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Detection of homozygous wildtype V1016V using allele-specific polymerase chain reaction in *Aedes albopictus*

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Abstract. *Setiawan AR, Fadila SZ, Sucipto TH, Fauziyah S, Madaniyah S, Dewi EC, Naw SW, Tukiran, Cahyaningrum SE. 2023. Detection of homozygous wildtype V1016V using allele-specific polymerase chain reaction in* Aedes albopictus. *Biodiversitas 24: 62-67. Aedes* sp. is a carrier of several viruses that can infect humans and cause diseases such as zika, yellow fever, chikungunya, and dengue fever. Symptoms of dengue infection vary, consisting of classic dengue fever (DD), dengue hemorrhagic fever (DHF), and dengue shock syndrome. Insecticide spray can be used to manage *Aedes* mosquitoes chemically. Insecticide substances target nervous system proteins. Voltage-gated sodium channels (VGSC) are rendered inactive by pyrethroid binding. Knockdown is a signal indicating an insect has been knocked down in response to a specific insecticide. However, using insecticides for a long time can cause mosquitoes to become resistant. The pesticide resistance of mosquitoes is known as knockdown resistance (kdr). This study aims to detect kdr mutations (V1016G) in two male *Aedes albopictus* mosquitos named A1 and A2 collected from settlements in Kranggan, Sawahan, Surabaya, East Java, Indonesia, using allele-specific polymerase chain reaction (AS-PCR) assay. RNA was extracted from the two mosquito samples using an RNA extraction kit. After that, the extracted RNA was tested for kdr mutations using the AS-PCR method. After assaying, both samples are homozygous wildtype (V1016V) because the results showed bands appearing from samples A1 and A2 at 60 bp. On the other hand, this study has the potential to serve as preliminary monitoring for the program controlling vectors.

Keywords: *Aedes albopictus*, AS-PCR, dengue, knockdown resistance, V1016V

INTRODUCTION

Aedes sp. is one of the vectors of many diseases. Hence it is important to monitor the spread of *Aedes* mosquitoes (Pratiwi et al. 2019; Islam et al. 2021). Numerous viruses that cause dengue fever, yellow fever, zika, and chikungunya, are carried by *Aedes* sp. (Kauffman et al. 2017; Vu et al. 2020; Nasir et al. 2022). *Aedes albopictus* is a zoonotic pathogen bridge vector that feeds on various hosts, is a substantial biting irritant with the potential to provide a serious health hazard to people, and This mosquito vector can transmit at least 22 arboviruses (Lwande et al. 2020). Basic dengue fever (DD), dengue hemorrhagic fever (DHF), and dengue shock syndrome are among the symptoms of dengue infection (Wibowo et al. 2010; Ranjit and Kissoon 2011; Sasmono et al. 2015). An infected female mosquito of the *Aedes* genus, particularly an *Aedes aegypti* or an *A. albopictus*, must be bitten to transmit the dengue virus (Ferreira-De-Lima and Lima-Camara 2018; Balaska et al. 2020; Ogunlade et al. 2021).

Since its discovery in Jakarta and Surabaya in 1968, dengue hemorrhagic fever (DHF) has been endemic in Indonesia, with cases rising yearly and dispersing to new regions (Dania 2016; Haryanto 2018; Faridah et al. 2020). According to Arisanti's research (2021), Indonesia had varying DHF cases between 2010 and 2019. The highest cases were 204,171 in 2016 (78.85 per 100,000 people) and 65,602 in 2018. The 10-year percentage of Indonesia being larva-free, 24.1-80.2%, does not meet the 95% objective. DHF transmission is still happening in Indonesia, as evidenced by the fact that there are still many instances of the disease each year and that markers of DHF morbidity are still high, above 49 per 100,000 people (Arisanti et al. 2021).

Efforts to eradicate DHF are carried out, among others, through prevention activities, discovery, patient reporting, disease observation, and epidemiological investigations, as well as outreach to the community (Setiati et al. 2006; Karyanti et al. 2014). According to Satoto et al. (2020), physical environmental elements may serve as ideal mosquito breeding grounds, including low ceilings and varying interior and outdoor temperatures. The transmission of DHF is correlated with the absence of a wire net in ventilation, poor illumination, and excessive humidity (Satoto et al. 2020). According to the vector distribution with entomological index, mosquitoes, larvae,

and eggs, all contribute to the spread of DHF. Insecticides and vector control are crucial for preventing diseases spread by mosquitoes (Balaska et al. 2020).

Use of mosquito nets, even during the day, insecticide-treated materials (ITM) like window coverings, mosquito repellent creams (including DEET, IR3535, or Icaridin), coils, and full-sleeved shirts and pants all help reduce mosquito bites (World Health Organization 2009). Larvicides in big breeding containers and insecticide spray can be used to manage pests chemically. Insecticides with organophosphorus bases (such as fenitrothion and malathion) and pyrethroids are some of the more popular options (bioresmethrin, cypermethrin). Additionally, the CYD-TDV vaccine, authorized for use in endemic areas of 20 different nations, is the first recombinant live tetravalent dengue vaccine (Tricou et al. 2020). Before the discovery of mosquitoes resistant to DDT and dieldrin in 1970, DDT and dieldrin were no longer used in Indonesia to control dengue vectors. In addition, pyrethroid and organophosphate pesticides were utilized to combat dengue vectors (Silalahi et al. 2022).

Bti and diflubenzuron appear to be potential alternative larvicides to suppress dengue vectors in water storage containers in Laos, according to a study by Marcombe (Marcombe et al. 2018). According to Gan's research, four key insecticide classes—organochlorines, organophosphates, pyrethroids, and carbamates—are frequently employed to reduce mosquito populations (Gan et al. 2021). The general populace regularly uses pyrethroid insecticides, such as cyfluthrin, lamdacyhalothrin, and alpha-cypermethrin, to suppress dengue vectors (Wuliandari et al. 2015). These substances target nervous system proteins. Voltage-gated sodium channels (VGSC) are rendered inactive by pyrethroid binding. Insects get paralyzed and die due to continuous depolarization of electrically stimulated cell membranes (Field et al. 2017).

Knockdown is a signal indicating an insect has been knocked down in response to a specific insecticide. Knockdown disturbs the insect's motor system when an insect is subjected to pesticides. If it is noticed that the insect is paralyzed and unable to walk normally, this condition is known as collapse (Athanassiou et al. 2021). Point mutations in the para-type sodium channel pyrethroid target site cause knockdown resistance (kdr), a well-studied method of pyrethroid pesticide resistance in numerous insect species (Bass et al. 2007). One of the processes causing pyrethroid resistance is the occurrence of knockdown resistance (kdr) mutations, which have been observed in some *A. albopictus* populations in southern China (Wu et al. 2021).

It has been shown that *A. albopictus* is resistant to pyrethroid insecticides. According to Al-Amin's (2020) research, which examined the prevalence of insecticide resistance in *A. aegypti* in Bangladesh, of the survivors of a permethrin-exposed *A. aegypti* mosquito population in Dhaka, Bangladesh, 37.8% (28/74) were mutant homozygotes (GG), and 29.7% (22/74) were wildtype homozygotes (VV). The most frequent genotype of mosquitoes killed by permethrin and deltamethrin was VV/CC/VV among phenotypically susceptible insects (Al-Amin et al. 2020). At codon 1016, both the wildtype (V/V) and mutant genotypes (V/I or I/I) were found, according to Sombié (2019) investigation into the pesticide resistance of *A. aegypti* in Ouagadougou, the capital of Burkina Faso. Compared to dead insects (deltamethrin, 32%; permethrin, 29%), the VV/CC genotype frequencies are relatively lower in living mosquitoes (11%; 16%) (Sombié et al. 2019). *Aedes albopictus* mosquitoes in Surabaya will be tested for the V1016V mutation as part of this study utilizing the molecular method known as polymerase chain reaction (PCR).

MATERIALS AND METHODS

Materials

The materials used in this study were mosquito samples, QIAamp® Viral RNA by Qiagen (Hilden, Germany), Promega GoTaq® Master Mix (Madison, Wisconsin, USA), 70% ethanol, Nuclease Free Water (NFW), ethidium bromide, agarose gel, and TAE buffer. The instruments used are micropipette, tip, tray, biosafety cabinet level-2, board marker, vortex mixer, freezer -80°C, SimpliAmp™ Thermal Cycler (Applied Biosystem, Thermo Fischer), minicentrifuge, centrifuge, and gel documentation.

Methods

Surabaya is Indonesia's second-biggest city and the largest in East Java, with a population of approximately 2.87 million people. This circumstance can lead to urbanization and influence the propagation of mosquito-borne diseases, including dengue. This investigation focuses on the *A. albopictus* mosquito collected in Kranggan, Surabaya, East Java, Indonesia. Figure 1 is a map showing the sampling site.

General procedure

This research was authorized by the Lembaga Penelitian dan Pengabdian Kepada Masyarakat Universitas Airlangga with approval number 24-934/UN3.14/PPd/2013. Mosquito samples were obtained from residential areas in Surabaya. Two mosquitoes were identified as male *A. albopictus* (A1 and A2). *Aedes albopictus* mosquitoes have mutations resistant to insecticides (Djiappi-Tchamen et al. 2021).

RNA isolation

After mosquitoes were identified, the RNA was isolated using the QIAamp® Viral RNA kit by Qiagen, Germany, according to the instructions on the kit. There are several reagents used in this process. Some of them are AVL buffers, which lyse virus particles while purifying viral nucleic acids and inhibiting the action of RNase and DNase enzymes by denaturing them. In addition, ethanol is also useful in precipitation, a technique commonly used to concentrate and remove salts of nucleic acid preparations (DNA or RNA) in aqueous solutions (Shaomianah 2020). Other reagents are buffer AW1 which functions to wash the spin column membrane as well as to disrupt the cell,

dissolve its components, and simultaneously denature endogenous RNase, while buffer AW2, which contains sodium azide, functions to wash the spin column membrane, sodium azide is a preservative and is used to prevent growth microbes.

V1016V kdr genotyping using allele-specific PCR/AS-PCR assays

Allele-specific PCR experiments were conducted to genotype mosquitoes for the V1016V genotype. After acquiring cDNA, AS-PCR experiments were used to genotype kdr mutant alleles to find mutations at codon 1016 (Stenhouse et al. 2013). The reverse and forward primers used are presented in Table 1. Reactions were carried out with the respective volumes as follows: RT-PCR Mastermix 5 µL, NFW 1 µL, forward primer (Gly1016f) 0.5 µL, and reverse primer (Gly1016r and Val1016r) 0.25 µL each, and RNA 3 µL. The reaction was then carried out using an RT-PCR Thermocycler in steps of 45 minutes at 55°C, 2 minutes at a temperature of 94°C; 35 cycles of 30 seconds at 94°C (denature), 30 seconds at 55°C (anneal), and 30 seconds at 72°C (extension); continued with 2 minutes 72°C for the final extension. PCR products were put into 1.5% agarose gel and run at 100 V for 30 minutes in TAE buffer. After electrophoresis was completed, the gel staining process was carried out using ethidium bromide (EtBr) and gel visualization was performed using gel documentation instrument.

RESULTS AND DISCUSSION

Mosquito identification

Two mosquitoes (A1 and A2) were identified as male *A. albopictus*. *Aedes aegypti* and *A. albopictus* mosquitoes appear identical at first glance. Both are morphologically dark, with white stripes on their legs and backs. However, *A. albopictus* is smaller than *A. aegypti* (Lwande et al. 2020).

Table 1. The oligonucleotide sequences utilized to amplify fragments of the VG gene

	Sequence (5-3')	Product size (bp)
Gly1016f	ACCGACAAATTGTTTCCC	
Gly1016r	GCGGGCAGGGCGGCGGGGGCGGGG CCAGCAAGGCTAAGAAAAGGTTAACTC	60
Val1016r	GCGGGCAGCAAGGCTAAGAAAAGGTTAATTA	80

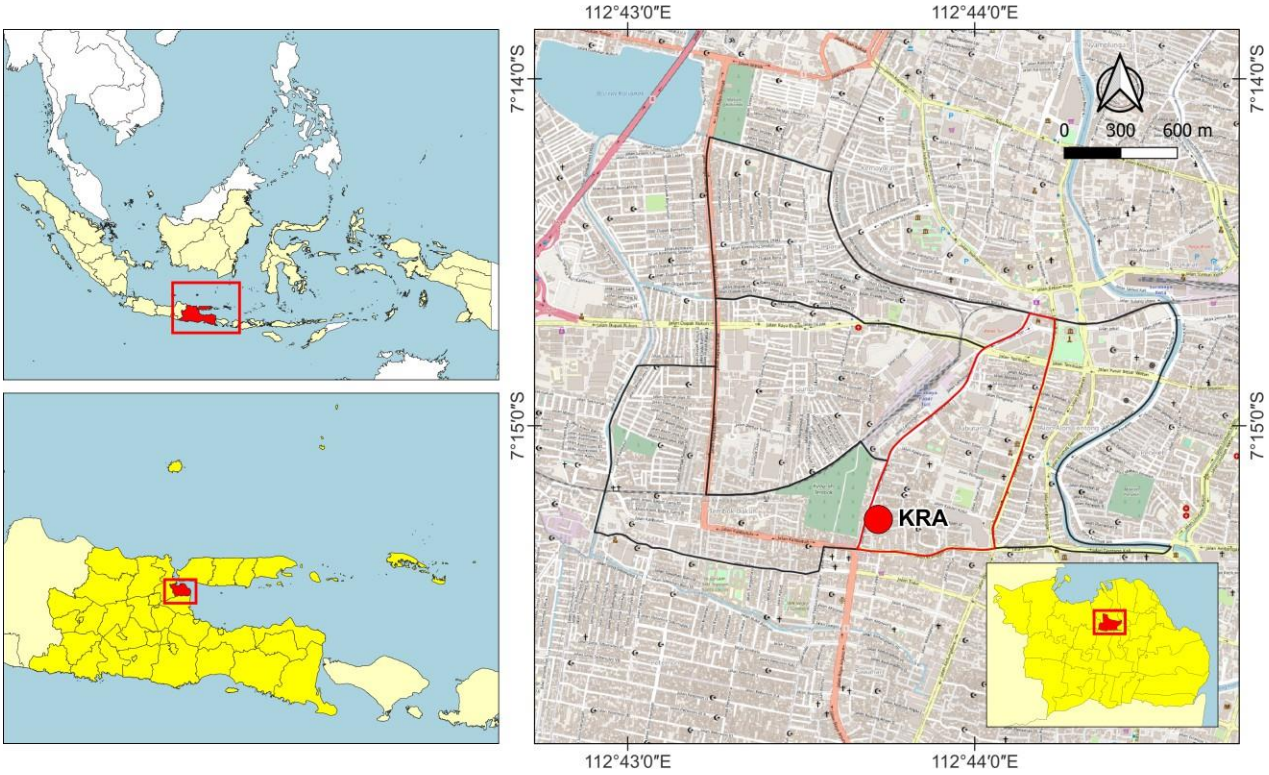

Figure 1. Geographical map of the city of Surabaya, East Java, Indonesia the sampling location is marked with a circle (KRA: Kranggan). Images created with QGIS Version 3.26.3

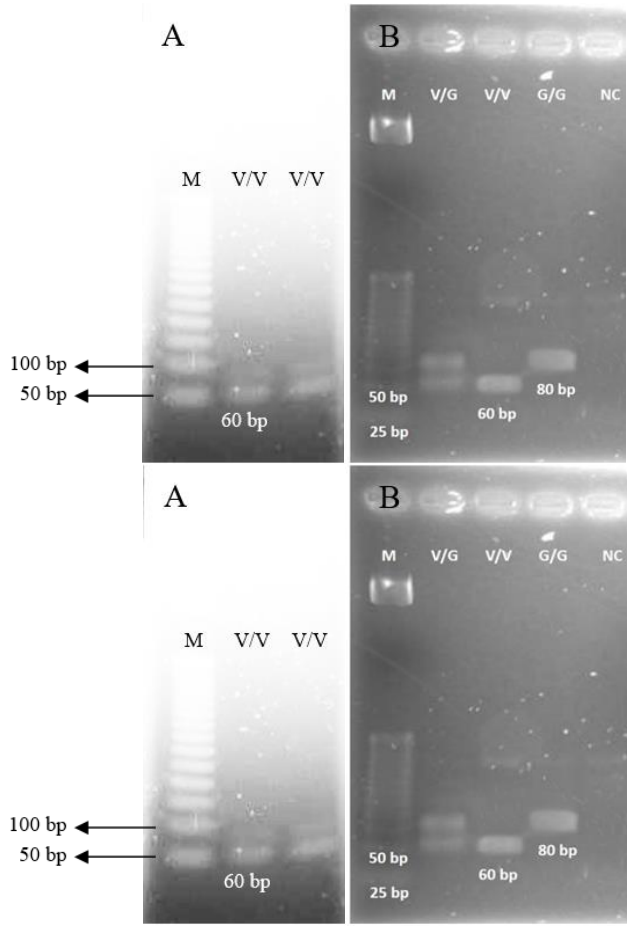

Figure 2. Result of PCR electrophoresis of the detection V1016V. The study result was compared to the reference. A. study result; B. reference figure (Fauziyah et al. 2021); M. Marker of 50 bp; V1016V: Homozygous Wildtype; G1016G: Homozygous mutant; V1016G: Heterozygous Mutant

Upon closer scrutiny, the dorsal mesonotum distinguishes the two species. *Aedes albopictus* has a white line on the dorsal medial thorax, but *A. aegypti* has a white curving line and two short lines in the middle. Moreover, the color of *A. albopictus* is darker than *A. aegypti* (Dania 2016).

AS-PCR results

The AS-PCR method is used to detect the genotype in the sample. The previous method, AS-PCR, was used to identify mutant alleles in mosquitoes (Stenhouse et al. 2013). With many mosquitoes, kdr screening utilizing the Allele-Specific PCR assay is a compassionate way to detect resistance (Yougang et al. 2020).

The results of the examination of the A1 and A2 mosquito samples are shown in Figure 2A. In the figure, it is found that the appearance of bands from samples A1 and A2 at 60 bp. The samples obtained are homozygous wildtype (V/V). According to Stenhouse et al. (2013), if the bands appear at 60 bp and 80 bp are heterozygous mutant (V/G), 80 bp are homozygous mutant (G/G), and 60 bp have homozygous wildtype (V/V) (Stenhouse et al. 2013).

Discussion

Dengue virus is transmitted to humans through mosquito bites by female *A. aegypti* as the primary vector and female *A. albopictus* as the secondary vector (Balaska et al. 2020; Ogunlade et al. 2021). One of the methods used to control the spread of dengue is to control vectors and their breeding sites. These controls can be carried out chemically (insecticides, fogging) or biologically (mosquito predators, releasing genetically modified mosquitoes) (Gan et al. 2021). Using pesticides is a simple and affordable method. However, prolonged exposure to insecticides can lead mosquitoes to develop insecticide resistance. Knockdown resistance (kdr) is the main mechanism of sodium channel insensitivity to insecticides such as DDT and pyrethroids. Kdr reduces the sensitivity of sodium channels to pyrethroids by reducing the target gene for pyrethroid insecticides (Wuliandari et al. 2015). Most kdr mutations are in the IS6, IIS6, and IIIS6 domains (Gan et al. 2021). Detecting kdr mutation is important because dengue fever is an endemic disease in Indonesia caused by the dengue virus. Therefore, it is necessary to determine kdr mutations in *A. albopictus* to study resistance monitoring programs and improve new strategies for vector management.

The method to detect kdr mutation in *A. albopictus* is Allele-Specific Polymerase Chain Reaction (AS-PCR). AS-PCR, also called Amplification Refractory Mutation System (ARMS), Allele-Specific Amplification (ASA), and PCR Amplification of Specific Alleles (PASA) (Bottema and Sommer 1993). AS-PCR uses the PCR method to detect single nucleotide polymorphism (SNP). AS-PCR was developed in 1989 by Newton. This method enables DNA polymerase to amplify the 3-primer end that complements the base in a variant or wildtype sequence (Putra et al. 2020). The fundamental of AS-PCR is primer extension, which only happens when the template's 3' end perfectly complements the primer (Lee et al. 2022).

AS-PCR methods can be used for genotyping because using two complementary reactions. One reaction uses AS-PCR primer specific to normal DNA sequence, and the other specific to mutant DNA sequence (Duta-Cornescu et al. 2009). The type of genotype can be determined by analyzing the PCR product. If only one band appeared in agarose gel, it is homozygote (either the one with the "wild type" primer or "mutant" primer) and if two bands appeared, it is heterozygote (Maksum et al. 2017). AS-PCR has many advantages, such as being simple, distinguishing homozygote and heterozygote, not requiring sequencing analysis, high sensitivity, being fast, inexpensive, and easy to design (Mahdieh and Rabbani 2013; Li et al. 2018).

This research is a preliminary study to detect kdr mutation in *A. albopictus* mosquitos. Two samples were identified as *A. albopictus* male mosquitos (A1 and A2). The results showed in figure 2A. Lane 1 is a 100 bp marker, lane 2 is sample A1, and lane three is sample A2. According to the picture, bands in lane 2 and lane 3 appeared only at 60 bp, indicating the samples are homozygous wildtype.

Homozygous wildtype detected at codon 1016. The frequency of the V/V genotype was lower in living mosquitoes (11% for deltamethrin and 16% for permethrin)

than in dead mosquitoes (32% for deltamethrin and 29% for permethrin) (Sombié et al. 2019). According to the Pareja-Loaiza study (2020), three genotypes (one of them is V1016V) were discovered in every *A. aegypti* mosquito along Colombia's Caribbean coast. Samples with genotype V410V were also found in the exact location. Triple homozygous wildtype genotypes (V410V/V1016V/F1534F) were only discovered in 13 of 27 combinations of tri-locus genotypes in 281 mosquitoes from Juan de Acosta and Barranquilla populations with frequencies value 0.04 and 0.08, respectively (Pareja-Loaiza et al. 2020).

In a separate study conducted by Wulandari (2020), 1314 samples of *A. aegypti* mosquitoes were gathered from 27 distinct places in the middle of Yogyakarta, and kdr mutation was performed. A total 1293 of 1314 samples were genotyped using the high-resolution melt (HRM) procedure. Those samples then detect kdr mutations in F1534C, V1016G, and S989P. The results were 1.08% homozygous wildtype (V1016V), 16.86% heterozygous mutant (V1016G), and 82.06% homozygous mutant (G1016G) (Wuliandari et al. 2020). Meanwhile, Al-Amin et al. (2020) in Dhaka showed that of the population exposed to permethrin, only 29.7% of the 74 samples of mosquitoes with the V/V genotype were still alive (Al-Amin et al. 2020).

From Zheng et al. (2022), no nonsynonymous mutations were identified in domain II codons 1011 and 1014, domain III codon 1532, or domain IV codon 1773 of the VGSC gene. Domain II contained a homozygous wildtype allele (V1016V) (GTA base sequence). Heterozygous wildtype (GTA/GTG) was only detected in one sample out of 659 total samples from Huangpu District; one of the three genotypes was wildtype homozygous V/V (GTA/GTA and wildtype heterozygous GTA/GTG). A heterozygous mutant (V1016G) was found in 15 populations. The mutant allele V1016G was present between 3.1% and 25.9% of the time in all 15 wild *A. albopictus* populations from Guangzhou. Most genotypes (82.8%) are homozygous wildtype (V/V) (Zheng et al. 2022). Furthermore, the study by Kushwah et al. (2020) in the Basavanagudi area of Bengaluru city showed that as many as 390 of the 572 (68.12%) samples of *A. aegypti* mosquitoes obtained with the V/V genotype (Kushwah et al. 2020).

In a study conducted by Gray et al. (2018) in Mérida, México, on *A. aegypti* mosquitoes, the V/V genotype was identified in several samples. Meanwhile, the majority of mosquito samples had I/I mutations. The percentage of V/V mosquitoes in Acim, Itzincab, and San Lorenzo was 17.14%, 11.89%, and 12.24%, respectively. When these samples were exposed to insecticides, 79.17%, 64.71 %, and 76.47% of mosquitoes with the V/V genotype died, respectively. The percentage of mosquitos with the V/V genotype that perished was higher than that of mosquitos with the V/I and I/I genotypes (Gray et al. 2018).

Dengue fever is still one of Indonesia's diseases with the highest cases. In addition to finding a medicine or vaccine, it is also essential to eradicate the dengue virus. AS-PCR determines the presence of a mutated mosquito population that can spread the dengue virus. Two mosquitoes (A1 and A2) were identified as male *A. albopictus*. The samples are checked for knockdown resistance using the AS-PCR methods. The obtained results indicate that both samples (A1 and A2) are homozygous wildtype and have not developed knockdown resistance due to the band's presence at 60 bp. This research is a preliminary study and needs further research on detecting kdr mutation with more samples according to statistical calculations.

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