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Research Article

Detection of Vgsc-L1014F Allele Knockdown-resistance Mutation in Male *Culex quinquefasciatus*

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ABSTRACT

The distribution of *Culex quinquefasciatus* as a vector for several tropical diseases in Indonesia could affect the transmission of various pathogens, including filariasis, West Nile, encephalitis, and Rift Valley fever. The use of insecticides to control mosquito populations is carried out massively. However, this effort is threatened by an increase in mosquito resistance to insecticides, in this case, pyrethroids that target Voltage-gated sodium channel (VGSC) as a complex protein in mosquito nerve cells that plays a role in the movement of sodium ions, where these compounds can have a knockdown effect and lead to death. The existence of a single nucleotide mutation in this specific codon results in knockdown resistance (kdr) in mosquitoes. This study aims to determine whether there is a kdr mutation in the Vgsc-L1014F target using the latest molecular method ETAS-PCR to type tri-allelic variation at Vgsc-1014 in *Cx. quinquefasciatus* samples collected in three areas in the cities of Surabaya and Sidoarjo, namely Wonocolo, Sukodono, and Wonoayu. The result showed ten male pool samples (C1-C10) of *Cx. quinquefasciatus* did not carry mutations or present as homozygous wild type (TTT/TTT), with a DNA band size of 181 bp. Hence, this preliminary study could be extended for further research to build vector control monitoring programs.

1. Introduction

Cx. quinquefasciatus, as a member of the *Culex pipiens* complex in tropical to sub-tropical regions, is a vector species for several diseases such as lymphatic filariasis, Japanese encephalitis, West Nile, and Rift Valley fever with potential fatalities if not treated. Consequently, this can lead to a high transmission rate of the pathogen to each host with a high biting rate of up to 100 bites/person/night (Weissenböck *et al.* 2010; Farajollahi *et al.* 2011; Kauffman & Kramer 2017; Talipouo *et al.* 2021). A study in Morocco showed that the *Cx. pipiens* complex became a strong suspect for vector when West Nile Virus (WNV) epizootics occurred in 1996 and 2003, with the number of horse deaths reaching 42 (Hubálek & Halouzka 1999; Bkhache *et al.* 2016). In 2021, the bite of the *Cx. quinquefasciatus* has reached 19,774 people in fifteen provinces in Indonesia (Setiyaningsih *et al.* 2021). The widespread distribution of the arbovirus, including in Surabaya, also resulted in the discovery of 12 WNV cases in early 2014 (Ikawati *et al.* 2015).

The primary strategy to control mosquito populations is spraying insecticides, which generally contain pyrethroids (permethrin, deltamethrin, and cypermethrin) (7,8) as a synthetic insecticide that is classified for digestive poisons by targeting the Voltage-Gated Sodium Channel (VGSC) protein in nerve cells

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(Rahayu *et al.* 2022), where Vgsc itself is a complex protein—scattered in the nervous system that plays a role as the channel for movement of sodium ions in cells. Pyrethroids will bind to the α subunit of Vgsc and cause prolonged open sodium channels or permanent depolarization due to increased activation (Bradberry *et al.* 2005; Du *et al.* 2016; Gajendiran & Abraham 2018). This modification causes muscle spasms and 'knockdown' or rapid paralysis and death of the insect (Hudayya & Jayanti 2012).

However, the occurrence of resistance to insecticides threatens human welfare as a significant impact of the existence of these disease vectors (Xu *et al.* 2005) with several mechanisms: a decreased cuticle penetration, behavioral changes, increased detoxification metabolism by the presence of glutathione S-transferase enzymes (GSTs) and multi-function oxidases P450s, also alteration or change in the target insecticide molecule (Lopes *et al.* 2019) which called SNP (single nucleotide polymorphism) or mutation (Lusiastuti *et al.* 2015) that occurs in one of the vgsc alleles at codon 1014 causes the amino acid leucine to become phenylalanine, thus potentially causing kdr (knockdown resistance) mutations, and effectiveness of the insecticide decrease, then mosquitoes can recover within hours or even survive (WHO 1980; Lusiastuti *et al.* 2015). The factors that influence resistance are the repeated use of insecticides, which can cause vector resistance and environmental pollution (Sunaryo 2014), the presence of biological factors (change of generations, monogamous or polygamous marriages) (Pradani *et al.* 2018), environmental factors such as a dirty place or lots of garbage, unplanned urbanization, inadequate solid waste management and also ineffective controls (Saleeza *et al.* 2011).

The L1014F is the most widespread mutation of *Cx. pipiens* complex (Scott *et al.* 2015). One of the results of a study in Brazil showed the presence of a kdr mutation in *Cx. quinquefasciatus* at L1014F is detected positive at a low frequency (4–7%) (Steinhagem *et al.* 2012). Research in two areas in Kupang, NTT, Indonesia showed that mosquito samples resistant to pyrethroids (0.25% permethrin), with details in the Kelapa Lima area at 7.06% and in Oebobo at 12.69% (Sunbanu *et al.* 2021). Martins in Central Uganda has been reported the mutation frequency of *Cx. quinquefasciatus* on Vgsc-L1014F by 62% (Silva Martins *et al.* 2017). A study by Norris (2011) in Macha, Zambia, shows that *Cx. quinquefasciatus* detected 7% were homozygous mutant Vgsc-L1014F (with a kdr frequency of 0.263) (Norris & Norris 2011).

In this study, the kdr-allele mutation detection of Vgsc-L1014F was carried out in samples of *Cx. quinquefasciatus* mosquitoes where it collected from several areas in Surabaya and Sidoarjo to find out whether there are mutations in these targets using the latest molecular method ETAS-PCR to type tri-allelic variation at Vgsc-1014, where this research is a preliminary study and requires further testing with a large number of samples which are more varied using kdr detection methods and other biological assays that are accurate based on statistical analysis. Therefore, it is essential to know as a follow-up step in developing monitoring programs on a national and global scale.

2. Materials and Methods

2.1. Materials

Ten pool male mosquito samples of *Cx. quinquefasciatus* was collected from several parts of Surabaya and Sidoarjo.

2.2. Mosquito Sampling

Ten pool male mosquito samples were collected from 3 different areas in Surabaya and Sidoarjo, where the populations of the two regions reached an average of 2.5 million people (Badan Pusat Statistik Kabupaten Sidoarjo 2020; Badan Pusat Statistik Kota Surabaya, 2022) with details: 1 sample from Wonocolo, six samples from Sukodono and two other samples from Wonoayu (Figure 1).

2.3. RNA Extraction

The *Cx. quinquefasciatus* samples were extracted using the Zymo Research Corporation Quick-RNA™ Miniprep Plus Kit (Zymo Research Corporation USA) to obtain a pure RNA solution. The purity of the RNA checked using a nanodrop spectrophotometer at A260/A280 nm ranged from 1.46-2.13.

2.4. Genotyping of L1014F with ETAS-PCR

The Engineered-Tail Allele-Specific PCR (ETAS-PCR) method was used for genotyping, followed by the digestion of endonuclease enzymes. In this method, DNA doubling in the 178 bp region (focused on the Vgsc-1014 locus) is done with four different primers (three forward primers and reverse primer), where the specific allele of the primer is designed to match one

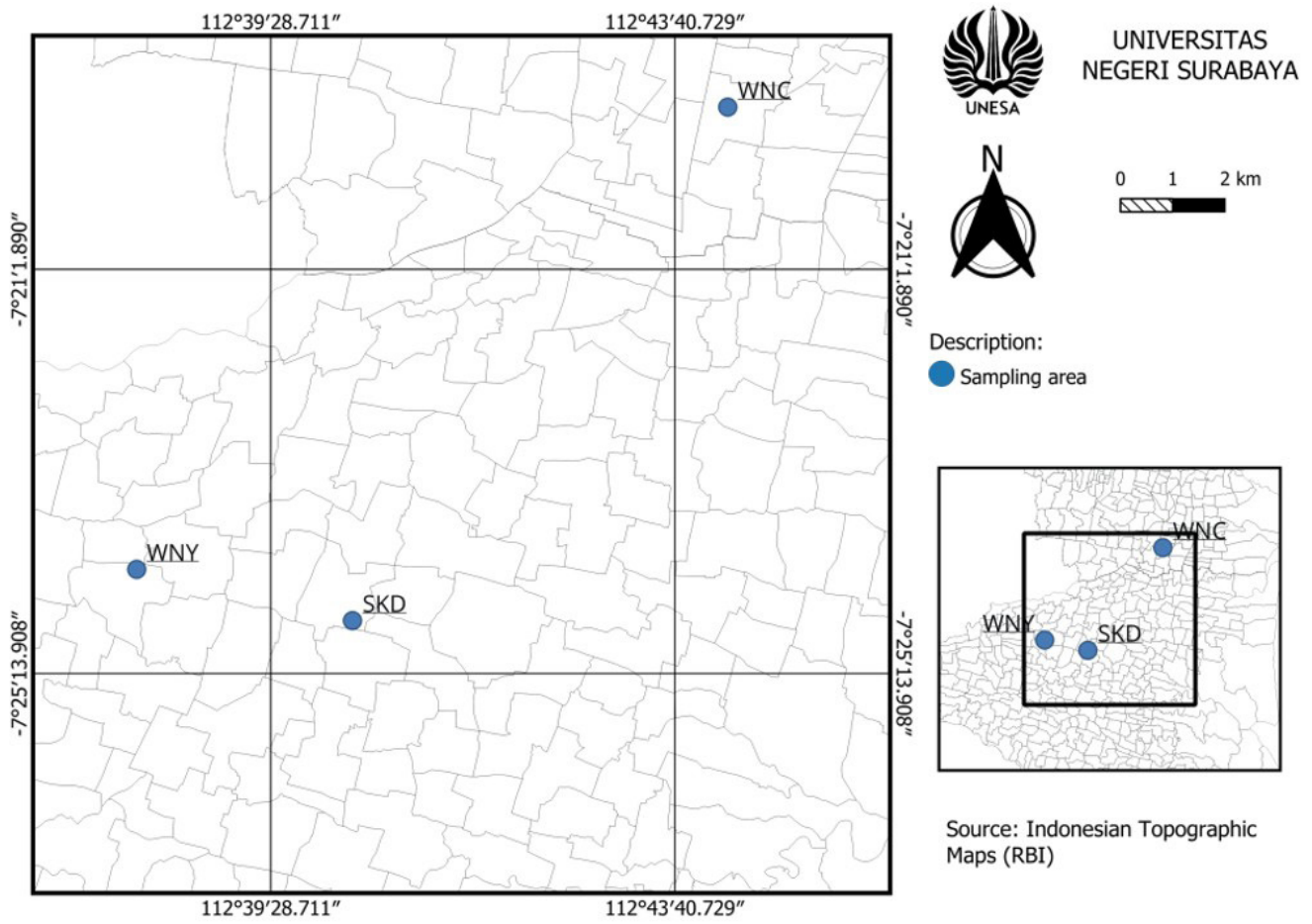

Figure 1. Geographical map of the city of Surabaya and Sidoarjo. The sampling area is marked with a blue circle, with details: WNY (Wonoayu), SKD (Sukodono), and WNC (Wonocolo). The map was designed using the QGIS 3.28.1 version

of the SNP alleles (TTA, TTT, TTC) in the last base of codon Vgsc-1014 (Martins *et al.* 2019). The preparation process with one reaction (20 µL) PCR reagents used 5 µL RNA template, 1 µL reverse oligonucleotide primer, and 1 µL of each forward primer, as shown in Table 1, 10 µL RT-PCR master mix, and 2 µL Nuclease Free Water (NFW). PCR conditions were run at one cycle of 55°C for 45 minutes (reverse transcription), 95°C for 5 minutes for one cycle, 40 cycles for the denaturation step at 94°C for 1 minute; annealing at 59°C for 1 minute; and extension at 72°C for 1 minute; 1 cycle for the final extension stage at 72°C for 10 minutes and 16°C.

The restriction process was carried out for cDNA that has been amplified by PCR using several reagents as follows: 12 µL NFW, 2 µL buffer, and 1 µL Eco32I restriction enzyme to cut at 56 bp with the help of three engineered tails on (5'-GATATC-3') from each forward primer, so that when the restriction result is 234 bp, each allele has a different fragment length, where TTT is 181 bp, TTA 206 bp, and TTC 231 bp (Martins *et al.* 2019). The incubation process was carried out at 37°C for 10 minutes.

After the PCR product had been successfully restricted, then it was visualized using electrophoresis using 1.5% Agarose gel for 30 minutes, stained with EtBr, and the DNA bands were read under the UV light.

3. Results

3.1. Mosquito Identification

Ten sample pools, where pool 1-pool 10 respectively, containing 10, 18, 15, 30, 16, 25, 20, 10, and 15 mosquitoes, were collected in several areas in Surabaya and Sidoarjo and identified as male *Cx. quinquefasciatus* with morphological characteristics of a dark brown body, two parallel hairy (thicker than female) antennae, and dark black eyes; there are no hairs on the spiracles or the post's spiracular. The length of the maxillary palps of the male mosquito is equal to or even longer than the proboscis. *Cx.*

Table 1. Vgsc-L1014F oligonucleotide primers. The restriction target by the Eco32I enzyme is underlined (Martins *et al.* 2019)

Primer	Sequence (5'-3')	Fragment size (bp)
Vgsc-1014/F-T	AGTAGCGGATAACAATTTCACACAGG AAGGGTTTTCCCAGTCACGACGTTGAT ATCGCCACCGTAGTGATAGGAAATATT	181
Vgsc-1014/F-A	AGTAGCGGATAACAATTTCACACAGGA TATCGAAGGGTTTTCCCAGTCACGACG TTGCCACCGTAGTGATAGGAAATTCA	206
Vgsc-1014/F-C	GATATCAGTAGCGGATAACAATTTCACA CAGGAAGGGTTTTCCCAGTCACGACGTT GCCACCGTAGTGATAGGAAACTTC	231

quinquefasciatus is known to have widespread resistance in various regions, including Africa and Asia.

3.2. ETAS-PCR/Vgsc-1014 Result

ETAS-PCR results for detecting tri-allele specific mutations in ten samples (C1-C10) are shown in Figure 2.

Based on the picture above, the appearance of the DNA band length in all samples is 181 bp, showing no mutations or homozygous wild-type.

4. Discussion

Resistance to pyrethroid-type insecticides can have a 'knockdown' effect, causing paralysis and death (Hemingway *et al.* 2004; Singh *et al.* 2010; Hidayahtullah *et al.* 2020) by targeting the Vgsc part, which plays a role in information throughout the nervous system is widespread in many insect species, one of which is the *Cx. quinquefasciatus* (Hemingway *et al.* 2004; Wondji *et al.* 2008). Research conducted in Sri Lanka found that resistance to pyrethroids reached 80% (Chandrasiri *et al.* 2020). A leucine change causes the most common resistance substitution phenotype at codon 1014 in the Vgsc S6 domain II region by reducing the affinity of the insecticide target site (O'Reilly *et al.* 2006; Dong *et al.* 2015; Diouf *et al.* 2020; Rahman *et al.* 2021).

The resistance level of *Cx. quinquefasciatus* against major insecticides demonstrated in a recent study in Cameroon detected resistance to permethrin (death rate: 14.25-66.05%), deltamethrin (death rate: 2.91-20.78%), DDT (death rate: 8.87-27.91%) (Nchoutpouen *et al.* 2019). Another study by Omotayoid *et al.* (2022) showed that *Cx. quinquefasciatus* experienced resistance to permethrin in Dutse and Kafin-Hausa, Nigeria, with a resistance level of 12.2% and 77.78%, respectively. Meanwhile, the resistance to Lambda-cyhalothrin was only detected in Kafin-Hausa, with a percentage of 83.95%. In contrast, in another city, namely Ringim, there was still an alleged resistance of 90% (Omotayoid *et al.* 2022). The high presence of L1014F resistance in *Cx. quinquefasciatus* in Bengal, India, resulted in a low percentage of mosquito mortality, including 7.69–36.36% for deltamethrin, 4–34.61% for lambda-cyhalothrin, 11.11–36.36% for permethrin, 0–34.61% for DDT, 0–18.18% for propoxur and 0–8% for malathion (Rai & Saha 2022).

Various methods have been carried out to detect the mutations at specific alleles. One commonly used method is AS-PCR (Allele-Specific PCR) (Morlan *et al.* 2009). Research carried out on *Aedes aegypti* mosquitoes has shown to have V/G and G/G mutations detected by the AS-PCR method (Widiastuti *et al.* 2015).

The ETAS-PCR has been developed as an easy-to-implement method to allow mutation detection of Vgsc. The ETAS-PCR/Vgsc-1014 method is a single PCR reaction followed by digestion steps employing endonuclease enzymes to cut three specific DNA fragment alleles. It is superior to the previous method, the AS-PCR method, which can only distinguish two allelic types (TTA and TTT) but not TTC-resistant alleles (Martins *et al.* 2019; Chandrasiri *et al.* 2020).

Based on the above methods, the DNA band of 181 bp was detected in all samples in this study. According to Martins *et al.* (2019), allelic mutations in Vgsc-L1014F are regarded as positive (heterozygous or homozygous mutant) if there are two DNA bands of 181 bp and 203 bp-length (TTT/TTA), 203 bp and 231 bp (TTA/TTC), 181 bp and 231 bp (TTT/TTC). ETAS-PCR/Vgsc-L1014F results on ten samples of *Cx. quinquefasciatus* collected in several areas in Surabaya and Sidoarjo did not detect any mutations/homozygous wild-type. The absence of mutations in L1014F in the mosquito sample could be due to the difference in the location of the target mutation. Other possibilities of the undetected mutations were that the mosquitoes might have other resistance mechanisms to

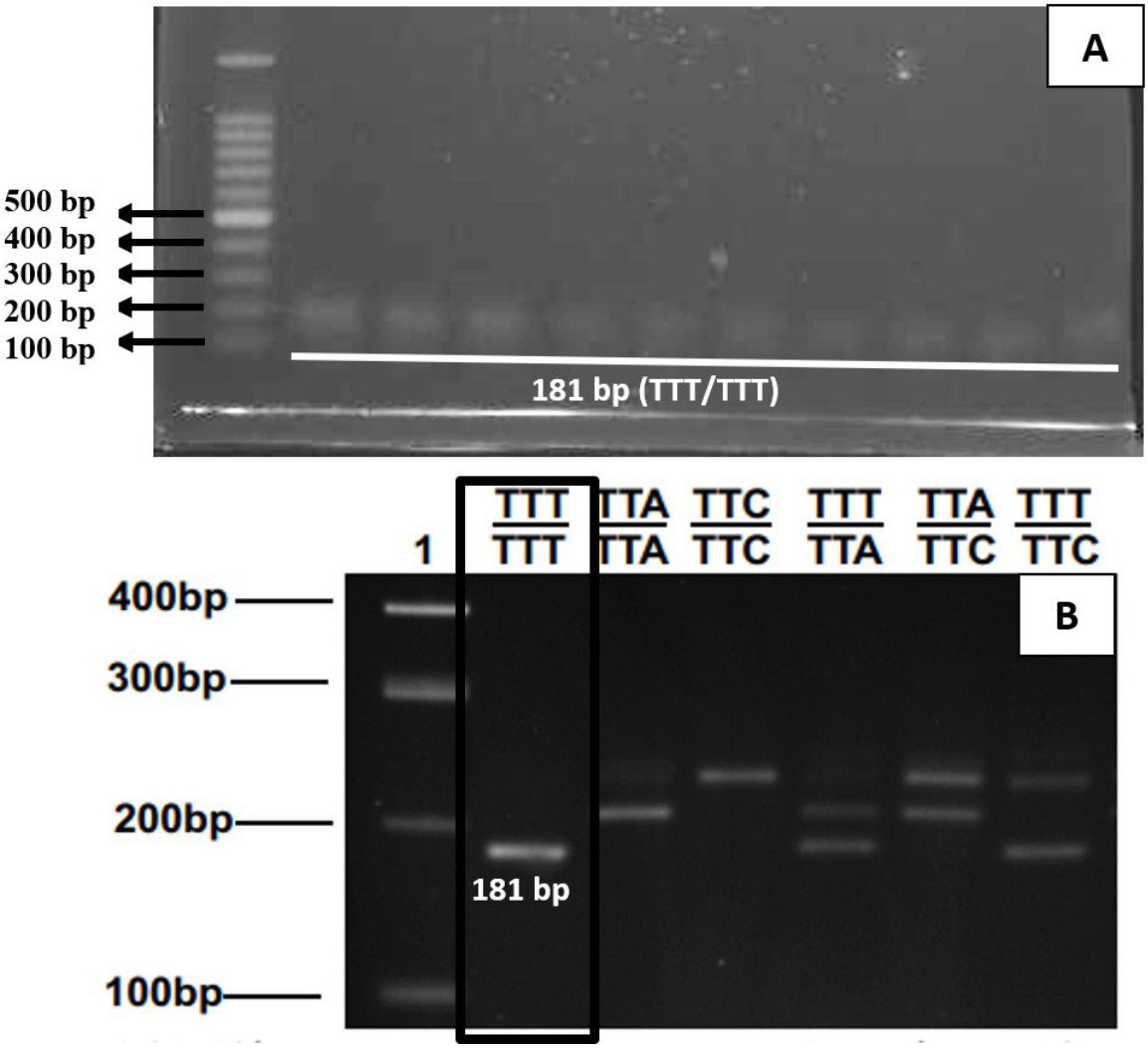

Figure 2. (A) ETAS-PCR results of samples C1-C10 on DNA length of 181 bp (homozygous wild-type TTT/TTT), (B) References (Martins *et al.* 2019)

insecticides that included detoxification metabolism, physiological resistance, and behavioral resistance (Pradani *et al.* 2018; Rahayu *et al.* 2022).

Molecular screening to detect the Vgsc-L1014F *Cx. quinquefasciatus* mutation, employing the ETAS-PCR method, has not yet been widely reported in Indonesia. In contrast, results on kdr mutations in the Vgsc-L1014F allele have been reported in various countries. A previous study by Martins *et al.* (2019) in the Tororo region, Uganda, showed a high frequency of detection of heterozygous wild type/allelic resistance among mosquitoes that were resistant to deltamethrin and DDT, where at least one allele had a resistance level ranging from 70 .37 to 85.78% (Martins *et al.* 2019). Another study by Norris (2011) in Macha, Zambia, detected that 54.5% was a homozygous wild-type occurring in a large proportion of *Cx. Quinquefasciatus* mosquito samples, 38.5% were heterozygous, and 7% were homozygous mutant Vgsc-L1014F (with a kdr frequency of 0.263). In addition, 46% of the total homozygous wild-type managed to survive, indicating that other resistance mechanisms besides kdr mutations are possible (Norris & Norris 2011). This also occurred in the results of a study by Yanola *et al.* 2015 with population samples taken from six regions, one of which was in Lampang, Thailand, where the homozygous wild type (L/L1014) was detected in 113 *Cx. quinquefasciatus* that died when given 0.05% deltamethrin. Another mechanism to detect a detoxification resistance mechanism in Chiang Mai showed a positive result of deltamethrin resistance in the larval stage of the samples after being given a P450 monooxygenase enzyme inhibitor (Yanola *et al.* 2015), which indicates that there is a resistance mechanism other than kdr mutation (Xu

et al. 2005; Sarkar *et al.* 2009). Thus, it must be considered an effective control of culex-borne diseases, including environmental management methods that are independent of insecticides use of larvicides (bacterial larvicides, benzoylureas, juvenile hormone mimics, organophosphates, spinosyns), especially in urban and semi-urban areas -with easy-to-reach habitats-, and the use of repellents that are applied to the skin as self-protection (World Health Organization 2022).

In conclusion, based on the detection of the Vgsc-L1014F allelic mutation using the ETAS-PCR method, ten pooled samples (C1-C10) of mosquitoes identified as male *Cx. quinquefasciatus* were not mutated or present as a homozygous wild type, with an 181 bp DNA band.

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