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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

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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 resistance (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.

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.

* Corresponding author.
E-mail address: lucaslow24@gmail.com (V.L. Low).

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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 *Cx. quinquefasciatus*, as the fogged insecticides, mainly pyrethroids would inadvertently contaminate *Cx. quinquefasciatus* breeding grounds such as polluted drains.

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′-CAA GGC TAA GAA AAG GTT AAG AAC-3′ with CD3 specific primer, 5′-CCA CCG TAG TGA TAG GAA ATT TA-3′ for the TTA (Leu) detection, CD4 specific primer, 5′-CCA CCG TAG TGA TAG GAA ATT TT-3′ for the TTT (Phe) detection or CD5 specific primer, 5′-CCA CCG TAG TGA TAG GAA ATT C-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, 12 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 TC-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 BigDyeH 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, Pahang, Penang, Perak and Perlis) differed

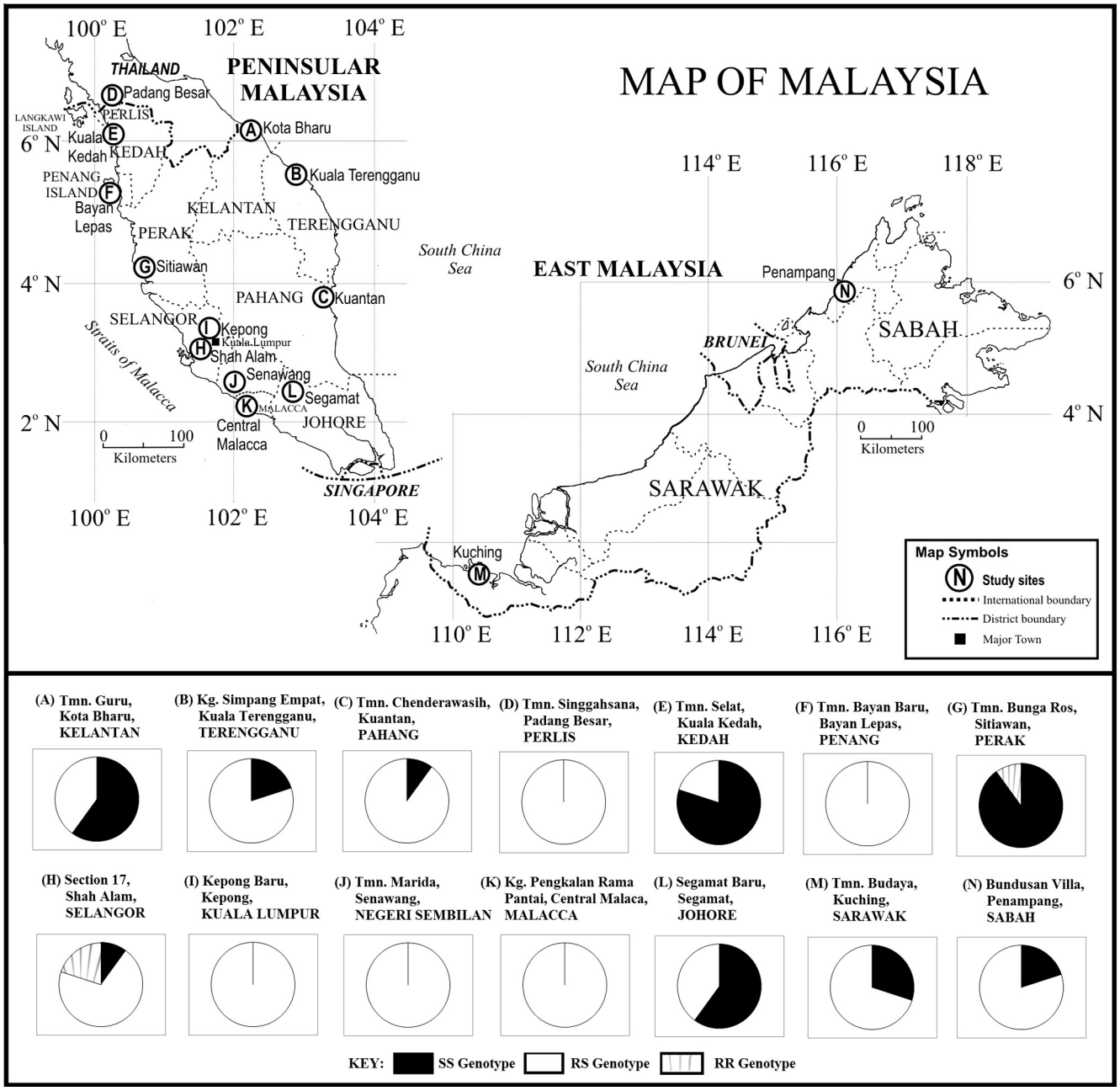

Fig. 1. Genotype distribution of *kdr* gene in *Culex quinquefasciatus* across all study sites in Malaysia. *Tmn. = Taman, Kg. = Kampung.

significantly ($P \leqslant 0.05$). 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 pyrethroids, 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

Table 1
Genotypes and frequency of *kdr* alleles in Malaysian *Culex quinquefasciatus*.

Localities		Genotype			Allele Frequency		HW
	n	SS	RS	RR	S	R	(*P*-value)*
Kelantan	10	6	4	0	0.80	0.20	1.00
Terengganu	10	2	8	0	0.60	0.40	0.17
Pahang	10	1	9	0	0.55	0.45	0.05
Perlis	10	0	10	0	0.50	0.50	0.01
Kedah	10	8	2	0	0.90	0.10	1.00
Penang	10	0	10	0	0.50	0.50	0.01
Perak	10	9	0	1	0.90	0.10	0.05
Selangor	10	1	7	2	0.45	0.55	0.52
Kuala Lumpur	10	0	10	0	0.50	0.50	0.01
Negeri Sembilan	10	0	10	0	0.50	0.50	0.01
Malacca	10	0	10	0	0.50	0.50	0.01
Johore	10	6	4	0	0.80	0.20	1.00
Sarawak	10	3	7	0	0.65	0.35	0.22
Sabah	10	2	8	0	0.60	0.40	0.17
Total	140	38	99	3	0.63	0.37	0.00

HW = Hardy–Weinberg test.
* The exact probability for rejecting Hardy–Weinberg equilibrium.

Table 2
kdr Genotypes detected by both AS-PCR and sequencing methods.

N	AS-PCR			Sequencing		
	TTA (SS)	TTA/T (RS)	TTT (RR)	TTA (SS)	TTA/T (RS)	TTT (RR)
40	21	16	3	24	13	3

[23]. However, this study does not exclude the involvement of metabolic mechanisms which can occur in the same populations, as observed by Djouaka et al. [26].

The present study reported the highest frequency of resistance *kdr* allele in Selangor population. One plausible explanation for this incidence could be permethrin and DDT resistance phenotypes evolved in this population, where the highest resistance ratio (3.81 folds) and low mortality rate (6.67%) were observed in our previous larval and adult bioassays, respectively [15]. In fact, a large number of dengue and chikungunya cases from the residential areas in Selangor have been persistently reported to the Ministry of Health, Malaysia. To control the outbreak of dengue and chikungunya fevers, permethrin fogging has been the preferred option since 1996 [13]. Consequently, the intense permethrin fogging activities for dengue vectors control has also exerted selective pressure on *Cx. quinquefasciatus* in these dengue and chikungunya endemic areas.

The findings of this study also indicated that RS genotype of 1014F mutation was the most predominant genotype and was well dispersed across majority of the study sites. A RR genotype was detected from two of 14 locations (i.e., Perak and Selangor). It has been reported that the absence of RR genotype in a population might alter the metabolic and developmental processes and consequently reduce its fitness-enhancing traits [27]. A previous study showed that high fitness cost has contributed to the rapid declination of the RR genotype after a few generations of insecticide-free conditions [28]. 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), probably due to the elimination of RR genotype in fitness cost evolution.

Attempts to determine the relationship between the frequency of *kdr* resistance allele with the insecticide susceptibility status in both larval and adult stages were made [15], but no association was found in either stage with regards to DDT and permethrin. Previous studies elsewhere have reported different relationship between the frequencies of the *kdr* resistant allele and the DDT and pyrethroids resistance phenotype. The insecticide resistance phenotype in several species of mosquito, house flies as well as the cockroach has been found to be correlated with the frequencies of *kdr* resistant allele [18,22,29–31]. Inversely, a number of studies also reported that no association was found between the *kdr* mutation and insecticide resistance phenotype in other insect species [23,32–34]. Given the lack of this association, it is possible that other detoxification mechanisms such as glutathione S-transferases and P450 monooxygenases could also be involved in DDT and pyrethroids resistance, respectively [4].

There have been many arguments about the accuracy of both PCR and sequencing methods for the detection of heterozygosity in an individual sample [35]. In the present study, we found that the results of DNA sequencing were not in agreement with the AS-PCR method. Similarly, previous studies also reported the incongruence results in both sequencing and AS-PCR methods [23,35].

We acknowledge that the estimation of single nucleotide polymorphism allele frequency could not be conclusively identified in the present study due to limited sample size. Therefore, for future study, additional sampling efforts with increased sample size from wider biogeographical areas should be carried out to provide a better understanding on the course of evolution in Malaysian *Cx. quinquefasciatus*. Nevertheless, the present study has demonstrated the first appearance of this widespread 1014F allele in Malaysian *Cx. quinquefasciatus* and documented the first field-evolved instance of knockdown resistance in insect species in Malaysia. This alarming case in the history of knockdown resistance development would pose a great challenge to both local authorities and researchers in the advancement of vector control management. It is possible that more than one resistance mechanism could confer DDT and permethrin resistance in these populations. Hence, the biochemical characterization of metabolic mechanism is currently in progress to unravel the actual mechanism(s) that contribute to the evolution of insecticide resistance.

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