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Original Article

First Detection of a Putative Knockdown Resistance Gene in Major Mosquito Vector, *Aedes albopictus*

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SUMMARY: The Asian tiger mosquito, *Aedes albopictus* (Skuse), is the major vector of Chikungunya fever and the secondary vector of dengue fever. We collected *Ae. albopictus* from Singapore and performed genotyping assay to detect mutations of the voltage-gated sodium channel, which is the target site of pyrethroid insecticides. We detected an amino acid substitution, F1534C, which is suspected to confer knockdown resistance (*kdr*) to pyrethroid insecticides. Of the collected mosquitoes, 53.8% were homozygous for this mutation, and the allele frequency of this mutation was estimated to be 73.1%. No *kdr* mutation was detected in the 5 other loci of domains II and IV. This is the first evidence for the presence of the *kdr* gene in *Ae. albopictus*, and our findings highlight the need for studying the global distribution of this allele in this important vector insect.

INTRODUCTION

Chikungunya fever is a re-emerging arthropod-borne disease caused by Chikungunya virus (CHIKV), which is an Alphavirus belonging to the Togaviridae family (1). The symptoms of Chikungunya fever are similar to those of dengue fever and include rash, sudden-onset high fever and joint pain, and occasionally, hemorrhage. More than a million cases of Chikungunya fever were reported during a recent outbreak, between 2005 and 2006, in the countries around the Indian Ocean (2). By June 2006, Reunion Island reported more than 266,000 estimated cases, which was equivalent to approximately one-third of the entire population of the island. The estimated number of deaths during the epidemic period in Reunion Island was 203, and the mortality was approximately 0.076% (3). Officially, 1.39 million cases of Chikungunya fever were reported from India during 2006 (2).

CHIKV is transmitted to humans by mosquitoes of the genus *Aedes*, particularly the yellow fever mosquito, *Aedes aegypti* (Linnaeus), which is a primary vector insect in Asia (4). However, the CHIKV strain responsible for the recent outbreak has amino acid substitution in the E1 envelope glycoprotein (A226V), whereby its transmissibility is increased (>100-fold) for another vector species, *Aedes albopictus* (5,6). It has been also reported that CHIKV orally infected by *Ae. albopictus* is rapidly delivered to the salivary glands of the mosquito and that the virus can be detected in the mosquito's saliva within 2 days post infection (7). Consequently, *Ae. albopictus* has attracted considerable attention as a major vector for CHIKV transmission (8).

Ae. albopictus was incriminated as the vector of CHIKV during the 2005 epidemic Reunion Island. Subsequent reports from India, Mayotte, Sri Lanka, Malaysia, Singapore, and Thailand reiterated the role of *Ae. albopictus* in the Chikungunya pandemic, which continues to persist in Southeast Asia (9–11). *Ae. albopictus* has also been implicated in dengue outbreaks in Hawaii, China, Seychelles, Mauritius, and more recently, France, where *Ae. aegypti* was absent or had limited presence (12–16). Although *Ae. albopictus* is not as efficient as *Ae. aegypti* in transmitting dengue, its potential role in transmission during a dengue epidemic cannot be ignored.

Ae. albopictus is distributed worldwide. Although native to Southeast Asia, Western Pacific islands, and islands of the Indian Ocean, it has spread East Asia, Pacific islands, Africa, the Middle East, Europe, and the Americas in the last few decades of the 20th century (12,17–19). In Japan, *Ae. albopictus* has been found to annually migrate to the northern areas due to global warming in the last few years (20). It develops more readily in rural and suburban areas, where it breeds in natural habitats and artificial containers. It has been reported that *Ae. albopictus* has displaced other mosquito species in some places and often competes with *Ae. aegypti* in the urban environment (12,21). In Singapore, which maintains a green urban environment and is therefore known as a garden city, *Ae. albopictus* is ubiquitous. Alhough this species has been frequently found in close association with greenery, it has also been found to breed in artificial containers along with *Ae. aegypti*.

Successful control of the vector mosquitoes is the key strategy for the prevention and control of epidemics, and the successful implementation of this strategy de-

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pends heavily on the use of insecticides, particularly pyrethroids, which constitute a major class of insecticides employed in the control of adult mosquitoes worldwide. However, continuous use of these chemicals has resulted in the development of resistance in some mosquito species, as observed in the case of other agricultural pest insects (22). The understanding of insecticide resistance, which is essential for the development of appropriate vector control programs, can be improved by genotyping of the key gene(s) responsible for conferring this resistance. The loss of sensitivity of the active site of the protein targeted by pyrethroids (i.e., the voltage-gated sodium channel [VGSC]), known as knockdown resistance (*kdr*), is one of the major mechanisms of pyrethroid resistance. Several point mutations responsible for the *kdr* phenotype have been reported in the *Ae. aegypti* VGSC gene, while so far no *kdr* gene has been reported for *Ae. albopictus*. Because of its wider range of natural habitats, including tree holes, rock pools, and bamboo stumps, in addition to outdoor artificial containers, *Ae. albopictus* is considered to be exposed to insecticides to lesser extent than *Ae. aegypti*, which is more closely associated with human habitation; therefore, *Ae. albopictus* is thought to be less likely to develop insecticide resistance (2). However, in Singapore, *Ae. aegypti* and *Ae. albopictus* share the same urban habitat, where a well-established mosquito control program is being implemented since 5 decades. Therefore, *Ae. albopictus* in Singapore should have considerable exposure to insecticides. In this study, we report a *kdr* mutation in the VGSC gene of *Ae. albopictus* collected from Singapore, which is the first evidence for the presence of this resistance mechanism in this species.

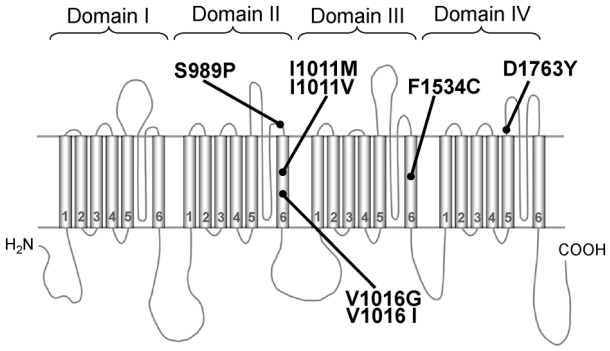

Fig. 1. Diagram of the locations of possible *kdr* mutations found in *Aedes aegypti*. Point mutations in the voltage-gated sodium channel protein so far reported from pyrethroid-resistant *Ae. aegypti* are indicated. Positions are numbered according to the amino acid sequence of the most abundant splice variant of the house fly sodium channel (GenBank accession nos. AAB47605 and AAB47604).

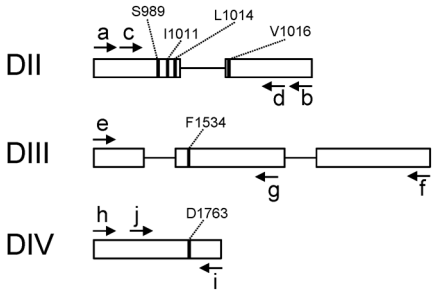

Fig. 2. Strategies for genotyping the *kdr* mutations. The partial genomic DNA encoding voltage-gated sodium channel (VGSC) in *Ae. albopictus* and primer positions are indicated. The open boxes and black lines indicate exons and introns, respectively. The black boxes indicate 6 loci of possible *kdr* mutations. Primers a (aegSCF20), b (aegSCR21), e (aegSCF7), f (aegSCR7), h (albSCF6), and i (albSCR8) were used to amplify VGSC gene and primers c (aegSCF3), d (aegSCR22), g (aegSCR8), and j (albSCF7) were used for sequencing. The sequence of each primer is described in Materials and Methods. The lengths of amplified DNA were approximately 480 bp, 740 bp, and 280 bp for domain II, III, and IV, respectively.

MATERIALS AND METHODS

Mosquitoes: Adults (24 males and 2 females) of *Ae. albopictus* were collected in the Jalan Bukit Merah area, Singapore, in March 2009, by the sweeping method. Jalan Bukit Merah is a typical residential area with high-rise apartment buildings surrounded by greenery.

Genotyping of the VGSC gene: Genomic DNA for PCR templates was prepared from individual mosquitoes using a REDExtract-N-Amp Tissue PCR kit (Sigma-Aldrich, St. Louis, Mo., USA). We targeted 6 amino acid loci to identify the candidate(s) of *kdr*: the most typical *kdr* substitution, L1014F (22), and 5 amino acid loci in VGSC associated with pyrethroid resistance in *Ae. aegypti*, namely S989P (23), I1011M or V (24), V1016G or I (24), F1534C (25,26), and D1763Y (27) (Fig. 1). Several splice variants are known to exist in the sodium channel cDNA; therefore, the positions of the amino acids may vary among the reported cDNA. Therefore, although a full-length sodium channel cDNA has been identified in *Ae. albopictus*, we numbered the amino acid position in our study according to the sequence of the most abundant splice variant of the house fly sodium channel (GenBank accession nos. AAB47605 and AAB47604). Partial DNA fragments of domains II, III, and IV were amplified by PCR using TaKaRa Ex Taq Hot Start Version (Takara Bio, Shiga, Japan) and 3 primer sets (Fig. 2): aegSCF20 (gacaatgtg gatcgcttccc) and aegSCR21 (gcaatctggcttgttaacttg) for domain II, aegSCF7 (gagaactcgccgatgaactt) and aegSCR7 (gacgacgaaatcgaacaggt) for domain III, and albSCF6 (tcgagaagtacttcgtgtcg) and albSCR8 (aacag caggatcatgctctg) for domain IV. The PCR products were purified using the MonoFas DNA purification kit I (GL Sciences Inc., Tokyo, Japan) and directly sequenced with the primers: aegSCF3 (gtggaacttcaccgactt ca) (forward primer for domain II), aegSCR22 (ttcac gaacttgagcgcgttg) (reverse primer for domain II), aegSCR8 (tagctttcagcggcttcttc) (reverse primer for domain III), and albSCF7 (aggtatccgaacgttgctgt) (forward primer for domain IV), using the ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, Calif., USA).

RESULTS AND DISCUSSION

We collected 26 adult mosquitoes (24 males and 2 females) of *Ae. albopictus* and genotyped 6 loci of VGSC, which may cause pyrethroid resistance. The F1534C mutation (TTC to TGC) at segment 6 of domain III was

Table 1. Genotypes of the voltage-gated sodium channel gene in *Ae. albopictus* collected from Singapore

	Loci of amino acids[1)]					
Aedes albopictus	953	975	978	980	1474	1703
Aedes aegypti	996	1018	1021	1023	1565	1794
Musca domestica	989	1011	1014	1016	1534	1763
#1	S/S	I/I	L/L	V/V	F/C	D/D
#2	S/S	I/I	L/L	V/V	C/C	D/D
#3	S/S	I/I	L/L	V/V	C/C	D/D
#4	S/S	I/I	L/L	V/V	C/C	D/D
#5	S/S	I/I	L/L	V/V	C/C	D/D
#6	S/S	I/I	L/L	V/V	C/C	D/D
#7	S/S	I/I	L/L	V/V	F/C	D/D
#8	S/S	I/I	L/L	V/V	C/C	D/D
#9	S/S	I/I	L/L	V/V	F/C	D/D
#10	S/S	I/I	L/L	V/V	C/C	D/D
#11	S/S	I/I	L/L	V/V	C/C	D/D
#12	S/S	I/I	L/L	V/V	C/C	D/D
#13	S/S	I/I	L/L	V/V	F/C	D/D
#14	S/S	I/I	L/L	V/V	F/C	D/D
#15	S/S	I/I	L/L	V/V	F/C	D/D
#16	S/S	I/I	L/L	V/V	C/C	D/D
#17	S/S	I/I	L/L	V/V	F/C	D/D
#18	S/S	I/I	L/L	V/V	F/C	D/D
#19	S/S	I/I	L/L	V/V	C/C	D/D
#20	S/S	I/I	L/L	V/V	C/C	D/D
#21	S/S	I/I	L/L	V/V	F/C	D/D
#22	S/S	I/I	L/L	V/V	F/F	D/D
#23	S/S	I/I	L/L	V/V	F/C	D/D
#24	S/S	I/I	L/L	V/V	C/C	D/D
#25	S/S	I/I	L/L	V/V	C/C	D/D
#26	S/S	I/I	L/L	V/V	F/F	D/D

[1)]: Positions are numbered according to the amino acid sequence of the voltage-gated sodium channel from the Asian tiger mosquito (*Aedes albopictus*, accession no. AY663384), Yellow fever mosquito (*Aedes aegypti*, accession no. ACB37024), and house fly (*Musca domestica*, accession nos. AAB47605 and AAB47604). Twenty-four adult males (#1–24) and 2 females (# 25 and 26) were used.

detected at a high frequency (Table 1, Fig. 3). On the other hand, no *kdr* mutation was detected at the other 5 loci of domains II and IV (Table 1). Of the 26 mosquitoes tested, 24 (92.3%) exhibited the F1534C mutation, and only 2 mosquitoes were homozygous for the F1534 wild-type allele. The frequency of the C1534 allele was 73.1% (38/52).

Pyrethroid insecticides can be classified into types I and II, according to the absence or presence of the α-cyano group in the alcohol moiety (28). Harris et al. reported that *Ae. aegypti* collected from the Cayman Islands of the Caribbean region showed a high level of resistance to the type I pyrethroid permethrin. Genotyping studies revealed that this population exhibited F1534C mutation at a high frequency (29). Additional studies clearly confirmed that F1534C mutation was strongly correlated with resistance to permethrin (29). More recently, an electrophysiological study demonstrated that the VGSC protein with F1534C mutation has reduced sensitivity to type I, but not type II, pyrethroids (30). In fact, the *Ae. aegypti* mosquitoes collected from Cayman Islands showed a remarkably higher resistance to permethrin than the 2 other type II pyrethroids, deltamethrin and lambda-cyhalothrin; this finding is consistent with the results of abovementioned electrophysiological study (29). Further, since the inheritance of the C1534 gene is recessive (29), heterozygous carriers of C1534 are expected to be phenotypically susceptible to type I pyrethroids. In this study, 53.8% of *Ae. albopictus* (14/26) were homozygous for F1534C mutation, which indicates that nearly half of the *Ae. albopictus* population at the collection site may be insensitive to type I pyrethroids.

In Singapore, fogging with permethrin, which is classified as a type I pyrethroid, was performed as part of the dengue control program from the 1980s to 1991; permethrin-resistant *Ae. aegypti* were detected during this period (31). Furthermore, an assortment of

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          D  H  V  G  K  A  Y  L  C  L  F  Q  V  A  T  F  K  G  W  I   1500
F1534     CGACCACGTGGGGAAGGCGTACCTGTGTCTGTTCCAGGTGGCAACGTTCAAGGGCTGGAT
C1534     ************************************************************

          Q  I  M  N  D  A  I  D  S  R  E                             1511
F1534     CCAGATCATGAACGATGCCATCGACTCGCGGGAGgtaagttctggatcttcgatcatcat
C1534     ***************C**************A**********t**a***c****a******

                                                 V  G  K  Q  P  I  1517
F1534      tccgaatgaacactaatcgatcgtttcccttgaacctttcacagGTGGGCAAGCAGCCGA
C1534      *******a*************************t*c*ct********************

            R  E  T  N  I  Y  M  Y  L  Y  F  V  F  F  I  I  F  G  S  F  1537
F1534     TTCGCGAGACCAACATCTACATGTACCTCTACTTCGTGTTCTTCATCATCTTCGGGTCGT
C1534     *C*********************************T****************G********
                                                              C

            F  T  L  N  L  F  I  G  V  I  I  D  N  F  N  E  Q  K  K  K  1557
F1534     TCTTCACCCTCAACCTGTTCATCGGTGTCATCATCGACAACTTCAACGAGCAGAAGAAGA
C1534     ************************************************************

            A  G  G  S  L  E  M  F  M  T  E  D  Q  K  K  Y  Y  N  A     1577
F1534     AAGCCGGTGGCTCGCTGGAAATGTTCATGACGGAGGATCAGAAAAAGTACTACAACGCA
C1534     ************************************************************
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Fig. 3. Deduced nucleotide and amino acid sequence around F1534C mutation. The partial sequences of the voltage-gated of sodium channel gene (domain III) of *Ae. albopictus* collected from Singapore are indicated. F1534 (type S) and C1534 (type R) indicate the sequence of wild type (i.e., pyrethroid susceptible) and F1534C mutated haplotypes, respectively. The black blocks indicate segment 6 of domain III. Asterisks indicate that they share the common nucleotides with type S. The lowercase letters indicate intron. The position of the amino acid substitution, F1534C, is also shown.

pyrethroids is being regularly used by pest control companies under private contracts. Widespread use of pyrethroids in this manner probably led to the local selection of the C1534 allele in *Ae. albopictus*, which coexists with *Ae. aegypti* and *Culex* in urban Singapore. Further application of type I pyrethroids will result in the fixation of the C1534 allele, which will considerably reduce the efficacy of the control of *Ae. albopictus* with this type of insecticide. This is the first report on a *kdr* mutation in *Ae. albopictus* and highlights the need to further study the global distribution of the C1534 allele in this important vector species.

kdr mutations have been detected in many pest insects, and the current study shows that *Ae. albopictus* is not an exception. The survey of the distribution of the F1534C mutation in epidemic areas of Chikungunya fever is imperative for the development of appropriate vector control strategies. The efficacy of vector control strategies may be improved by replacing previously used insecticides by newer ones determined on the basis of genotyping studies of VGSC, thereby prolonging the lifespan of active ingredients. During the last 3 decades, *Ae. albopictus* has invaded the Americas, African regions, and more than 13 European countries (2,32–35), and therefore, researchers investigating vector control in these countries need to pay more attention to the spread of *Ae. albopictus* with the *kdr* mutation reported in this paper. Additionally, we need to monitor metabolism-based insecticide resistance in *Ae. albopictus* (36). We are currently evaluating the effect of the C1534 VGSC allele on the resistance of *Ae. albopictus* against a wide range of insecticides.

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Conflict of interest None to declare.

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