Original Research Report

DETECTION OF KNOCKDOWN-RESISTANCE HOMOZYGOUS MUTANT C1534C USING ALLELE-SPECIFIC POLYMERASE CHAIN REACTION IN *Aedes albopictus* AND *Aedes aegypti*

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ABSTRACT

Dengue hemorrhagic fever (DHF) has been endemic in Indonesia for decades. Insecticides are necessary to manage the transmission of the dengue virus. However, prolonged use of insecticides can lead to insecticide resistance. This study aimed to examine the genotype of mosquitoes using the allele-specific polymerase chain reaction (ASPCR) method. The ASPCR method was chosen for genotype detection due to its high sensitivity, affordability, and ease of design. Five mosquitoes were collected from human habitation in four different areas of Surabaya, Indonesia, namely Kranggan, Ulul Azmi Mosque, Ploso, and Kalijudan. Among them, three samples were identified as *Aedes albopictus* (A1, A2, and A5) and two samples were identified as *Aedes aegypti* (A3 and A4). The frequency of resistant alleles was analyzed using the Hardy-Weinberg package in RStudio version 2023.03.1. This study revealed that two mosquitoes carried homozygous mutant alleles with a band of 113 bp and three mosquitoes carried homozygous wild-type alleles with a band of 93 bp. Cysteine-to-cysteine (C/C) mutations and phenylalanine-to-phenylalanine (F/F) mutations at codon 1534 were observed in *Aedes aegypti* and *Aedes albopictus* mosquitoes. The homozygous mutant alleles were found in Kranggan, Surabaya, Indonesia. Further research is required to assess insecticide resistance and knockdown resistance (*kdr*)-like mutation by collecting more representative samples from larger areas within the region of Surabaya. Nevertheless, this study can be used as a reference for vector control and early prevention of dengue fever.

Keywords: Dengue; *Aedes albopictus*; *Aedes aegypti*; knockdown resistance; allele-specific polymerase chain reaction (AS-PCR)

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Highlights:
1. This research analyzed a previously understudied subject in Surabaya, Indonesia, and discovered knockdown resistance (*kdr*) mutations in *Aedes albopictus*.
2. The findings of this study can prompt further research, including bioassay testing and the development of more potent insecticides.

INTRODUCTION

Aedes mosquitoes are carriers of several arboviruses that can infect humans. These mosquitoes cause illnesses such as chikungunya, yellow fever, zika, and dengue fever (Vu et al. 2020). Female *Aedes aegypti* mosquitoes are the primary vector responsible for spreading viruses to humans. On the other hand, female *Aedes albopictus* mosquitoes serve as the secondary vector (Balaska et al. 2020). Indonesia is quite well known for its tropical weather and high humidity. These factors greatly

contribute to the increased danger of mosquito-borne viral illnesses, including dengue hemorrhagic fever (Maula et al. 2018).

Aedes albopictus is the Latin name for mosquitoes with smaller physical characteristics and greater flying range than *Aedes aegypti*. *Aedes aegypti* and *Aedes albopictus* differ morphologically in the location of the back (mesonotum) (Rahayu & Ustiawan 2013). *Aedes aegypti* has a line-shaped back with two curved lines and two straight white lines, while *Aedes albopictus* has only one white stripe on the mesonotum. In terms of physical appearance, *Aedes albopictus* mosquitoes commonly have a darker color than *Aedes aegypti* (Dania 2016).

There are four distinct serotypes of dengue virus (DENV), i.e., DENV-1, DENV-2, DENV-3, and DENV-4. DENV infection can be asymptomatic, and the illness may manifest as dengue fever (DF), dengue hemorrhagic fever (DHF), or dengue shock syndrome (DSS) (Wardhani et al. 2017, Soegijanto et al. 2021). In Indonesia, dengue hemorrhagic fever was initially reported in Jakarta and Surabaya in 1968. Since then, cases have been increasing annually and spreading to new areas in Indonesia (Soegijanto et al. 2021). Currently, dengue cases have been reported in 34 Indonesian provinces. Surabaya had the highest number of cases of dengue virus infection in East Java during 2010–2013 (Wardhani et al. 2017, Putri et al. 2019).

The use of insecticides is essential to prevent the spread of DENV. People have been using insecticides to prevent the spread of diseases caused by dengue vectors, which have the potential to become endemic in many countries (Balaska et al. 2020). Initially, dichlorodiphenyltrichloroethane (DDT) and dieldrin were used to control dengue vectors in Indonesia. However, the use of these chemicals was terminated in 1970 because of the resistance developed in mosquitoes. Organophosphate and pyrethroid pesticides have been employed as alternatives to manage dengue vectors (Silalahi et al. 2022).

Insecticides that prevent dengue fever in Indonesia become less effective as mosquitoes develop resistance. Insects exposed to insecticides for a particular period produce insecticide-resistant offspring due to selection pressure across generations (Amelia-Yap et al. 2018). Resistance to pesticides in mosquitoes is referred to as knockdown resistance (*kdr*). Mutations in sodium channel genes cause a decrease in the sensitivity of these genes (Kushwah et al. 2015, Wuliandari et al. 2015). Resistance to pyrethroid pesticides in *Aedes albopictus* and *Aedes aegypti* has been observed. The first identification of a *kdr* mutation in *Aedes albopictus* was reported in Singapore in 2009. The investigation discovered that 24 of the 26 mosquitoes tested positive for F1534C mutation (Auteri et al. 2018).

Another study conducted in Makassar, Indonesia, found similar findings. The study revealed that a number of *Aedes aegypti* mosquitoes contained F1534F, F1534C, and C1534C mutations (Hamid et al. 2017). Mutations were detected in mosquitoes according to studies conducted in several countries, such as Malaysia, Costa Rica, Brazil, India, China, Japan, France, Italy, and the United States. Research conducted in a number of regions in Indonesia also yielded similar results (Fauziyah et al. 2021). In this study, the purpose was to determine whether the obtained samples carried mutations or not by employing the allele-specific polymerase chain reaction (AS-PCR) method.

MATERIALS AND METHODS

Adult mosquitoes (n=5) were collected from residential areas in Surabaya, Indonesia (Figure 1). Three mosquitoes were identified as *Aedes albopictus*, while the other two were identified as *Aedes aegypti*. The mosquito samples were labeled as A1, A2, A3, A4, and A5. The samples A1, A2, and A5 were *Aedes albopictus* mosquitoes, while the samples A3 and A4 were *Aedes aegypti* mosquitoes. The RNA from the samples A1–A5 was extracted using QIAamp® Viral RNA Kit (Qiagen, Germany) in accordance with the manufacturer's instructions (Setiawan et al. 2023).

Table 1. AS-PCR steps for detecting mutations in the samples.

Step	Temperature and time	Cycle
Initial denaturation	94 °C in 2 minutes	1
Denaturation	94 °C in 30 seconds	
Annealing	60 °C in 30 seconds	35
Extension	72 °C in 30 seconds	
Elongation	72 °C in 2 minutes	1

The mutant *kdr* allele was generated using an AS-PCR assay. The PCR reaction was carried out by referring to a study by Atencia et al. (2016). The volume of the PCR reaction was 22 µL, which contained 12.5 µL of green PCR master mix, 7.5 µL of nuclease-free water, 0.5 µL each of forward primers (Cys1534f and Phe1534f), and 1 µL of reverse primer (Cys1534r). The AS-PCR steps are presented in Table 1.

The use of primers in this study was based on a study by Atencia et al. (2016). A thermal cycler was used

to carry out a PCR reaction. The AS-PCR method was chosen because it can distinguish between homozygous and wild-type alleles. Furthermore, this method has advantages, such as being fast, inexpensive, highly sensitive, and easy to design (Setiawan et al. 2023).

Table 2. The oligonucleotide sequences utilized to amplify fragments of the FC gene.

Primer sequence		Product (bp)
Cys1534f	5'GCGGGCAGGGCG GCGGGGGCGGGGC CTCTACTTTGTGTT CTTCATCATGTG3'	113
Phe1534f	5'GCGGGCTCTACTT TGTGTTCTTCATCA TATT3'	93
Cys1534r	5'TCTGCTCGTTGAA GTTGTCGAT3'	

The first step in performing AS-PCR according to the guidelines was designing a specific primer to amplify different target regions between normal alleles (wild type) and mutant alleles. Specific primers must be designed to bind only to the mutated region of the mutant allele (Yang et al. 2017). The second step was optimizing PCR conditions, including temperature, time, and primary concentration, to obtain optimal results. These parameters were checked as needed during the initial trials and modifications (Lorenz 2012). The next step was electrophoresis and analysis of the results. Electrophoresis was performed following a PCR assay to separate the DNA fragments on an agarose gel. The DNA bands that emerged were then analyzed to determine the presence of mutant alleles (Lee et al. 2016).

It was important to note that the size and intensity of the band corresponded to a mutant or normal allele. The final step was interpreting the results based on the results of the DNA banding patterns and determining the presence of mutant and normal alleles in the tested samples (Mahdieh & Rabbani 2013). Observation of the differences in DNA banding patterns between the positive control, the negative control, and the tested samples is necessary for determining the AS-PCR result accurately.
According to Table 2, the F1534C gene could be associated with mutations in *Aedes* sp. mosquitoes and might cause resistance to permethrin-based insecticides. This mutation might occur at position 1534 in the gene encoding the targeted neurotoxin receptor (Fan & Scott 2020). AS-PCR was the laboratory method used to detect the presence of specific alleles in the DNA samples. In the context of F1534C, AS-PCR was used to identify the presence of F1534C mutation in *Aedes* sp. Mosquitoes (Darawi et al. 2013).

The AS-PCR method utilized specific primers designed to bind selectively to the normal (wild type) or mutational allele (F1534C) (Stenhouse et al. 2013). If a mutational allele was present, a primer designed for the mutational allele would amplify the DNA fragment. By using AS-PCR, researchers could detect the presence of the F1534C mutation in *Aedes* sp. mosquitoes by comparing the resulting DNA banding patterns to the positive and negative controls (Yang et al. 2017). This method could help monitor and research the spread of these mutations in mosquito populations and understand insecticide resistance. This method could also detect the mutation of the F1534C type in *Aedes* sp. mosquito gene.

In the study by Atencia et al. (2016), the different bands indicated the genotypes of *Aedes aegypti* and *Aedes albopictus*. If the band had appeared at 93 bp, the genotype was homozygous wild type (F/F or F1534F). If the band appeared at 113 bp, the genotype was homozygous mutant (C/C or C1534C). If the band appeared in two locations (93 bp and 113 bp), the sample was a heterozygous mutant. The frequency of the resistant alleles was analyzed using the Hardy-Weinberg package through RStudio version 2023.03.1+446.

Table 3. AS-PCR results and distribution of species according to sampling sites.

Sampling sites	*A. aegypti*	*A. albopictus*	Genotype			Allele frequency	
			SS (%)	SR (%)	RR (%)	S	R
Kranggan	0	2	0 (0)	0 (0)	2 (100)	0	1
Ploso	1	0	1 (100)	0 (0)	(0)	1	0
Ulul Azmi mosque	1	0	1 (100)	0 (0)	(0)	1	0
Kalijudan	0	1	1 (100)	0 (0)	(0)	1	0
Total	2	3	3	0	2		

SS: F1534F (susceptible allele/homozygous wild type); SR: F1534C (susceptible resistant/heterozygous mutant); RR: C1534C (resistant resistant/homozygous mutant).

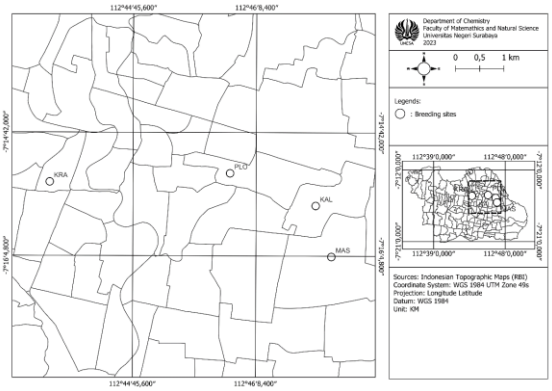

Figure 1. Geographical map of Surabaya, with a focus on four breeding sites (KRA: Kranggan; PLO: Ploso; MAS: Ulul Azmi Mosque, Universitas Airlangga; KAL: Kalijudan). QGIS version 3.26.3 was used to create this figure.

RESULTS

A total of five samples, which consisted of three *Aedes albopictus* and two *Aedes aegypti* mosquitoes, were tested. These two species of mosquitoes had differences in the dorsal mesonotum that could be detected with the naked eye. The thorax of the *Aedes aegypti* samples had a white curving line and two short white lines in the middle. On the other hand, the *Aedes albopictus* samples had a thorax with only a white stripe. In addition, the color of the *Aedes albopictus* samples was darker than that of the *Aedes aegypti* samples.

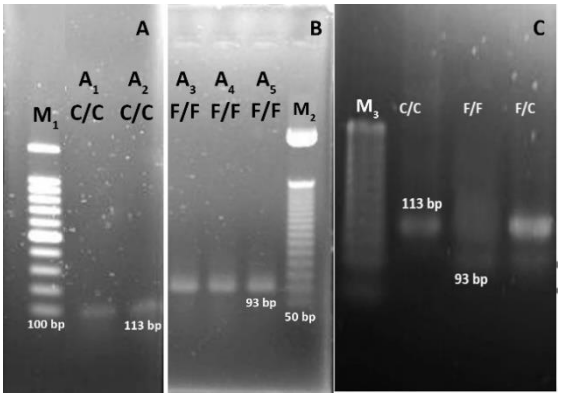

Figure 2. The electrophoresis results of the PCR, with (A) and (B) representing the study results and (C) representing the reference figure.
M_1=100 bp marker; M_2=50 bp marker; M_3=25 bp marker; C/C=homozygous mutant; F/F=homozygous wild type; F/C=heterozygous mutant.

Different bands from the analysis results indicated the *Aedes aegypti* and *Aedes albopictus* genotypes. The genotype was homozygous wild type (F/F or F1534F) if the band appeared at 93 bp, while it was homozygous mutant (C/C or C1534C) if the band appeared at 113 bp. On the other hand, the genotype was heterozygous mutant if the band was present in two locations (93 bp and 113 bp). The results of this study are presented in Figure 2. The figure showed that two samples contained point mutations. In contrast, the other three samples did not carry any mutations. Two samples (A1 and A2) tested positive for the homozygous C1534C mutation, while the other three samples (A3, A4, and A5) tested positive for the homozygous wild type F1534F.

Table 3 presents the distribution of species and allele frequencies. Two samples from Kranggan (C/C frequency=100%) and one from Ploso (F/F frequency=100%) were *Aedes albopictus*. The remaining samples from Ulul Azmi Mosque and Kalijudan were *Aedes aegypti* and had the genotype F/F (100%). Heterozygous mutants (F/C) were not detected in all areas. The total frequency of the F allele was 100%, while the frequency of the C allele was also 100%.

The provided data offered an overview of the AS-PCR results for the C1534C and F1534F mutations in the mosquito samples that were analyzed. This research's findings would help to improve our understanding of the presence and distribution of these mutations in *Aedes albopictus* and *Aedes aegypti* mosquito populations.

DISCUSSION

This article highlights the detection of the *kdr* mutant allele in mosquito samples collected from the study sites. The use of the AS-PCR method in this study was in line with previous studies by Stenhouse et al. (2013) and Lee et al. (2016), which also utilized the same method to identify mutant alleles in mosquitoes. This method can provide rapid, accurate, and cost-effective genotyping results.

A previous study by Fauziyah et al. (2021) also examined samples of *Aedes aegypti* from several sites by extracting RNA from these mosquitoes and analyzing the point mutations. In this study, two bands appeared at 113 bp, indicating that the samples carried homozygous mutations. On the other hand, three bands appeared at 93 bp, indicating that the samples were homozygous wild type. The percentage of homozygous mutant in the samples collected from Kranggan was 100%. Meanwhile, the percentage of homozygous wild-type allele in samples collected from Ploso, Ulul Azmi Mosque, and Kalijudan was also 100%. There was no heterozygous mutant (F/C) detected in the samples. A heterozygous mutant is the product of a genetic mutation in the neurotoxin target receptor gene of *Aedes* sp. mosquitoes. Changes in the amino acid phenylalanine (F) to cysteine (C) at position 1534

are the cause of this mutation (Zhu et al. 2019). It results in the presence of two distinct alleles, a wild-type allele with the amino acid phenylalanine (F) and a mutant allele with the amino acid cysteine (C), at position 1534 within the same gene (Saingamsook et al. 2017). Therefore, the F-to-C mutation at position 1534 can be referred to as F/C, which indicates a variation of the normal and mutant alleles at that position.

A study by Zheng et al. (2022) examined the pattern of *kdr* mutations in mosquitoes collected from several districts in Guangzhou, China. The results showed that homozygous F1534F wild-type mutations were found in 15 field populations of *Aedes albopictus* collected from 11 districts. Four alleles were identified at codon 1534 of domain III, i.e., F/C, F/F, F/L, and F/S. These alleles corresponded to the mutant alleles F1534C, F1534L, and F1534S, as well as the wild-type allele F1534F. A wild homozygous F/F (TTC/TTC) genotype was observed, and two cases of CTC were found in Conghua district. TTG was the primary base sequence of the F1534L allele (Zheng et al. 2022). Another study by Hamid et al. (2017) performed F1534C allele-specific PCR and confirmed F to C point mutation spread in domain III voltage-gated sodium channel (VGSC) genes. The homozygous CC and FC heterozygous frequencies in the resistant phenotypes were found to be 0.21 and 0.25, respectively.

In India, a novel T1520I point mutation with a frequency of 0.13 and an F1534C mutation with a frequency between 0.40 and 0.79 have been identified (Kushwah et al. 2015). The F1534C *kdr* mutation in susceptible and phenotypically resistant mosquito specimens was further discovered using 200 successful amplifications and partial sequencing of the VGSC genes. In northern West Bengal, India, the population under study consisted of 81% homozygous (1534F/F), 12.5% heterozygous (1534F/C), and 6% homozygous (1534C/C) samples carrying the F1534C *kdr* mutation (Modak & Saha 2022). On the other hand, samples of sensitized (dead) mosquitoes showed C/C mutations with a frequency of 0.13. While the C-containing allele was more common in heterozygotes, the CC allele was relatively rare in the sensitive sample group. In Denpasar, Indonesia, the overall frequency of C in mosquitoes was lower than in the F of Domain III sequences, of which the database was collected in GenBank. The F1534C mutations and the F1534F wild types were found, with accessions no. KY078304 and KY078303 (Hamid et al. 2017).

Selection pressure on mosquito vector insecticide target sites has been reported and subsequently detected globally in the principal mosquito vector species, i.e., the *Aedes* mosquitoes. It results in the incidence and evolution of mutations at one or more locations in the relevant gene (Sokhna et al. 2013). Multiple studies have demonstrated that mutations impact the sensitivity of mosquitoes to a variety of insecticides. The presence of *kdr* mutations poses a risk of pyrethroid and DDT resistance (Smith et al. 2018, Chen et al. 2019). There are two primary methods that mosquito vectors have developed to resist the lethal impact of insecticides. These methods involve either an increase in the insensitivity of insecticide target sites caused by genetic point mutations or an increase in the activity of enzymes responsible for detoxifying insecticides (Liu 2015). Understanding the molecular mechanisms behind pesticide resistance can help prevent the spread of dengue fever. Pyrethroid insecticides, commonly used in spraying or fogging, can disrupt the central and peripheral nervous systems of mosquitoes or insects. The disruptions may cause convulsions, paralysis, and death (Davies & Williamson 2009). However, the number of mosquitoes increases instead as a result of the inability to manage pyrethroid-resistant mosquitoes.

Strength and limitations

Due to the limited number of samples collected from only a few areas in Surabaya, Indonesia, the genotype variation in the population could not be represented. Further research is required to collect more representative samples from more areas in the Surabaya region and to investigate the *kdr* mutation. However, this study remains significant as a prompt for *kdr* mutation detection and the development of new vector management strategies.

CONCLUSION

Homozygous mutant alleles were identified in *Aedes albopictus* mosquitoes collected from Kranggan, Surabaya, Indonesia. The insecticide resistance status needs to be assessed, and new vector control strategies should be implemented accordingly. It is anticipated that the findings of this study will serve as a reference for future research and contribute to the development of synthetic or natural insecticides that are more effective for dengue vector control.

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Conflict of interest

None.

Ethical consideration

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Author contribution

SZF was responsible for manuscript preparation as well as data collection and analysis. ARS was responsible for manuscript preparation, data collection, and grammar checking. SF was responsible for manuscript preparation and data confirmation. SM and ECD were responsible for manuscript preparation and data collection. SWN collected the data and drafted the manuscript. SEC validated the data and contributed to the preparation of the manuscript. TT was responsible for data validation and manuscript preparation. THS was responsible for the conceptualization, data collection, and investigation.

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