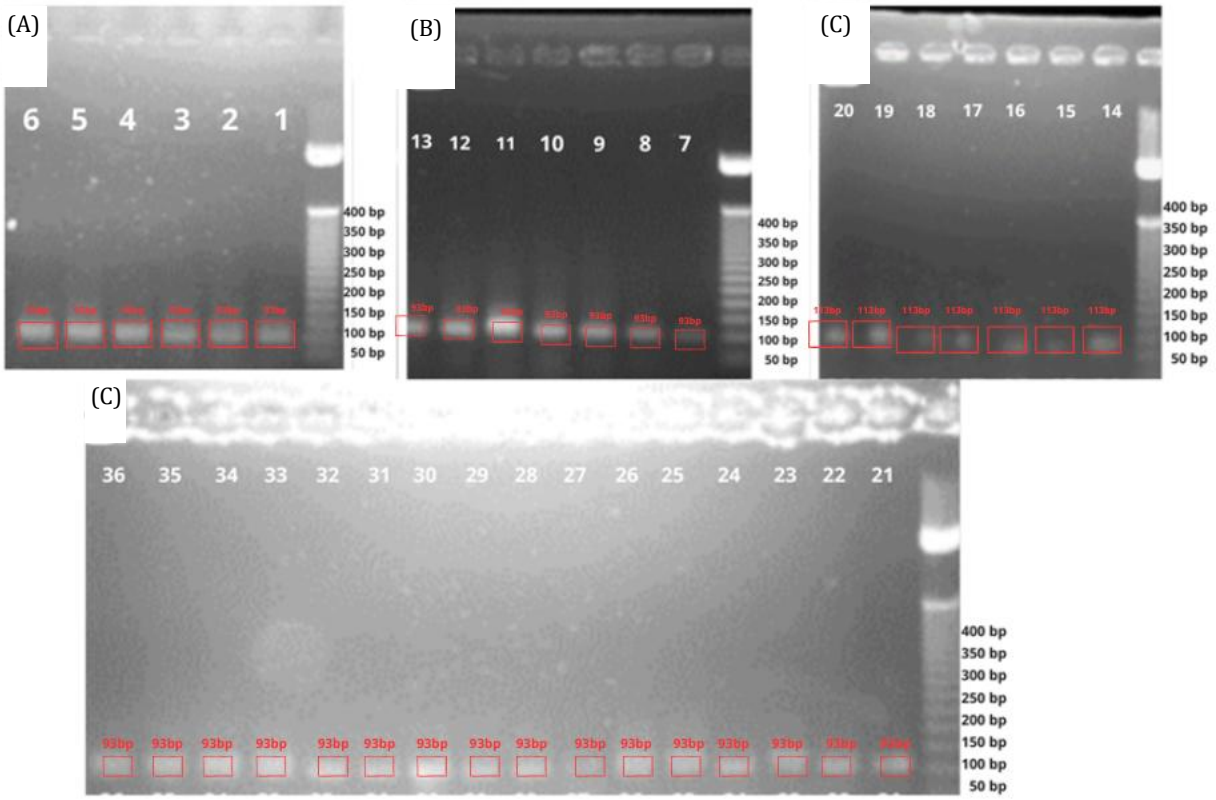

Figure 6. The sample codes used were as follows: (A) 1-6 [homozygote wild type]; (B) 7-13 [homozygote wild type]; (C) 14-20 [homozygote mutant]; and (D) 21-36 [homozygote wild type].

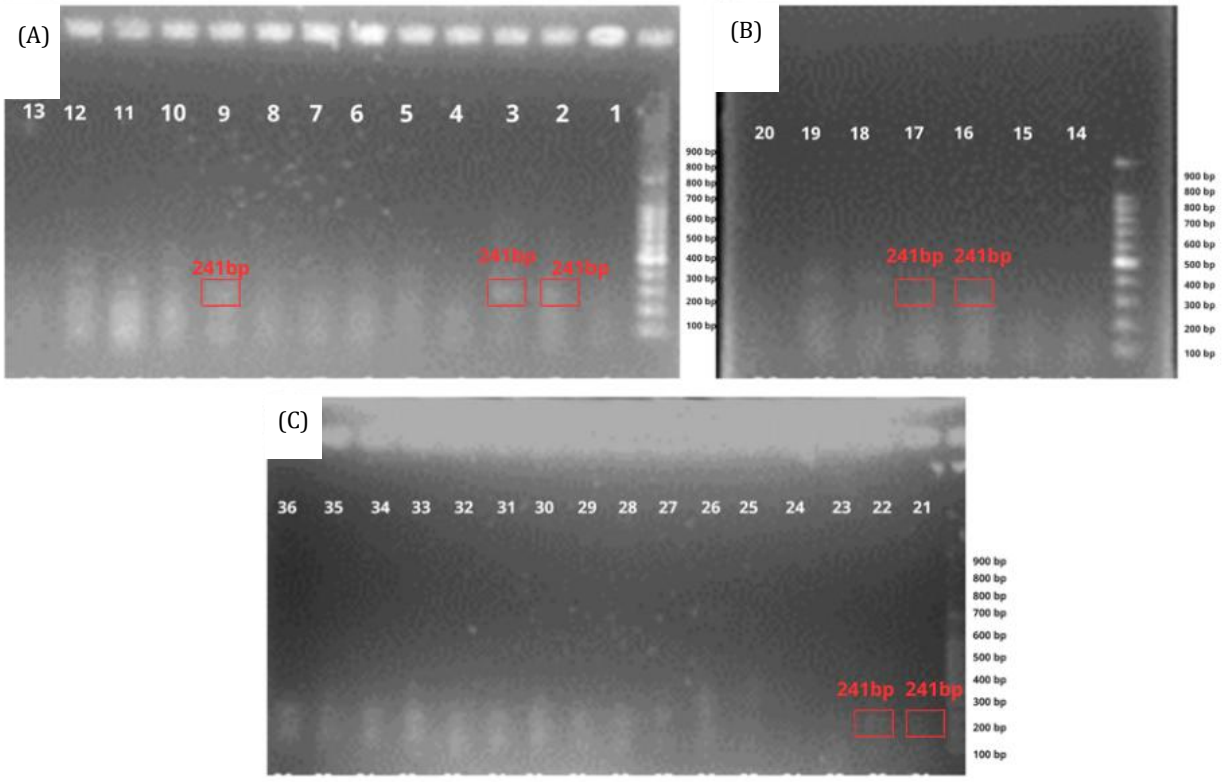

Figure 7. (A) Sample code: 1-13 [2.3.9 positive JEV]. (B) Sample code: 14-20 [16.17 positive JEV]. (C) Sample code: 21-36 [21.22 positive JEV] compared with the 100 bp marker

In addition to detecting the JEV, this study also detected the WNV. As a results from Figure 8, UV gel documentation revealed that the *Aedes* mosquitoes collected in the Mulyorejo area, Surabaya, were all negative for WNV because no sample showed a ribbon at 482 bp (39). Therefore, for the sake of urgency, there are still no cases of WNV in this area, but the spread of this virus will continue to be examined further in Indonesia, especially in disease-prone areas in Surabaya.

In this study, mosquito mutations were also detected in 8 samples where previous research used allele-specific polymerase chain reaction (AS-PCR) did not detect V1016G and F1543C mutations in mosquitoes isolated in urban parks Surabaya (14). This study also found that 7 sample in Mulyorejo indicating the presence of Dengue virus with type 2, it relevant to research has been conducted by Kotaki et al., (38) that isolated DENV-2 from 2008 to 2014. This study confirms that in previous studies that found JEV in Bali Island, there was also a JEV virus that was transmitted by the Aedes mosquito vector in Mulyorejo.

The number of Dengue cases in Surabaya is relatively lower than in 2018, and long-term use of insecticides also causes *Aedes* mosquitoes to mutate to develop immunity from the pesticide (12). However, the latest discovery revealed that the *Aedes* vector also has the potential to harbor other viruses, such as JEV. This study can be used as a basis for further development in handling the high number of cases of Dengue in Indonesia in 2022, which requires finding the most effective way to reduce the spread of Dengue virus by *Aedes* mosquito vectors (2). Some types of viruses found are DENV-2 according to research conducted by Kotaki et al., (38) which, in mosquito isolation research during 2008-2014, found 42 strains of DENV-2, most of which were from the Surabaya strain.

These high numbers of cases may be due to the ineffectiveness of the insecticides used, such as pyrethroids and DDT insecticides. Visualization via gel documentation revealed that mutations at two codons occurred in some mosquito samples: the mutation at codon 1534 changed phenylalanine to cystine, and the mutation at codon 1016 changed valine to glycine (12). This was in line with the increasing number of mosquitoes affected by the reduced performance of mosquito eradication, where mosquitoes had knockdown resistance and did not die completely (39).

Figure 9 shows the *Aedes* sp. Characterization began with the collection of 36 samples from the study area. All samples were morphologically identified and subjected to RNA extraction, followed by RT-PCR assays for DENV, JEV, and WNV detection. Seven samples were positive for DENV serotype 2, while no WNV-positive samples were found. Subsequent mutation analysis of VG and FC genes revealed variations in genotype distribution, including homozygous wild-type, heterozygous mutant, and homozygous mutant profiles.

Further development is needed to develop insecticides that can utilize the synthesis of herbal compounds to increase the effectiveness of mosquito control but also limit the use of highly toxic materials to minimize the possible impact on humans. This research also revealed the potential for high cases of Dengue to increase the susceptibility to JEV infection, as many findings revealed that the Japanese encephalitis virus circulated in several regions in Indonesia and was transmitted by *Aedes* mosquito vectors (16). Therefore, the high incidence of Dengue virus infection correlates with increased JEV virus concentration, but it remains unknown whether this virus causes more severe cases of infection.

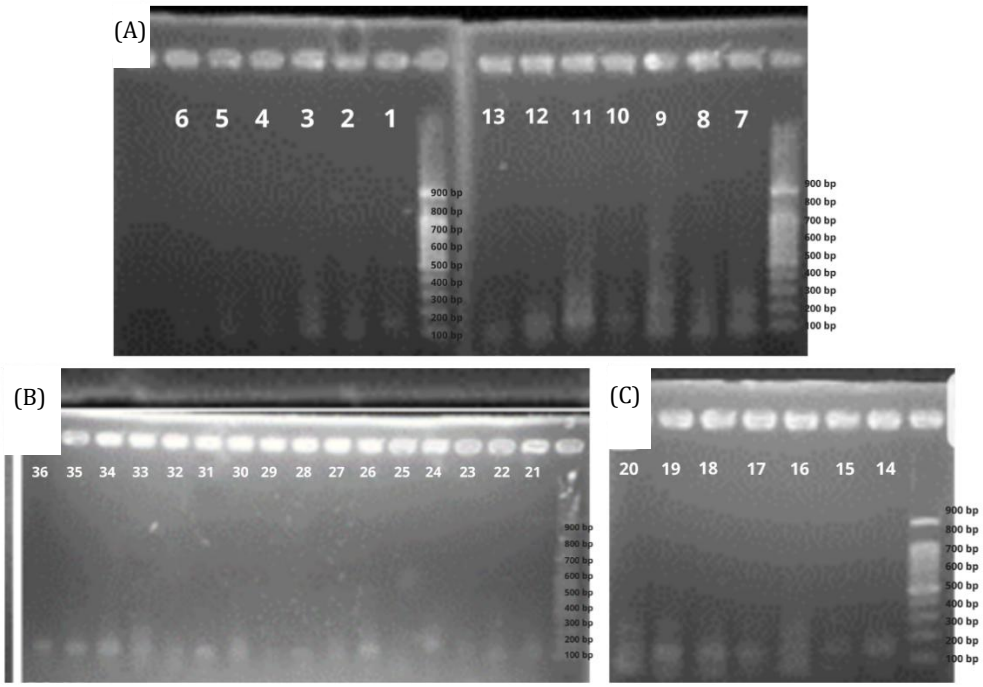

Figure 8. (A) Sample code: 1-13 (negative). (2) Sample code: 14-20 (negative). (3) Sample code: 21-36 (negative), compared with the 100 bp marker

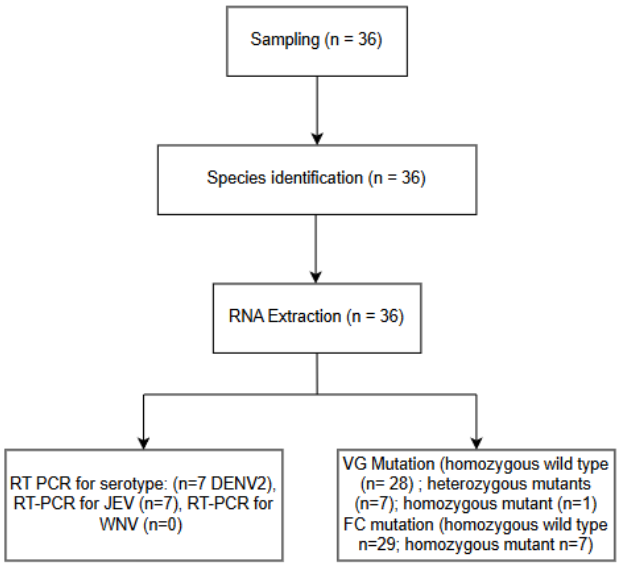

Figure 9. Experimental outline of *Aedes* sp. characterization

4. CONCLUSIONS

The study revealed that *A. albopictus* and *A. aegypti* larvae isolated from the urban park in Mulyorejo, Indonesia, tested positive for DENV-type 2 in 7 samples. Additionally, this study identified that *Aedes* mosquitoes in this location also have the potential to harbor Japanese encephalitis virus (JEV), with 7 samples testing positive for JEV, and 2 samples positive for double infections (DENV and JEV). However, the potential for West Nile virus (WNV) transmission remains low, as all samples tested negative for WNV vectors. These findings highlight the need for the development of antiviral agents targeting both Dengue virus and JEV, while also considering the effectiveness and safety of insecticide doses. In particular, the use of herbal-based alternatives should be explored to mitigate the potential harmful effects of chemical insecticides on humans' health.

Author contributions: AGP, THS: Conceptualization. MRHK, SF: Methodology. H, THS, PW: Validation. YD: Formal analysis. NNH: Investigation. ARW, NK: Resources. AAIDCR: Data curation. AGP: Writing—original draft preparation. MRHK: Writing—review and editing. ARW: Visualization. PW: Supervision. THS: Project administration. H: Funding acquisition. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest: None.

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