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Detection of Voltage-Gated Sodium Channel (VGSC) L1014F knockdown-resistance (*Kdr*) mutation of *Culex quinquefasciatus* mosquitoes from Surabaya, Indonesia

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Abstract. *Listiandari IF, Sucipto TH, Fadila SZ, Setiawan AR, Fauziyah S, Madaniyah S, Dewi EC, Ihsan AS, Herdyastuti N, Agustini R, Saputri RD, Hariyono. 2025. Detection of Voltage-Gated Sodium Channel (VGSC) L1014F knockdown-resistance (*Kdr*) mutation of* Culex quinquefasciatus *mosquitoes from Surabaya, Indonesia. Biodiveritas 26: 1354-1359. Culex quinquefasciatus* mosquitoes are a genus of mosquito that can transmit dangerous viruses to humans through their bites, causing diseases in humans such as lymphatic filariasis, chikungunya, Japanese encephalitis, West Nile fever, encephalitis, and St. Louis encephalitis. Therefore, vector control is needed to suppress the spread of these diseases. An easy and inexpensive method to control the vector is using insecticide. Long-term use of insecticide causes the mosquitoes to gain resistance to it. The phenomenon of mosquitoes becoming resistant to insecticides is referred to as knockdown resistance (*Kdr*). *Kdr* occurs due to a mutation in the Voltage-Gated Sodium Channel (VGSC), which is the target site of the insecticide. This mutation leads to the reduced sensitivity of the sodium channel to pyrethroid insecticides. The primary objective of this study was to detect the presence of the *Vgsc*-L1014F *Kdr* mutation in *C. quinquefasciatus* mosquitoes. Samples were obtained from six different locations in Surabaya, East Java, Indonesia. This was determined using a technique known as allele-specific PCR (AS-PCR). DNA was extracted from 23 samples of *Culex* spp. mosquitoes samples, and the VGSC-L1014F mutation was detected using the AS-PCR technique. The results of this study indicated 13 positive cases for the TTT/TTA mutation and 10 negative cases.

Keywords: AS-PCR**,** *Culex quinquefasciatus*, *Kdr* mutations, insecticide resistance, VGSC-L1014F

INTRODUCTION

Diseases spread by arthropods are a significant contributor to infectious diseases worldwide. Viruses that are transmitted by arthropods to humans are called arboviruses, short for arthropod-borne viruses (Goic et al. 2016; Brugman et al. 2018). Arboviruses are transmitted through the bite of blood-feeding arthropods (Mavian et al. 2019). One of these blood-feeding arthropods is the mosquito. Mosquitoes are the most prolific vectors because they can spread dangerous infections to humans. *Anopheles*, *Culex*, and *Aedes* are the three genera of mosquitoes that can spread pathogens to humans (Dahmana and Mediannikov 2020). Mosquitoes from the genus *Culex quinquefasciatus* Say 1823 can spread illnesses such as lymphatic filariasis, chikungunya, Japanese encephalitis, West Nile fever, encephalitis, and St. Louis encephalitis (Franklinos et al. 2019; Dahmana and Mediannikov 2020).

One of the diseases spread by *C. quinquefasciatus* is Japanese Encephalitis (JE), and *Culex* spp. mosquitoes are the primary vector in the spread of Japanese encephalitis in Indonesia. The *Culex* spp. mosquitoes, which are more active at night, transmit JE by sucking blood from animals and humans. The risk of JE transmission may increase due to the high density of *Culex* spp. and available reservoirs. This is especially true in areas with high agricultural and livestock cultures (Kardena et al. 2021). According to reports, there were 40 JE cases in 2015, 43 cases in 2016, 6 cases in 2017, 6 cases in 2018, and 10 cases in 2019. This data was taken from 11 provinces in Indonesia (Panjinegara et al. 2024). The infectious agent that causes Japanese Encephalitis (JE) is the Japanese Encephalitis Virus (JEV). JEV is a virus from the genus *Flavivirus* that attacks the central nervous system. This causes brain inflammation, permanent impairment, and even death in those affected (Kardena et al. 2021).

Several attempts have been made to limit the number of infections by *Culex* spp., such as immunization. However, insecticide vector control can also be implemented (Silva et al. 2019). The most widely used class of insecticide is

pyrethroid, as they are inexpensive, have low toxicity to mammals, and exhibit high insecticidal activity (Gray et al. 2018). This insecticide is easy to find in shops or supermarkets in both big cities and rural areas. Most of the active ingredients in Indonesian insecticides are from the group of synthetic pyrethroids, like d-allethrin, transfluthrin, and deltamethrin (Sunaryo and Widiastuti 2020). Pyrethroid pesticides are neurotoxins targeting the voltage-gated sodium channel's receptor site. Pyrethroid pesticides distrupt the insect's nervous system by affecting sodium ion channels, causing a continuous action potential. The pyrethroid insecticides bind to the VGSC protein, which controls nerve impulses. As a result, continual nerve impulse stimulation will induce hyperexcitation and convulsions in the insects (Ahamad and Kumar 2023). However, prolonged use of insecticides can be harmful to human health. This is because it can lead to the emergence of insecticide resistance in insect populations (Saleh et al. 2021).

Resistance occurs when mosquitoes or insects can survive exposure to insecticides that would normally be lethal to the wild population. This ability is passed down from generation to generation, enabling them to survive concentrations of insecticides that are lethal to wild populations (Chandrasiri et al. 2020). Resistance mechanisms can be divided into two groups: decreased sensitivity of the target site and increased metabolic detoxification of insecticides (Dang et al. 2017). Resistance due to decreased sensitivity of the target site has been the focus of much research. The insensitivity of the target site in insects can lead to insecticide resistance, which is explained by the mechanism known as knockdown resistance (*Kdr*). The target site insensitivity appears due to mutations in the amino acid sequences at VGSC of nerve cell membranes. When mutations occur in the primary target of insecticides, VGSC, they can result in reduced sensitivity of these channels to pyrethroid-type insecticides, thus causing knockdown resistance (Kushwah et al. 2015; Wuliandari et al. 2015).

In *C. quinquefasciatus*, the most common target site insensitivity is the L1014F *Kdr* mutation in the *vgsc* gene, conferring resistance to pyrethroid (Chandrasiri et al. 2020). The L1014F mutation is common in segment six domain 2 (IIS6) at position 1014 of VGSC found in *C. quinquefasciatus* worldwide. The *C. quinquefasciatus* mosquito's L1014F mutation can be found using various techniques. Although direct sequencing is the gold standard for mutation detection, it is costly and impractical for large mosquito samples due to its time and resource-intensive nature. Several techniques detect the L1014F mutation, including allele-specific PCR (AS-PCR). This technique is simple, inexpensive, and gives the same sensitivity and specificity as direct DNA sequencing (Chamnanya et al. 2022).

The resistance of *Culex* spp. to pyrethroids has been reported previously. A study by Okafor et al. (2023) in *Culex* spp. mosquitoes obtained from Nigeria found the *Kdr* L1014F mutation. Silva et al. (2019) reported on the occurrence of VGSC-1014 mutations in a study of wild *Culex* spp. from Africa and South America (Silva et al. 2019). Additionally, the study by Chamnanya et al. (2022) in five regions in Thailand showed that there had been an L1014F mutation in *Culex* spp. mosquitoes. This study aims to detect the VGSC-L1014F mutation in *C. quinquefasciatus* mosquitoes obtained from Surabaya, East Java, Indonesia, using the allele-specific polymerase chain reaction (AS-PCR) method.

MATERIALS AND METHODS

Study area

Samples were collected from several sampling sites in Surabaya, East Java, Indonesia, including Kranggan, Ketintang, Ploso, Ulul Azmi Mosque, Gubeng, and Wisma Permai (Figure 1). Using QGIS Version 3.26.3 software, the sampling sites were displayed using Indonesian topographic maps. Figure 1 displays the circular sampling area maps (i) KRA: Kranggan; (ii) PLO: Ploso; (iii) MAS: Ulul Azmi Mosque; (iv) WIS: Wisma Permai; (v) GUB: Gubeng; and (vi) KET: Ketintang.

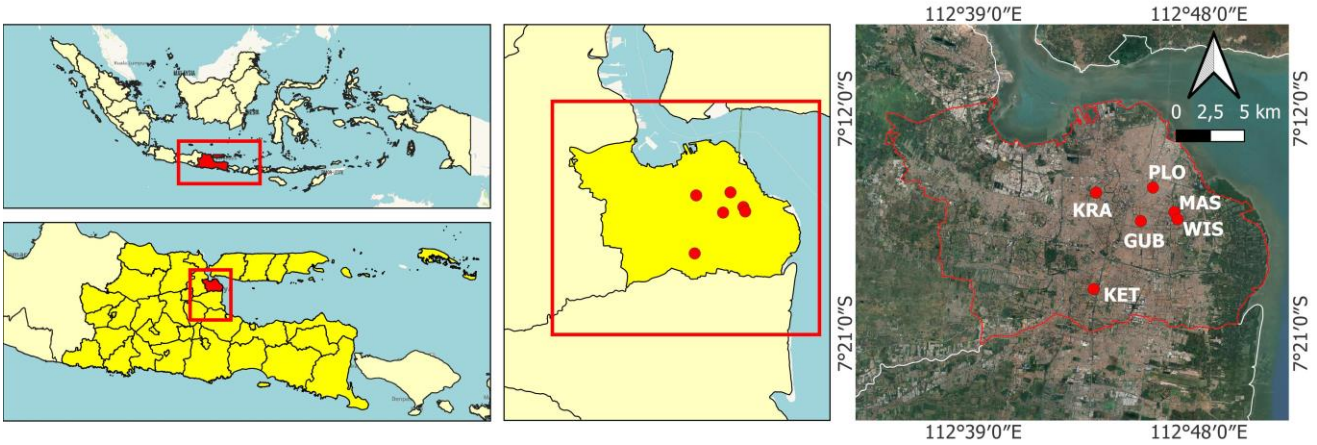

Figure 1. Displays the sampling area maps marked with circles (KRA: Kranggan; PLO: Ploso; MAS: Ulul Azmi Mosque; WIS: Wisma Permai; GUB: Gubeng; KET: Ketintang). Image created with QGIS Version 3.26.3

Sampling

Purposive sampling was conducted in the mentioned districts in 2019. The sampling points were determined according to the presence of stagnant water, vegetation, and other environmental conditions conducive to breeding sites for adult *C. quinquefasciatus* mosquitoes. Surveyors inspected every area. If adult mosquitoes were found, they were collected and transferred to the laboratory for further processing. The collected samples were pooled and stored at -80°C. The mosquitoes obtained were identified by species using the *C. quinquefasciatus* mosquitoes identification key from the Ministry of Health of the Republic of Indonesia (Direktorat Peningkatan Mutu Tenaga Kesehatan 2023).

DNA extraction

The samples were suspended in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS). Mosquito cells were then lysed using ultrasonic cell disruption to extract the genetic material. Subsequently, the lysate was filtered, and the filtrate was utilized for DNA extraction. DNA extraction was performed using the QIAamp DNA Mini Kit (Qiagen GmbH, Hilden, Germany), following the manufacturer's protocol. Briefly, the entire body of a single mosquito was homogenized in a microcentrifuge tube containing 80 µL of PBS using a pestle for 30 sec. Then pipette 20 µL QIAGEN Protease (or proteinase K) into a 1.5 mL microcentrifuge tube. Add 180 µL Buffer ATL to the sample. Incubate at 60°C for 30 minutes. After incubation, add 200 µL buffer AL to the sample, then vortex for 15 seconds. Add 200 µL ethanol 96% and mix by vortexing for 15 seconds, then centrifuge briefly. After spinning down, the mixture is put into a QIAamp Mini spin column (2 mL collection tube) and centrifuged at 8,000 rpm for 1 minute. Discard the 2 mL collection tube containing the filtrate and replace it with a new 2 mL collection tube.

After replacing, add 500 µL Buffer AW1, then centrifuge at 8000 rpm for 1 minute. Discard 2 mL of the collection tube containing the filtrate and replace it with a new 2 mL collection tube. Add 500 µL of buffer AW2, then centrifuge at 13,000 rpm for 3 minutes. Discard 2 mL of the collection tube containing the filtrate and replace it with a new 2 mL collection tube-centrifuge again at 13,000 rpm for 1 minute. Transfer the QIAamp Mini spin column to a 1.5 mL microcentrifuge tube. Add 60 µL of Buffer AE or distilled water. Incubate at room temperature (15-25°C) for 1 minute, then centrifuge at 8,000 rpm for 1 minute, yielding 50 µL of DNA template is obtained.

VGSC-L1014F mutation assay

The VGSC-L1014F mutation assay was conducted using allele-specific PCR (AS-PCR). After obtaining DNA, AS-PCR was performed according to the method of Silva et al. (2019). The PCR was performed in 12.5 µL, consisting of 2,5 µL DNA sample, 6.25 µL green PCR master mix, 2.75 µL nuclease-free water, 0.5 µL reverse primer (VGSC1014/R), and 0.5 µL of each forward primer (VGSC1014/F-T, VGSC1014/F-A, VGSC1014/F-C). The nucleotide sequence of the primers is presented in Table 1. The reaction was carried out in a PCR thermocycler, with 1 cycle at 95°C in 5 minutes (initial denaturation), 40 cycles at 94°C in 1 minute (denaturation) at 59°C in 1 minute (annealing), at 72°C in 1 minute (extension); and 1 cycle at 72°C in 10 minutes (final elongation).

After PCR, restriction digestion was performed using the Eco32I enzyme. A total of 7 µL PCR was mixed with 6 µL nuclease-free water, 1 µL FastDigest buffer, and 0.5 µL Eco32I restriction enzyme. The mixture was incubated at 37°C for 10 minutes. Agarose gel (1,5%) electrophoresis was carried out at 100 V for 30 minutes following the incubation period. The gel was stained for 30 minutes using ethidium bromide, and visualization was performed under UV light using a gel documentation system. The Eco32I enzyme was chosen because it recognizes a specific sequence (5'-GATATC-3') in the engineered tail of the primer. This cutting produces DNA fragments of different lengths, namely TTT (181 bp), TTA (206 bp), and TTC (231 bp) so that it allows allele analysis using simple methods such as agarose gel electrophoresis, without the need for sophisticated technology such as sequencing (Silva et al. 2019).

RESULTS AND DISCUSSION

The results of the study are presented in Figure 2.A, while Figure 2.B is from the literature sources. There were 13 positive results for TTT/TTA mutations, which were indicated by the appearance of DNA bands measuring 181 and 206 bp, and 10 negative results, which were indicated by the absence of DNA bands. Figure 2.A shows that the sample has the TTT/TTA mutation (bands appear at 181 bp and 206 bp), which is consistent with the TTT/TTA mutation in the electrophoresis results reported in the literature in Figure 2.B. From Figure 2.B, lane 1 is a 100 bp DNA ladder. Lanes 2, 3, and 4 show homozygous individuals for each codon: TTA (206 bp), TTC (231 bp), and TTT (181 bp). Lanes 5 and 6 show heterozygous individuals for TTT/TTA (181 and 206 bp), TTA/TTC (206 bp and 231 bp), and TTT/TTC (181 bp and 231 bp).

Table 1. The sequences of nucleotides used in the reverse and forward primers (Silva et al. 2019)

Primer	Sequence (5-3')	Product (bp)	Codon change
VGSC1014/R	GATCGGTATGAACTGTTTGTTTACATC		
VGSC1014/F-T	AGTAGCGGATAACAATTTCACACAGGAAGGGTTTTCCCAGTCACGACGTT GATATCGCCACCGTAGTGATAGGAAATATT	181	TTT
VGSC1014/F-A	AGTAGCGGATAACAATTTCACACAGGATATCGAAGGGTTTTCCCAGTCAC GACGTTGCCACCGTAGTGATAGGAAATTCA	206	TTA
VGSC1014/F-C	GATATCAGTAGCGGATAACAATTTCACACAGGAAGGGTTTTCCCAGTCAC GACGTTGCCACCGTAGTGATAGGAAACTTC	231	TTC

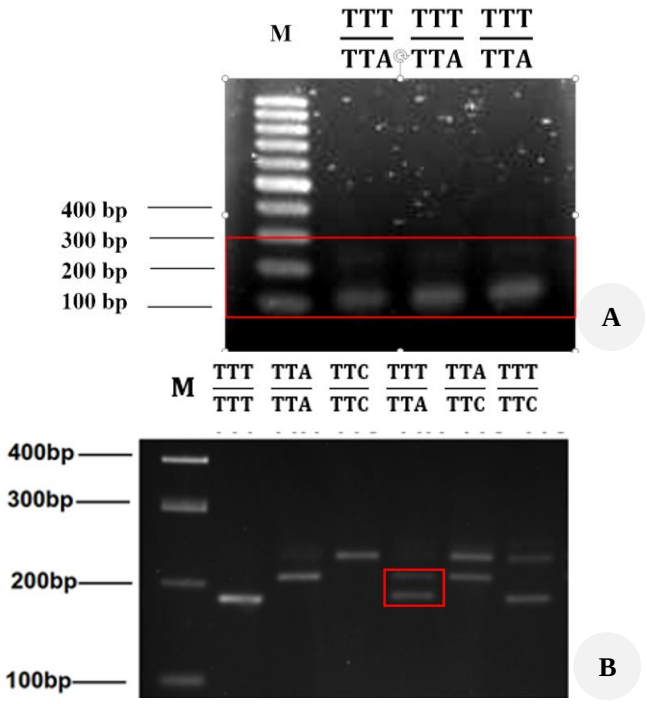

Figure 2. The results of the PCR electrophoresis for the detection of the VGSC-L1014F mutation. The results were compared with the reference. A: study result; B: reference figure (Silva et al. 2019); M: 100 bp marker; TTT/TTT, TTA/TTA, TTC/TTC: homozygous; TTT/TTA, TTA/TTC, TTT/TCC: heterozygous

Discussion

Culex quinquefasciatus, the southern house mosquito, is a global vector of several human and veterinary diseases (Chamnanya et al. 2022). *Culex* spp. mosquitoes are more numerous at night than during the day (Kardena et al. 2021). *Culex* spp. mosquitoes are inactive and rest more in dark places, such as corners of rooms or shelters during the day, and are more commonly found indoors than outdoors. *Culex* spp. mosquitoes can develop optimally at 22-32°C temperatures with humidity >60% (Panjinegara et al. 2024). *C. quinquefasciatus* mosquitoes have a blood-feeding behavior, which includes biting birds and mammals, making them potential carriers of dangerous arboviruses, such as lymphatic filariasis, chikungunya, Japanese encephalitis, West Nile fever, encephalitis, and St. Louis encephalitis (Silva et al. 2017). In Indonesia, *Culex* spp. mosquitoes are the main vector in the spread of Japanese Encephalitis. In this study, mosquitoes were collected from 6 locations in Surabaya city to ensure local diversity of the mosquito species/species group, and the habitat characteristics for each mosquito species/species group were considered.

There are two ways to control mosquito-borne diseases (i) the first is by vaccinating and administering medication to infected people, and (ii) the second is by controlling mosquito vectors with chemical insecticides. Using chemical insecticides to eradicate mosquitoes is still the most efficient primary method. The major insecticide classes include pyrethroids, Organophosphates (OPs), carbamates, and organochlorines (Ore et al. 2023). The most frequently used insecticides for mosquito vector control by people in Surabaya are pyrethroid-based. Pyrethroids interact with Voltage-Gated Sodium Channel (VGSC) in the mosquito nervous system. VGSC is the physiological target of pyrethroid insecticides and is integral to the insect nervous system. The sodium channel protein consists of four homologous domains (DI-DIV) each of which comprises six transmembrane segments (S1-S6) connected by intracellular and extracellular loops (Clarkson et al. 2021). Pyrethroid molecules bind to this protein, stabilize the ion-conducting active state, and thus disrupt normal nervous system function, producing paralysis ('knock-down') and death. However, amino acid substitutions at key positions within the protein alter the interaction with insecticide molecules, increasing the dose of insecticide required for knock-down, known as knock-down resistance or *Kdr* (Lee et al. 2020). The *Kdr* mutation represents a form of resistance to pyrethroid insecticides (Lee et al. 2020), mainly due to changes in one or more amino acids in the mosquito's VGSC, which leads to change in the conformation of the VGSC, reducing the ability of the pyrethroid molecule to bind as efficiently and further avoiding the toxic effects of the pyrethroids (Zhao et al. 2023).

When vector control agencies spray adult mosquitoes with pyrethroids, mosquitoes with a *Kdr* mutation or similar tend to have a higher survival rate than mosquitoes that do not have this mutation (Amelia-Yap et al. 2018). As a result, mosquitoes resistant to pyrethroids can survive and reproduce even when exposed to insecticides, reducing the effectiveness of vector control and increasing the risk of arbovirus transmission to humans (Dusfour et al. 2019). The current spread of pyrethroid resistance in *C. quinquefasciatus* may be due to increased selection pressure caused by the massive deployment of pyrethroid (indoor residual spraying, thermal fogging, and use of household insecticides) in vector control across the country, in conjunction with the use of pyrethroid-based insecticides in agricultural pest control (Chamnanya et al. 2022). Pyrethroids can be associated with the incidence of mutations in codon 1014 of the VGSC gene (Rai and Saha 2022). The behavior of people in Surabaya in using pyrethroid household insecticides can contribute to mutations in the VGSC gene codon 1014 *Culex* spp. mosquitoes.

The mechanism for mutations in the resistance-related VGSC gene is the change of one nucleotide base, resulting in the substitution of leucine with phenylalanine at residue 1014 (Mack et al. 2021). VGSC gene mutations cause *Kdr* allele polymorphisms, namely the *Kds* (knockdown susceptible), *Kdr-w* (leucine to phenylalanine mutation in codon 1014), and *Kdr-e* (leucine to serine mutation in codon 1014) alleles (Thiaw et al. 2018). There are three allelic variations at the locus VGSC-1014: TTA allele coding leucine (L) for wildtype phenotype, and TTT and TTC alleles coding phenylalanine (F) for a resistant phenotype (Chamnanya et al. 2022). In this study, *Culex* spp. samples taken at six different locations in Surabaya were identified as having heterozygous resistance (TTT/TTA-L1014F/L1014). This resulted in the amino acid leucine being substituted with the amino acid phenylalanine at residue 1014 of the voltage-gated sodium channel, which may have

contributed significantly to the insecticide resistance recorded in the vector population from this area. In addition, other genetic factors and environmental influences can also play an important role in the development of resistance, such as insecticides/pesticides usage in agriculture, the presence of anthropogenic or natural xenobiotics, and biotic interactions between vectors and other organisms, may affect both the overall mosquito responses to pyrethroids and the selection of resistance mechanisms (Bharadwaj et al. 2025). This study supports the finding that in cases of resistance of *C. quinquefasciatus* mosquitoes to pyrethroid insecticides, one of the resistance mechanisms often occurs through mutations in the VGSC gene, such as the VGSC-L1014F mutation (Delannay et al. 2018). The L1014F mutation is found worldwide in *C. quinquefasciatus* mosquitoes and other insect species and has been functionally confirmed to be responsible for *Kdr* in an *in vitro* expression system. Many studies of the VGSC-L1014F mutation have been conducted on *C. quinquefasciatus* populations in several countries such as Thailand (Chamnanya et al. 2022), Texas (Lee et al. 2020), Korea (Jeon et al. 2024) and China (Region 2023).

The L1014F mutation in *C. quinquefasciatus* mosquitoes can be found using various techniques. Although direct sequencing is the gold standard, it is costly and impractical for large mosquito samples. The L1014F mutation can be found using a variety of methods, such as allele-specific PCR (AS-PCR), Hot Oligo Ligation Test (HOLA), ABI Prism SNaPshot Multiplex, Single probe/Melting curve analysis using Real-time PCR, Pyrosequencing, and the TaqMan assay. Our study successfully developed the allele-specific PCR (AS-PCR) technique for L1014 detection in *C. quinquefasciatus*. The Allele-Specific PCR Assay (AS-PCR) can be implemented to detect *Kdr* mutant and provide rapid, accurate, and cost-effective genotyping. Rapid results and the precise target can be used to figure out the VGSC gene of mosquitoes in a population (Fauziyah et al. 2021). This method has the same sensitivity and specificity as direct DNA sequencing, is easy to use, and is reasonably priced. However, it is not appropriate to analyze a large number of mosquitoes due to its time-consuming nature and the inability of gel electrophoresis to swiftly determine genotyping findings (Chamnanya et al. 2022).

Continuous use of pyrethroids will cause a low level of susceptibility by mosquitoes to pyrethroids so that the spread of insect-borne diseases can increase. In addition, exposure to pyrethroids can cause oxidative stress, inflammation, and DNA damage (Zhu et al. 2020). Research on exposure to pyrethroid insecticides has been conducted in the United States which states that environmental exposure to pyrethroid insecticides is associated with an increased risk of death from cardiovascular disease and cancer. Observations conducted over 14 years in adults stated that 246 deaths occurred, including 41 related to cardiovascular disease and 52 related to cancer (Bao et al. 2020).

This study successfully identified the mutation TTT/TTA in the VGSC genes (L1014F alleles) in *Culex* spp. mosquitoes in Surabaya. These mutations indicate that *Culex* spp. Mosquitoes are not sensitive to pyrethroid insecticides. *Culex* spp. can be a vector of JEV and filariasis, so the control of *Culex* spp. Mosquito vectors need to be considered using insecticides instead of pyrethroid groups. These findings provide new insights into the challenges faced in controlling disease vectors. The discovery of this mutation is important for local health services to enable them to review the use of existing insecticides and consider other alternatives. In addition, the routine monitoring of insecticide resistance needs to be improved to detect changes in mosquito populations and anticipate potential increases in resistance. With these steps, the effectiveness of *Culex* spp. mosquitoes control can be increased while reducing the risk of spreading vector-borne diseases.

The study shows that the AS-PCR method can differentiate and detect the VGSC-L1014F mutation in the *C. quinquefasciatus* mosquito gene obtained from six places in Surabaya. The results of the study show that there were 13 positive results for the TTT/TTA mutation and 10 negative results for the sample code.

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