First molecular genotyping of voltage gated sodium channel alleles in *Culex quinquefasciatus* populations in Malaysia

V.L. Low \(^{a,*}\), C.D. Chen \(^{a}\), P.E. Lim \(^{a,b}\), H.L. Lee \(^{c}\), T.K. Tan \(^{d}\), Yvonne A.L. Lim \(^{d}\), M. Sofian-Azirun \(^{a}\)

\(^{a}\) Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia

\(^{b}\) Institute of Ocean and Earth Sciences, University of Malaya, 50603 Kuala Lumpur, Malaysia

\(^{c}\) Medical Entomology Unit, WHO Collaborating Centre for Vectors, Institute for Medical Research, Jalan Pahang, 50588 Kuala Lumpur, Malaysia

\(^{d}\) Department of Parasitology, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia

**Abstract**

A nationwide investigation was performed to detect the presence of 1014 mutation(s) in voltage gated sodium channel (*kdr*) gene of *Culex quinquefasciatus* from 14 residential areas across 13 states and a federal territory in Malaysia. Molecular genotyping of *kdr* mutation was performed via a modified three tubes allele-specific-polymerase chain reaction (AS-PCR) and direct sequencing of *kdr* gene. Based on the results of AS-PCR, homozygous susceptible (SS) genotype was found in nine out of 14 populations with 38 individuals from a total sample size of 140. Heterozygous (RS) genotype was most predominant (99 individuals) and distributed across all study sites. Homozygous resistant (RR) genotype was detected in Perak (one individual) and Selangor (two individuals). The resistance *kdr* allele frequencies ranged from 0.1 to 0.55, with the highest being detected in *Cx. quinquefasciatus* population from Selangor. This study has documented the first field-evolved instance of 1014F mutation in Malaysian mosquitoes and the findings of this study could be utilized in the implementation of strategic measures in vector control programs in Malaysia.

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1. Introduction

Globally, the evolution of multiple or cross insecticide resistance in medically and agriculturally important insect pests is a major limiting factor in the advancement of vector/pest control management [1,2]. In the last few decades, organochlorine insecticides (i.e., DDT) have been heavily used in pest control programs [2]. However, while the ultimate or progressively evolving DDT resistance in insect pests were documented, in recent decades, pyrethroid-based insecticides have been introduced as alternatives to DDT [3]. Both pyrethroids and DDT attack the voltage-gated sodium channel of insects leading to the development of knockdown resistance when there is an excessive use of either class of insecticide [4].

Knockdown resistance is not a new phenomenon and is an increasing problem in every part of the world. Knockdown resistance has been the subject of research interest among researchers for more than 50 years and intensive research efforts have unraveled the underlying mechanisms that conferred knockdown resistance at a molecular level [5]. Over the years, knockdown resistance have been extensively reported in a number of insect pests (i.e., mosquitoes, cockroaches, ticks, lice, house flies, horn flies, fruit flies, white flies, aphids, beetles, and moths), as reviewed by Soderlund and Knipple [5], Hemingway et al. [4] and Liu et al. [6].

In Malaysia, mosquitoes are important insect vectors/pests and the application of insecticides remains the main method of control in mosquito control programs [7]. Specifically, *Culex quinquefasciatus* Say is the most abundant Malaysian pest mosquito [8,9]. Insecticide resistance towards DDT and pyrethroids in Malaysian *Cx. quinquefasciatus* have been frequently reported [10–15]. However, in Malaysia, research efforts have mainly focused on the biochemical characterization of enzyme-based metabolic mechanisms [12,14]. Indeed, there is a lack of evidence of insecticide resistance conferred by mutations in the voltage gated sodium channel in Malaysian mosquitoes as well as other insect species in Malaysia.

According to our previous report [15], both WHO larval and adult bioassays revealed that Malaysian *Cx. quinquefasciatus* has developed a wide spectrum of insecticide resistance towards DDT and permethrin. In particular, DDT resistance was expressed most frequently, as 0% knockdown was recorded from 12 out of 14 of the populations [15]. In this context, it is of paramount importance to investigate the knockdown resistance at a molecular level and thereby attempting to determine the prevalence of the *kdr* mutation in *Cx. quinquefasciatus* populations from all states and a federal territory in Malaysia.

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* Corresponding author.

E-mail address: lucaslow24@gmail.com (V.L. Low).
2. Materials and methods

2.1. Ethical notes

This research was regulated by the Medical Review & Ethics Committee (MREC), Ministry of Health Malaysia. No specific permits were required for this study. This study also did not involve endangered or protected species.

2.2. Mosquito strains

The selection criteria for the study sites were based on the frequency of reports of dengue cases and fogging activities, as dengue is the most prevalent mosquito-borne viral disease in Malaysia. Specific mosquito control programs mainly target Aedes and not Culex mosquitoes. However, widespread fogging against dengue vectors would also exert selective pressure on mosquitoes. However, widespread fogging against dengue vectors would also exert selective pressure on mosquitoes. Therefore, widespread fogging against dengue vectors would also exert selective pressure on mosquitoes.

A nationwide Culex larval survey was carried out at 14 dengue endemic residential areas across 11 states and a federal territory (i.e., Kuala Lumpur) in Peninsular Malaysia and two states in East Malaysia (Fig. 1). Details of the studied study sites and sample collections have been described elsewhere [15]. Field-collected larvae were transported to the laboratory and reared to adulthood for identification using taxonomic keys [16]. In the present study, a total of 140 adults Cx. quinquefasciatus with 10 individual mosquitoes representing each of the 14 study sites were randomly selected.

2.3. DNA extraction

Prior to DNA extraction, abdomens were dissected out of the mosquito samples to avoid contamination. DNA was extracted from each specimen using i-genomic CTB DNA Extraction Mini Kit (iNtRON Biotechnology Inc., Kyungki-Do, South Korea). All isolation steps were performed according to manufacturer instructions.

2.4. Detection of kdr mutation by allele-specific (AS)-PCR method

A modified three tubes AS-PCR method [17–18] was performed to detect the presence of 1014F and 1014S alleles. Three separate PCR reactions were conducted by using the mixture of CD1 primer, 5’-AAC TTC ACC GAC TTC ATG CAC-3’ and CD2 primer, 5’-GCA GGC TAA GAA AAG GTT AAG AAC-3’ with CD3 specific primer, 5’-CCA CGG TAG TGA TAG GAA ATT TA-3’ for the TTA (Leu) mutation, CD4 specific primer, 5’-CCA CGG TAG TGA TAG GAA ATT TT-3’ for the TIT (Phe) detection or CD5 specific primer, 5’-CCA CGG TAG TGA TAG GAA ATT TC-3’ for the TCA (Ser) detection. The ratio of the primer mixture was CD1:CD2:CD3/4/5 = 3:10:7. The control product of 490-bp was amplified from primers CD1 and CD2 while the 370-bp fragment was the kdr-specific allele from primers CD3, CD4, and CD5.

The amplification of sodium channel region was performed in a final volume of 25 μL containing 25–50 ng genomic DNA of mosquito, 2 μL of ExPrime Taq Master Mix (GENETBIO Inc., Daejeon, South Korea) and 2 μL of primer mixture. PCR was carried out using a Bio-rad MyCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA). The PCR conditions included an initial denaturation of 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s (denaturation), 60 °C for 30 s (annealing), 72 °C for 45 s (extension) and a final extension at 72 °C for 10 min [18].

The amplified fragments were electrophoresed on 2% agarose gel pre-stained with SYBR Safe (Invitrogen, Carlsbad, CA) in TAE buffer.

2.5. Detection of kdr mutation by sequencing method

A subset of 40 individual samples from the 140 samples tested was screened for kdr mutation by direct sequencing. We designed new primers based on our cloned sequences (KC189872 and KC189873): JKDR_F, forward primer, 5’-GGA TCG AAT CCA TGT GGG ACT-3’ and JKDR_R, reverse primer, 5’-TGC ACC TTT AGG TGT GGA CCT CTC-3’.

The amplification of sodium channel region was performed in a final volume of 50 μL containing 5 μL 10× buffer, 2.5 mM of each dNTP, 10 pmoL of each forward and reverse primer, 1.5 U Taq polymerase (iNtRON Biotechnology Inc., Kyungki-Do, South Korea), and 25–50 ng genomic DNA of mosquito. PCR was carried out using Bio-Rad MyCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA). The PCR conditions included an initial denaturation of 94 °C for 5 min, followed by 40 cycles of 94 °C for 45 s (denaturation), 59 °C for 45 s (annealing), 72 °C for 45 s (extension) and a final extension at 72 °C for 5 min.

The amplified fragments (~285 bp) were electrophoresed on 2% agarose gel pre-stained with SYBR Safe™ (Invitrogen, Carlsbad, CA) in TAE buffer. The PCR products were purified with MEGAquick-spin PCR & Agarose Gel DNA Extraction System (iNtRON Biotechnology Inc., Kyungki-Do, South Korea).

The purified PCR products were sent to a commercial company for DNA sequencing in both directions. Samples were sequenced using BigDye™ Terminator v3.1 Sequencing Cycle Kit (Applied Biosystems, Foster City, CA) and analyzed on an ABI PRISM 377 genetic analyzer (Applied Biosystems, Foster City, CA).

Sequencing data were analyzed and edited using ChromasPro 1.5 (Technelysium Pty Ltd., Qld, Australia) and BioEdit 7.0.9.0. [19]. The sodium channel sequences were preliminarily aligned using the CLUSTAL X program [20] and subsequently aligned manually. Representative sequences of the sodium channel gene of Cx. quinquefasciatus in this study were deposited in GenBank under the accession numbers KC189872–KC189889.

2.6. Statistical analysis

The frequencies of kdr allele were determined by Hardy–Weinberg Equilibrium, using GenePOP (ver 3.4) software [21].

3. Results

The AS-PCR method demonstrated the presence of the classical 1014F mutation in all of the wild populations of Cx. quinquefasciatus tested, while the 1014S mutation was not detected across all study sites in Malaysia (Fig. 1 and Table 1). Overall, the SS genotype was found in a majority of the study sites (nine out of 14) with 38 individuals from a total sample size of 140. The RS genotype was detected across all study sites and was most predominant with 99 individuals from a total sample size of 140. Of 14 populations, two populations (i.e., Perak and Selangor) indicated the presence RR genotype with three individuals. It is of interest that the SS genotype was not detected in five populations (i.e., Kuala Lumpur, Malacca, Negeri Sembilan, Penang and Perlis).

The genotype frequencies at kdr locus from seven populations (i.e., Johore, Kedah, Kelantan, Sabah, Sarawak, Selangor and Terengganu) conformed to the Hardy–Weinberg expectations at the 95% confidence level (P > 0.05). Inversely, the genotype frequencies at kdr locus from another seven populations (i.e., Kuala Lumpur, Malacca, Negeri Sembilan, Penang, Perak and Perlis) differed...
significantly ($P \leq 0.05$). The resistance kdr allele frequencies ranged from 0.1 to 0.55, with the highest being detected in Cx. quinquefasciatus population from Selangor (Table 1).

The results of DNA sequencing of 40 individual samples revealed only the presence of the 1014F mutation, while no other mutations were detected. Of these 40 individual samples, 24 were assigned as SS genotype, 13 as RS genotype and three as RR genotype. However, the results of DNA sequencing were not in complete agreement with AS-PCR method (Table 2). Of 40 samples, three individuals were assigned as SS genotype, but not RS genotype, which was contrasted with the AS-PCR results.

4. Discussion

The distribution of 1014 mutation(s) in Cx. quinquefasciatus, at varying frequencies has been reported worldwide [18,22–25]. In the current study, the classical knockdown resistance, 1014F mutation at varying frequencies was detected from all populations, while the 1014S mutation and other mutations reported previously were not detected in Malaysian Cx. quinquefasciatus. It has been documented that mosquitoes with 1014F mutation contributed high levels of resistance against both DDT and pyrethroïds, while the 1014S mutation contributed high levels of resistance against DDT but low levels of resistance against pyrethroids [17]. Based on our previous report, the Malaysian Cx. quinquefasciatus populations displayed high levels of resistance against DDT but relatively low levels of resistance (or susceptible) against permethrin [15].

We propose that the widespread 1014F mutation occurred in Malaysian Cx. quinquefasciatus has resulted in the development of high DDT resistance. Likewise, a recent study also indicated that Indian Cx. quinquefasciatus with 1014F mutation demonstrated high DDT resistance but was susceptible against deltamethrin.

Fig. 1. Genotype distribution of kdr gene in Culex quinquefasciatus across all study sites in Malaysia. *Tmn. = Taman, Kg. = Kampung.
endemic areas. The pressure on activities for dengue vectors control has also exerted selective pressure since 1996 [13]. Consequently, the intense permethrin fogging has been the preferred option areas in Selangor have been persistently reported to the Ministry of Health, Malaysia. In the present study, it was observed that there was an excess of RS genotype recorded in most of the populations (i.e., Perlis, Penang, Kuala Lumpur, Negeri Sembilan and Malacca), was an excess of RS genotype recorded in most of the populations (i.e., Perlis, Penang, Kuala Lumpur, Negeri Sembilan and Malacca), probably due to the elimination of RR genotype in fitness cost (i.e., Perlis, Penang, Kuala Lumpur, Negeri Sembilan and Malacca), was an excess of RS genotype recorded in most of the populations (i.e., Perlis, Penang, Kuala Lumpur, Negeri Sembilan and Malacca), probably due to the elimination of RR genotype in fitness cost (i.e., Perlis, Penang, Kuala Lumpur, Negeri Sembilan and Malacca), probably due to the elimination of RR genotype in fitness cost (i.e., Perlis, Penang, Kuala Lumpur, Negeri Sembilan and Malacca), probably due to the elimination of RR genotype in fitness cost (i.e., Perlis, Penang, Kuala Lumpur, Negeri Sembilan and Malacca), probably due to the elimination of 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