The F1534C voltage-sensitive sodium channel mutation confers 7- to 16-fold resistance to pyrethroid insecticides in Aedes aegypti

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Abstract

BACKGROUND: Recent outbreaks of dengue and Zika have emphasized the importance to effectively control Aedes aegypti, which vectors the viruses causing these diseases. Pyrethroid insecticides are primarily used to control adult A. aegypti, especially during disease outbreaks. However, pyrethroid resistance in A. aegypti is an increasing problem. Mutations in the voltage-sensitive sodium channel (Vssc) are a common mechanism of pyrethroid resistance. The F1534C mutation is common and distributed globally in A. aegypti populations, but previous studies disagree about the role of this mutation in conferring resistance to pyrethroid insecticides.

RESULTS: We isolated a congenic strain (1534C:ROCK) which was closely related to a susceptible strain Rockefeller (ROCK), but was homozygous for the 1534C Vssc allele. We determined resistance levels against eight insecticides that target the VSSC: six pyrethroids, DDT and DCJW (the bioactivated metabolite of indoxacarb). The resistance levels ranged from 7- to 16-fold, and resistance was inherited as an incompletely recessive trait. We also found a novel 367I+1520I+1534C allele, in mosquitoes from Thailand. The T1520I mutation did not increase pyrethroid resistance beyond what was conferred by the F1534C mutation alone.

CONCLUSION: The F1534C Vssc mutation is common in A. aegypti populations and confers 7- to 16-fold resistance to pyrethroids, DDT, and DCJW in Aedes aegypti. These resistance levels are considerably less than previously reported for the S989P+V1016G mutations. Our results provide useful information for resistance management, specifically the levels of resistance conferred by the most common Vssc mutation in A. aegypti.

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Keywords: Insecticide resistance; Vssc; F1534C; pyrethroids; DDT

1 INTRODUCTION

Aedes aegypti (L), an anthropophilic mosquito, is a major vector of dengue, yellow fever, chikungunya, and Zika, which are four major prevalent arboviruses causing human suffering.\textsuperscript{1,2} Accelerating globalization of trade and travel, climate change and urbanization are expanding the distribution of A. aegypti, making it a more serious risk to human health.\textsuperscript{3} Pyrethroid insecticides have been commonly used to manage adult A. aegypti. However, the extensive use of pyrethroids for the last three decades has caused the evolution of resistance and reduced efficacy of current insecticide-based control strategies.\textsuperscript{4,5} The voltage sensitive sodium channel (VSSC) is the target site of pyrethroid insecticides. There is a single copy of Vssc in most insect species,\textsuperscript{6} and mutations in the Vssc, collectively referred to as knockdown resistance (kdr), are a major mechanism of pyrethroid resistance in A. aegypti.\textsuperscript{7-9}

To date, at least 11 Vssc mutations have been identified, individually or in combination, in A. aegypti populations. Alleles that have been identified are 410L, 982W, 1011V/M, 1016I/G, 1534C, 410L+1016I, 410L+1534C, 410L+1016I+1534C, 923V+1011M, 989P+1016G, 989P+1016G+1534C, 1011M+1016G, 1016I+1534C, 1016G+1534C, 1016G+1763Y and 1520I+1534C.\textsuperscript{8,10-13} Amino acid positions of these mutations are numbered based on the house fly VSSC (Genbank accession number: AAB47604). Eleven of these mutations have been evaluated electrophysiologically by heterologous expression of Vssc in Xenopus oocytes.\textsuperscript{14-17} Nine mutations or combinations of mutations (V410L, V1016G, S989P+V1016G, S989P+V1016G+F1534C, V1016G+D1763Y, V1016I+F1534C, I1011M, F1534C and T1520I+F1534C) decrease VSSC sensitivity against pyrethroids and another two (I1011V and V1016I) do not change VSSC sensitivity to the pyrethroids (Table 1). Unfortunately, the levels of resistance conferred by these different alleles are unknown, except for the 989P+1016G allele which confers 21- to 107-fold resistance to pyrethroids, >2000-fold resistance to DDT and 13.4-fold resistance to the oxadiazine insecticide DCJW (the bioactive metabolite of indoxacarb).\textsuperscript{20}

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Table 1. Summary of the Vssc mutations found in *Aedes aegypti* that have been examined via heterologous expression and electrophysiological studies for sensitivity to pyrethroids

| Allele          | Sensitivity of the VSSC to: | References |
|-----------------|-----------------------------|------------|
|                 | Permethrin                  | Deltamethrin | References |
| V410L           | ↓                           | ↓           | 15          |
| V1016G          | ↓                           | ↓           | 14          |
| S989P+V1016G    | ↓                           | ↓           | 14          |
| S989P+V1016G+F1534C | ↓                           | ↓           | 14          |
| V1016G+D1763Y   | ↓                           | ↓           | 14          |
| V1016+F1534C    | ↓                           | ↓           | 14          |
| I1011M          | ↓                           | 0           | 14          |
| F1534C          | ↓                           | 0           | 14          |
| T1520+F1534C    | ↓                           | 0           | 17          |
| I1011V          | 0                           | 0           | 17          |
| V1016I          | 0                           | 0           | 17          |
| S989P††         | 0                           | 0           | 17          |
| T1520††         | 0                           | 0           | 17          |
| D1763Y††        | 0                           | 0           | 14          |

† and 0 indicate that sensitivity of the VSSC to the pyrethroid is decreased or unchanged, respectively.

†This mutation has been studied in oocytes, but is only found to co-occur with other mutations in field populations of *A. aegypti*.6,18,19

The V410L, S989P, V1016G/I and F1534C mutations are common, but vary geographically in *A. aegypti*.4,13 V410L has been detected in the Americas, and V1016I has been detected only in Asia.4,13,24,25 V1016G mutations have been found in Asia4,13,24,25 and the Americas.10,13 F1534C is widely distributed nearly every pyrethroid resistant *A. aegypti* population has alleles with the F1534C mutation, and in some resistant populations the F1534 allele is undetectable.13,26,27 However previous studies disagree about the role of F1534C mutation in conferring resistance to different pyrethroid insecticides. These previous studies have used two experimental approaches. The first approach involves electrophysiological analysis of VSSCs expressed in oocytes and indicates that F1534C decreases the sensitivity to permethrin, but not to deltamethrin.14,16 The second approach involves comparison of the frequencies of F1534C in dead (susceptible) vs. alive (resistant) mosquitoes from field populations after exposure to a single concentration of a pyrethroid. This approach has generated inconsistent results. Specifically, some studies found the F1534C mutation was correlated with permethrin26 and deltamethrin28 resistance. In contrast, some findings showed that the F1534C mutation was not associated with permethrin18,29 or deltamethrin20 resistance. We undertook the current studies to clarify these results.

Characterizing the role of the F1534C mutation in pyrethroid resistance is optimally studied in a strain having this mutation, but no other resistance mechanisms. Therefore, in this study, we isolated a congenic strain which was closely related to a susceptible strain, but contained the F1534C Vssc mutation, and determined resistance levels against eight insecticides that target the VSSC: six pyrethroids, DDT and DCJW. The levels of resistance in different hybrids also were examined. This information will aid in our understanding of the pyrethroid resistance evolution and inform resistance management practices in this important disease vector.

2 MATERIALS AND METHODS

2.1 Mosquitoes

One susceptible strain (Rockefeller, ROCK) and two pyrethroid resistant strains (BKK and UBN) were used in this study. The insecticide-susceptible ROCK strain has been reared in laboratories without exposure to insecticides for decades. BKK and UBN were originally collected from Bangkok and Ubon Ratchathani, Thailand in 2018, respectively.26,13 These strains were pooled to obtain enough mosquitoes and chosen because they had a high frequency (>0.8) of the F1534C mutation.

We first isolated a strain that was homozygous for 1534C (Thai1534C strain), so that full length cDNA sequences could be determined and all non-synonymous mutations identified. The procedure used for isolating the Thai1534C strain is illustrated in Fig. 1. In short, F1534C homozygotes from BKK and UBN males (see Vssc genotyping) were crossed en masse with unmated ROCK females, and F1 males and females were crossed. The F2 males and unmated females were genotyped, and individuals that were homozygous for 1534C were reared en masse. The resultant strain was named Thai1534C.

In order to isolate a strain that was congenic to ROCK, and had the desired 1534C Vssc allele, the following procedures were used. BKK and UBN adult males, which were homozygous for F1534C (see Vssc genotyping), were crossed en masse with unmated ROCK females. F1 males were backcrossed with unmated ROCK females and BC1 males were genotyped for the presence of the F1534C mutation. BC1 males that were heterozygous for F1534C were backcrossed to unmated ROCK females. This process was repeated for the BC2 and BC3 generations. At BC6 both males and unmated females were genotyped and individuals that were heterozygous for F1534C/1534C were reared en masse. Males and unmated females from the next generation were genotyped and individuals that were homozyous for 1534C were reared
en masse. In addition to 1534C homozygotes, these mosquitoes were found to have 0.4% F1534/1534C, 6.7% F367I/367I and 11% T1520I/1520I heterozygous individuals (no F1534, 367I or 1520I homozygotes were detected), so another genotype selection was done (see Vssc genotyping) on 130 females and 74 males to ensure that the final strain was homozygous for 1534C and was free of the F367I and T1520I mutations. This strain was named 1534C:ROCK (Fig. 1).

Mosquitoes were reared at 27 °C (±1 °C) with 70–80% relative humidity, and a photoperiod of 14 L: 10 D. Adults were maintained on 10% sugar water in cages ~35 × 25 × 25 cm holding ≤1000 mosquitoes. Larvae were reared in 27.5 × 21.5 × 7.5 cm containers with ~2 L deionized water and fed Cichlid Gold fish food pellets (Hikari, Hayward, CA, USA). Food pellets were given daily as needed.

2.2 Vssc genotyping

Genomic DNA was isolated from one hind leg of unmated adults using an alkali extraction method as follows. The legs were placed in individual wells of a 96-well PCR plate (BioRad, Hercules, CA, USA) containing three 2.3-mm diameter zirconia/silica beads (BioSpec Products, Bartlesville, OK, USA) and 10 µL 0.2 M NaOH. The samples were homogenized on a vortex mixer for 2 min and then incubated at 70 °C for 10 min. Then 10 µL of neutralization buffer (360 mM Tris–HCl, pH 7.5 and 10 mM EDTA) and 80 µL of ddH2O were added to each well. PCR was carried out using 2 µL gDNA, 12.5 µL GoTaq® Green Master Mix 2x (Promega, Madison, WI), 8.5 µL ddH2O, and 2 µL of 10 µM forward and reverse primer mix. The primers and thermocycler conditions are shown in Table 2.

Vssc genotypes were determined using Sanger sequencing and allele-specific polymerase chain reaction (ASPCR). For Sanger sequencing genotyping for F367I, T1520I and F1534C in 1534C:ROCK and Thai1534C, PCR products were treated with Sanger sequencing genotyping for F367I, T1520I and F1534C (6 µL:ROCK and Thai1534C, PCR products were treated with ExoSAP treated PCR product, 1 µL primer, 11 µL ddH2O at the Cornell University Biotechnology Resource Center. ASPCR was used to determine T1520I and F1534C genotypes in UBN, BKK and BC1 to BC4. Each ASPCR was evaluated on a 1% agarose gel and was scored as homozygous susceptible (ASPCR band produced only with susceptible primers), homozygous resistance (ASPCR band only with kdr primers) or heterozygous (ASPCR band with both susceptible and kdr primers). Samples were always run alongside DNA from an individual with a known genotype (determined by Sanger sequencing) for validation.

2.3 Cloning and sequencing of the Vssc cDNA

Total RNA from pools of 15 male Thai1534C mosquitoes was extracted using TRIzol® (Invitrogen, Carlsbad, CA, USA) and gDNA was removed by treating the samples with DNase I using TURBO DNase (Life Technologies Co., Carlsbad, CA, USA) as per the manufacturer’s instructions. cDNA was synthesized using Promega GoScript Reverse Transcription System (Promega) according to the manufacturer’s instructions. To identify the entire Vssc cDNA, four overlapping segments of Vssc (covering the entire cDNA sequence) were obtained by PCR (Fig. 2) with specific primer pairs designed based on the A. aegypti Vssc sequence from the Liverpool pool strain (accession number XM_021852349). PCR was carried out using 1 µL cDNA, 10 µL 5X PrimeSTAR GXL buffer, 4 µL dNTP mixture, 1 µL PrimeSTAR GXL DNA Polymerase (Takara Bio Inc., Shiga, Japan), 33 µL ddH2O, and 2 µL of 10 µM forward and reverse primer mix (Table 3). PCR cycling conditions were as follows: 95 °C for 3 min, followed by 35 cycles of PCR (98 °C for 10 s, 60 °C for 15 s and 68 °C for 2 min) and an extension at 68 °C for 10 min. The amplified segments of the expected size were run on a 1% agarose gel and were isolated using the Promega Wizard SV Gel and PCR Clean-up System (Promega). Purified DNAs were incubated at 72 °C for 30 min with dNTP and Taq polymerase (Thermo Fisher Scientific) to add a single deoxyadenosine (A) overhang to the 3'-ends of PCR products. These were cloned using the pGEM-T Vector System (Promega) and JM109 Escherichia coli competent cells (Promega) according to the manufacturer’s instructions. Positive colonies were confirmed by PCR with the specific primers for each segment. Positive colonies were grown in liquid media and plasmid isolation was performed using Wizard plus SV miniprep DNA purification system according to the manufacturer’s instructions (Qiagen). Four to five individual clones for each segment were sequenced at the Cornell University Biotechnology Resource Center and the sequences were aligned using MegAlign applications of Lasergene 7 (DNASTar, Madison, WI).

| Table 2. Vssc primers used for genotyping individual Aedes aegypti |
| Mutation | Primer | Sequence | Purpose |
| --- | --- | --- | --- |
| F367I | 367-F | AGTTTCGATACTCTGCGAGTGGG | PCR amplification |
| | 367-R | TCTTCCGGGTCTTTCCTGGAG | PCR amplification |
| F1534C | 1534com-F | GGAGAAGACTACGTGGGAGA | Allele-specific common primer and PCR amplification |
| | 1534com-R | CGGCCATGAAATTGAGAATAGC | Allele-specific outer primer and PCR amplification and sequencing |
| | F1534-R | GCCGTAAGAGACCAGGCAG | Allele-specific susceptible genotype |
| | F1534-C | GCCGTAAGAGACGCACCGC | Allele-specific resistance genotype |
| T1520I | 1520com-F | GGAGAAGACTACGTGGGAGA | Allele-specific outer primer and PCR amplification |
| | 1520com-R | CGGCCATGAAATTGAGAATAGC | Allele-specific common primer and PCR amplification and sequencing |
| | T1520-F | GCCGCGTATCCCGAGAC | Allele-specific susceptible genotype |
| | T1520-C | GCCGCGTATCCCGGAGAT | Allele-specific resistance genotype |

1 Thermocycler conditions: 94 °C for 3 min, 35 × (94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s) and 72 °C for 10 min.
2 Thermocycler conditions: 94 °C for 3 min, 35 × (94 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s) and 72 °C for 10 min.
3 The methods were previously reported.14
2.4 Insecticides
A total of eight insecticides that target the VSSC and one cytochrome P450 (CYP) inhibitor were used in this study: cyhalothrin (90.2%, ICI Americas, Wilmington, DE, USA), cypermethrin (94.6%, ICI Americas), DDT (dichlorodiphenyltrichloroethane, 98%, Sigma-Aldrich, St. Louis, MO, USA), deltamethrin (99.5%, ICI Americas), DDT (dichlorodiphenyltrichloroethane, 98%, Sigma-Aldrich, St. Louis, MO, USA), deltamethrin (99.5%,

Table 3. Primers used for determination of the Vssc cDNA sequences used in this study

| Primer name and sequence (5’→3’) | Amplification region |
|----------------------------------|----------------------|
| **Segment-1**                    |                      |
| AaS1F: GACAATGACCGAAGACCTCCGAT  | 1–1302 bp            |
| AaS1R: GAAGAGGCGCGCGAGGAA       |                      |
| **Segment-2**                    |                      |
| First PCR                        |                      |
| AaS2F: ACGCCTCTTTGTGGGAAC       | 973–3203 bp          |
| AaS2R: CAAGTTAAACAGCCAGATTGC    |                      |
| Nested PCR                       |                      |
| AaS2NF: TATCTGCCTTTCGCTAATGACCC| 1097–3203 bp         |
| AaS2R: CAAGTTAAACAGCCAGATTGC    |                      |
| **Segment-3**                    |                      |
| First PCR                        |                      |
| AaS3F: TCCGTTCCTTTTGGCACC       | 2973–5070 bp         |
| AaS3R: ACCTGTTCGATTTGGCTCGTC    |                      |
| Nested PCR                       |                      |
| AaS3F: TCCGTTCCTTTTGGCACC       | 2973–4804 bp         |
| AaS3R: GAAGAAGCGCGCTGAAGCTA     |                      |
| **Segment-4**                    |                      |
| First PCR                        |                      |
| AaS4F: GGAGAACCTACGTGGGAGAAC    | 4443–6390 bp         |
| AaS4R: TTCTGTCGGCTTGAATCTGA     |                      |
| Nested PCR                       |                      |
| AaS4NF: GAGAAGCGCGCTGAGTTCTGGA| 4459–6390 bp         |
| AaS4R: TCAGACATCGCGCTAGCTGGA    |                      |
| T7: TAATACGACTCACTATAGGG         | Sequencing for segment 1,2,3,4 clones |
| SP6: ATTAGGTGACACTATA            | Sequencing for segment 1,2,3,4 clones |
| AaS2seq: AACATTTGGCACTTGCAGCTG   | Sequencing for segment 2 clones |
| AaS3seq: ATAAAAGCCCAATGCAGTCACA | Sequencing for segment 3 clones |
| AaS4seq: ATTTTAGGTTCGCTCCTATGAGGG | Sequencing for segment 4 clones |

Figure 2. Diagram of the A. aegypti Vssc cDNA. (A) The relative positions of the primers on the Vssc cDNA. (B) Representative clones for each segment of the Vssc cDNA. The numbers in parentheses indicate the times the sequence was detected. c/d and l are mutually exclusive exons. j, n and a are optional exons, i, m, b, f’ and h are alternative 5'3' splice site variants.
Chem Service), DCJW (98%, DuPont, Wilmington, DE, USA), etofenprox (96.3%, Mitsui Toatsu, Tokyo, Japan), flumethrin (100%, Bayer CropScience), permethrin (99.5% pure, 24.1% cis, 75.8% trans, Chem Service), piperonyl butoxide (PBO) (90%, Sigma-Aldrich). All insecticides were diluted in analytical grade acetone (VWR, Radnor, PA, USA).

2.5 Insecticide bioassays

Topical adult bioassays were performed as described Smith et al.20 In short, a 0.22 μL drop of insecticide in acetone was applied to the thorax of 3- to 7-day-old mated females using a Hamilton PB-600 repeating dispenser equipped with a 10-μL syringe. Controls were treated with acetone only. Six doses were used per bioassay with at least three killing between 0 and 100%. There were 20 mosquitoes tested per dose per replicate. Mosquitoes were given a cotton ball saturated with distilled water and held at 25 °C. Four replicates over at least 2 days and two cages were done per strain for each insecticide. Mortality was defined as mosquitoes that were ataxic after 24 h. For DCJW, mortality was recorded at 72 h due to the slow action of this insecticide. Probit analysis,32 as adapted for R (http://cran.r-project.org/) use (available at https://github.com/JuanSilva89/Probit-analysis) using Abbott’s correction33 for control mortality, was used to calculate the LD50 and the 95% confidence intervals (CI). Resistance ratios (RR) were calculated by dividing the LD50 of 1534C:ROCK by the LD50 of ROCK controls included an acetone only application twice, and an acetone only plus a synergist application.

2.6 Inheritance of resistance

Because the F1534C and S989P+V1016G are widely distributed in Asian A. aegypti populations,6,7 two crosses were set up to evaluate the inheritance of resistance: 1534C:ROCK ♀ × ROCK ♂ and 1534C:ROCK ♀ × KDR:ROCK ♂ (containing S989P+V1016G).20 Each cross was performed using ~200 adult males and ~400 unmated females. Reciprocal crosses were not needed because kdr resistance is on chromosome 3 (i.e., not sex-linked nor due to maternal factors35) in A. aegypti. Insecticide bioassays of F1 hybrids were done as described above. Permethrin and deltamethrin were used to determine the inheritance of resistance because they have the minimum and maximum RR value in 1534C:ROCK among tested pyrethroid insecticides, respectively. The degree of dominance (D) was calculated using the formula given by Stone 1968.36

2.7 Are F367I and T1520I associated with pyrethroid resistance?

The Vssc cDNA sequences showed the presence of both F367I and T1520I mutations in 1534C:ROCK, using bioassays with the CYP inhibitor PBO. These bioassays were performed as described above, except that 2.5 μg PBO was applied to each mosquito 2 h prior to the permethrin application. For these experiment the mosquitoes were anesthetized on ice twice, once for PBO application and once for permethrin application. Controls included an acetone only application twice, and an acetone only plus a synergist application.

Figure 3. Resistance ratios (RRs) for permethrin +PBO, six non-synergized pyrethroids, DCJW and DDT as determined for the 1534C:ROCK strain. Values for the KDR:ROCK (989P+1016G)20 strain are given for comparison purposes. Error bars indicate the minimum and maximum 95% confidence intervals for the RRs of each insecticide.
3 RESULTS

3.1 Cloning and sequencing of the Vssc cDNA
To identify the amino acid substitutions in Vssc of the Thai1534C strain, the complete coding region was cloned and sequenced using four overlapping segments. Clones from each of the segments were variable, owing to mutually exclusive exons, optional exons, alternative 5′/3′ splice sites and nucleotide polymorphisms (Fig. 2). Clones containing either exon c or d (mutually exclusive exons) were found. Clones with mutually exclusive exon I were found, but none contained mutually exclusive exon k. Clones containing optional exons j, n and a were also found. In addition, clones containing 5′ or 3′ splice variants of exons 11 (i), 14 (m), 18 (b), 23 (f) and 24 (h) were found. Three nonsynonymous substitutions (F367I, T1520I and F1534C) were also identified, compared with the Liverpool A. aegypti Vssc sequence (accession number XM_021852349). The T1520I mutation was found only in clones also having the F1534C mutation, although the F1534C mutation was found in clones that lacked T1520I. All of the sequences have been deposited at GenBank (accession no. MN365028-MN365034). Genomic DNA sequences of eight individual mosquitoes from Thai1534C confirmed the presence of these amino acid substitutions. These mosquitoes were homozygous for F1534C, four were homozygous for 1520I, four were heterozygous for T1520I, five were homozygous for F367I and three were heterozygous for F367I. The frequencies of the F1534C, T1520I and F367I mutations were 1.0, 0.75 and 0.19 respectively.

3.2 Characterization of the 1534C:ROCK strain
All 1534C:ROCK mosquitoes are homozygous for T1520 (and thus F367) (see Section 3.4) and 1534C (n = 90, genotype results not shown). There was no significant difference between the RR values for permethrin and for PBO+permethrin in 1534C:ROCK (Fig. 3), confirming that 1534C:ROCK did not have CYP-mediated resistance.

3.3 Pyrethroid resistance in the 1534C:ROCK strain
The 1534C:ROCK strain was resistant to pyrethroids with RR values ranging from 7 (permethrin) to 16 (deltamethrin) (Fig. 3). The RR for deltamethrin was significantly higher (approximately two-fold) than for permethrin or cypermethrin. The 1534C:ROCK strain was 11.2-fold resistant to DDT, but had no cross-resistance to DCJW (Table 4).

We examined the levels of pyrethroid resistance in two hybrids. Crosses of the ROCK and 1534C:ROCK strains revealed that the inheritance of F1534C resistance was incompletely recessive to permethrin (D = −0.84) and deltamethrin (D = −0.90). Hybrids from 1534C:ROCK × KDR:ROCK (having the S989P+V1016G mutations (Smith et al20) strains had permethrin and deltamethrin LD50 values that were not significantly different from the 1534C:ROCK strain (Table 4).

3.4 Are the F367I and T1520I mutations associated with pyrethroid resistance?
We genotyped 71 individual Thai1534C mosquitoes for the F367I, T1520I and 1534C mutations. Five 367I/1520I/1534 genotypes were found: FF/TT/CC, FF/II/CC, FI/II/CC, FF/TI/CC and FI/TI/CC. This strongly suggested that only three types of alleles (F/T/I C) were present in the Thai1534C strain and that 367I co-occurred with 1520I.

Given that the F367I, and T1520I mutations were present in the Thai1534C strain, we evaluated if these mutations increase the pyrethroid resistance caused by F1534C alone by using a dose

### Table 4. Toxicity of eight insecticides that target the VSSC against susceptible (ROCK) and resistant (1534C:ROCK) strains of *Aedes aegypti*

| Insecticide   | Strain     | LD50 (95% CI) | Slope (SE) | n  |
|---------------|------------|---------------|------------|----|
| Cyhalothrin   | ROCK       | 0.068 (0.054–0.086) | 2.2 (0.2) | 540 |
| Cyhalothrin   | 1534C:ROCK | 0.82 (0.74–0.93) | 2.5 (0.2) | 540 |
| Cypemethrin   | ROCK       | 0.094 (0.083–0.105) | 2.7 (0.2) | 580 |
| Cypemethrin   | 1534C:ROCK | 0.72 (0.68–0.77) | 2.3 (0.1) | 520 |
| Deltamethrin  | ROCK       | 0.007 (0.006–0.008) | 3.3 (0.4) | 660 |
| Deltamethrin  | 1534C:ROCK | 0.11 (0.09–0.14) | 3.0 (0.4) | 680 |
| Deltamethrin  | 1534C:ROCK × KDR:ROCK | 0.007 (0.007–0.009) | 3.3 (0.4) | 540 |
| Deltamethrin  | 1534C:ROCK × KDR:ROCK | 0.084 (0.061–0.116) | 2.1 (0.3) | 600 |
| Etofenprox    | ROCK       | 2.08 (1.41–3.06) | 2.6 (0.6) | 500 |
| Etofenprox    | 1534C:ROCK | 19.54 (15.94–23.97) | 2.5 (0.3) | 560 |
| Flumethrin    | ROCK       | 0.72 (0.53–0.99) | 2.0 (0.3) | 520 |
| Flumethrin    | 1534C:ROCK | 9.44 (6.33–14.09) | 2.1 (0.4) | 600 |
| Permethrin    | ROCK       | 0.87 (0.74–1.02) | 3.3 (0.4) | 560 |
| Permethrin    | 1534C:ROCK | 6.10 (4.58–8.13) | 2.6 (0.4) | 680 |
| Permethrin    | ROCK×1534C:ROCK | 1.02 (0.87–1.19) | 2.9 (0.3) | 560 |
| Permethrin    | 1534C:ROCK × KDR:ROCK | 10.14 (7.21–14.26) | 2.3 (0.4) | 600 |
| Permethrin + PBO | ROCK       | 0.09 (0.08–0.11) | 2.3 (0.2) | 560 |
| Permethrin + PBO | 1534C:ROCK | 1.18 (1.04–1.34) | 1.6 (0.1) | 560 |
| DCJW          | ROCK       | 2.09 (1.71–2.56) | 2.6 (0.3) | 760 |
| DCJW          | 1534C:ROCK | 1.32 (1.00–1.73) | 3.0 (0.4) | 840 |
| DDT           | ROCK       | 14.92 (11.83–18.81) | 3.9 (0.7) | 480 |
| DDT           | 1534C:ROCK | 167.56 (142.56–196.94) | 1.7 (0.1) | 600 |

aStrain with 989P+1016G kdr allele.20
bLD50 in units of ng/mosquito.
of permethrin or deltamethrin + PBO to discriminate the susceptible (dead) and resistant (alive) phenotypes (Fig. 4). For the F367I mutation, there was no significant difference in the genotypes of the alive vs. dead after permethrin (Fisher’s exact test, $P = 1$, OR = 1.20, 95% CI = 0.31–4.78) or deltamethrin (Fisher’s exact test, $P = 1$, OR = 0.89, 95% CI = 0.14–5.64) treatment, indicating the F367I mutation does not confer any additional level of resistance to individuals having the 1534C allele (unless the trait is fully recessive). For the T1520I mutation, there was also no significant difference (Fisher’s exact test) in the genotypes of the alive vs. dead after permethrin (Fisher’s exact test, $P = 1$, OR = 1, 95% CI = 0.55–1.82) or deltamethrin (Fisher’s exact test, $P = 0.65$, OR = 1.16, 95% CI = 0.64–2.10). The 1534C allele is one of the most common kdr alleles in A. aegypti populations globally, and our results revealed that this allele confers 7–16 fold level of resistance to pyrethroids (Fig. 3). Similarly, the PMD-R strain of A. aegypti ( homozygous for 1534C) was 13-fold resistant to deltamethrin and an Aedes albopictus (Skuse) strain homozygous for the F1534C mutation was 5- and 11-fold resistant to permethrin + PBO and deltamethrin + PBO, respectively. Furthermore, these results are consistent with previous studies which reported an association of the F1534C mutation with permethrin or deltamethrin resistance. Collectively these results strongly support a role of the F1534C mutation in conferring 7- to 16-fold resistance to pyrethroids. In contrast, some studies failed to detect an association of F1534C with resistance to permethrin, deltamethrin or other pyrethroid resistant mechanisms (e.g. enhanced metabolic detoxification).

4 DISCUSSION

Insects have multiple voltage-sensitive sodium channel (Vssc) transcripts, and these different Vsscs (produced by differential exon use and RNA editing) can have different electrophysiological and pharmacological properties. A total of four mutually exclusive exons (c/d and k/l), two optional exons (j and a), five 5’/3’ splice site variants (i, b, e, f and h) are known. In A. aegypti, three other splice variants (called m, n and f) have been detected. We detected all the optional exons and alternative splice site variants, except e and f. The splice variants e and f are not a major contributor for pyrethroid resistance, because the Vssc cDNA from the susceptible strain SMK (GenBank: BAP46858) also lacks e and f (Fig. 2).

The 1534C allele is one of the most common kdr alleles in A. aegypti populations globally, and our results revealed that this allele confers 7–16 fold level of resistance to pyrethroids (Fig. 3). Similarly, the PMD-R strain of A. aegypti ( homozygous for 1534C) was 13-fold resistant to deltamethrin and an Aedes albopictus (Skuse) strain homozygous for the F1534C mutation was 5- and 11-fold resistant to permethrin + PBO and deltamethrin + PBO, respectively. Furthermore, these results are consistent with previous studies which reported an association of the F1534C mutation with permethrin or deltamethrin resistance. Collectively these results strongly support a role of the F1534C mutation in conferring 7- to 16-fold resistance to pyrethroids. In contrast, some studies failed to detect an association of F1534C with resistance to permethrin, deltamethrin or other pyrethroid resistant mechanisms (e.g. enhanced metabolic detoxification).
In this study, we found a novel F367I mutation that co-occurred with the T1520I mutation in individuals that also were homozygous 1534C. A T1520I mutation co-existing with F1534C was detected in an Indian A. aegypti population.15 Our results showed the T1520I did not increase permethrin or deltamethrin resistance over the F1534C mutation alone. However, there were no 367I homozygous individuals detected, so we are not able to rule out a role for the F367I mutation in pyrethroid resistance.

Heterologous expression (usually oocytes) and electrophysiological studies have been used to evaluate how changes in one or more amino acids alters VSSC sensitivity to pyrethroids, but these results are not always consistent with what is found in vivo. For example, based on heterologous expression/electrophysiology studies kdr-his was predicted to give more protection to deltamethrin than kdr.41 Yet our results, and similarly, heterologous expression/electrophysiological results indicated the F1534C reduced the channel sensitivity to permethrin, but not to deltamethrin.14,16 Yet our results, and similarly, heterologous expression/electrophysiological results indicated the F1534C reduced the channel sensitivity to permethrin, but not to deltamethrin.14,16 Yet our results, and similarly, heterologous expression/electrophysiological results indicated the F1534C mutation are resistant to both permethrin and deltamethrin (Fig. 3). Furthermore, heterologous expression/electrophysiological results indicated that the T1520I + F1534C mutations increased insensitivity to permethrin compared to the F1534C mutation alone.17 However we found no increased survival in mosquitoes that had T1520I+F1534C relative to F1534C alone (Fig. 4). Thus, the measurements of VSSC insensitivity in heterologous expression/electrophysiological studies are not always congruent with resistance levels measured in living insects.

The 1534C and 989P+1016G alleles are widely distributed in Asian populations,4,13 but 1534C confers less pyrethroid resistance than the 989P+1016G allele (Fig. 3). In fact pyrethroid resistance conferred by the F1534C allele is 2- to 10-fold less than the 989P+V1016G allele (Fig. 3). This is consistent with a previous study that showed permethrin selection favored the 989P +V1016G over the F1534C allele.39 The F1534C allele also provided less protection to DDT (compared to the 989P+V1016G) and no cross-resistance to DCW.

Consistent with what has been observed for other kdr alleles,53 resistance due to the F1534C allele was inherited as an incompletely recessive trait (Table 4).20 However, the 989P+V1016 +1534C/989P+1016G+F1534 hybrid (1534C:ROCK X kdr:ROCK) had similar levels of resistance as the 1534C homozygotes, which were 2.8-fold and 3.4 fold lower than the 989P+1016G homozygotes against permethrin and permethrin, respectively (Table 4). The 989P+V1016+1534C/989P+1016G+F1534 genotype is common in A. aegypti from Asia.30,35 Given that 989P+1534C/989P+1016G+F1534 individuals more resistant than the 989P+V1016+989P+1016G heterozygote may help explain why the 1534C allele is relatively common.

In summary, we determined the levels of resistance conferred by F1534C to six pyrethroid insecticides, DDT and DCW. RR values ranged from 7 (permethrin) to 16 (deltamethrin). The RR values are from 2- (deltamethrin) to >200-fold (DDT) lower than previously reported for the 989P+1016Gallele. The 989P +V1016+1534C/989P+1016G+F1534 heterozygotes have similar levels of resistance as the 1534C homozygotes. We found a novel 367I+1520I+1534C allele in addition to 1534C and 1520I+1534C in A. aegypti populations from Thailand. However, T1520I does not increase the permethrin or deltamethrin resistance caused by F1534C alone. Future studies are required to determine if F367I may increase T1520I+F1534C resistance levels. Our results reveal it is important to understand the levels of resistance conferred by the different resistance alleles found in natural populations.

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SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

REFERENCES

1. Leta S, Beyene TJ, De Ciercq EM, Amenu K, Revie CW and Kraemer MUG, Global risk mapping for major diseases transmitted by Aedes aegypti and Aedes albopictus. Int J Infect Dis 67:25–35 (2018).
2. Souza-Neto JA, Powell JR and Bonizzoni M, Aedes aegypti vector competence studies: a review. Infect Genet Evol 67:191–209 (2019).
3. Kraemer MUG, Reiner RC Jr, Brady OJ, Messina JP, Gilbert M, Pigott DM et al., Past and future spread of the arbovirus vectors Aedes aegypti and Aedes albopictus. Nat Microbiol 4:854–863 (2019).
4. Smith LB, Kasai S and Scott JG, Pyrethroid resistance in Aedes aegypti and Aedes albopictus: important mosquito vectors of human diseases. Pestic Biochem Physiol 133:1–12 (2016).
5. Moyes CL, Vontas J, Martins AJ, Ng LC, Koo SY, Dusfour I et al., Contemporary status of insecticide resistance in the major Aedes vectors of arboviruses infecting humans. PLoS Negl Trop Dis 11:e0005625 (2017).
6. Silva J and Scott JG, Conservation of the voltage-sensitive sodium channel protein within the Insecta. Insect Mol Biol 29:9–18 (2020).
7. Scott JG, Life and death at the voltage-sensitive sodium channel: evolution in response to insecticide use. Annu Rev Entomol 64:243–257 (2019).
8. Hsu J-C, Feng H-T, Haymer DS and Chen Y-H, Molecular and biochemical mechanisms of organophosphate resistance in laboratory-selected lines of the oriental fruit fly (Bactrocera dorsalis). Pestic Biochem Physiol 100:57–63 (2011).
9. Du Y, Nomura Y, Zhvorov B and Dong K, Sodium channel mutations and pyrethroid resistance in Aedes aegypti. Insects 7:60 (2016).
10. Murcia O, Henríquez B, Castro A, Koo S, Young J, Márquez R et al., Presence of the point mutations Val1016Gly in the voltage-gated sodium channel detected in a single mosquito from Panama. Parasit Vectors 12:62 (2019).
11. Granada Y, Mejía-Jaramillo A, Strode C and Triana-Chavez O, A point mutation V419L in the sodium channel gene from natural populations of Aedes aegypti is involved in resistance to λ-cyhalothrin in Colombia. Infects 9:23 (2018).
12. Saavedra-Rodríguez K, Maloof FV, Campbell CL, Garcia-Rejon J, Lenhart A, Penilla P et al., Parallel evolution of vgsc mutations at domains IS6, IIS6 and IIIS6 in pyrethroid resistant Aedes aegypti from Mexico. Sci Rep 8:6747 (2018).
13. Fan Y, O’Grady P, Yoshimizu M, Ponlawat A, Kaufman PE and Scott JG, Evidence for both recombination and mutations in the evolution of kdr alleles in Aedes aegypti. PLoS Negl Trop Dis (In Press) (2020).
14. Du Y, Nomura Y, Satar G, Hu Z, Nauen R, He SY et al., Molecular evidence for dual pyrethroid-receptor sites on a mosquito sodium channel. Proc Natl Acad Sci U S A 110:11785–11790 (2013).
15. Haddi K, Tomé HV, Du Y, Valbon WR, Nomura Y, Martins GF et al., Detection of a new pyrethroid resistance mutation (V410L) in the sodium channel of Aedes aegypti: a potential challenge for mosquito control. Sci Rep 7:46549 (2017).
16. Hirata K, Komagata O, Itoikawa K, Yamamoto A, Tomita T and Kasai S, A single crossing-over event in voltage-sensitive Na+ channel genes may cause critical failure of dengue mosquito control by insecticides. PLoS Negl Trop Dis 8:e3085 (2014).
17. Chen M, Du Y, Wu S, Nomura Y, Zhu G, Zhvorov BS et al., Molecular evidence of sequential evolution of DDT- and pyrethroid-resistant
The F1534C Vssc mutation confers ≥16-fold resistance to pyrethroids

18 Kushwah RBS, Dykes CL, Kapoor N, Adak T and Singh OP, Pyrethroid-resistance and presence of two knockdown resistance (kdr) mutations, F1534C and a novel mutation T1520I, in Indian Aedes aegypti. PLoS Negl Trop Dis 13:e0007432 (2019).

19 Chang C, Shen WK, Wang TT, Lin YH, Hsu EL and Dai SM, A novel amino acid substitution in a voltage-gated sodium channel is associated with knockdown resistance to permethrin in Aedes aegypti. Insect Biochem Mol Biol 39:272–278 (2009).

20 Smith LB, Kasai S and Scott JG, Voltage-sensitive sodium channel mutations S989P+V1016G in Aedes aegypti confer variable resistance to pyrethroids, DDT and oxadiazines. Pest Manag Sci 74:737–745 (2017).

21 Kawada H, Higa Y, Futami K, Muranami Y, Kawashima E, Osei JHN et al., Discovery of point mutations in the voltage-gated sodium channel from African Aedes aegypti populations: potential phylogenetic reasons for gene introgression. PLoS Negl Trop Dis 10:e0004780 (2016).

22 Seixas G, Grigoraki L, Weetman D, Vicente JL, Silva AC, Pinto J et al., Insecticide resistance is mediated by multiple mechanisms in recently introduced Aedes aegypti from Madeira Island (Portugal). PLoS Negl Trop Dis 11:e0005799 (2017).

23 Sombié A, Saiki E, Yaméogo F, Sakurai T, Shirozu T, Fukumoto S et al., High frequencies of F1534C and V1016I kdr mutations and association with pyrethroid resistance in Aedes aegypti from Somgandé (Ouagadougou), Burkina Faso. Trop Med Health 47:2–8 (2019).

24 Al Nazawi AM, Aqili J, Alzahrani M, McCall PJ and Weetman D, Combined target site (kdr) mutations play a primary role in highly pyrethroid resistant phenotypes of Aedes aegypti from Saudi Arabia. Parasit Vectors 10:161 (2017).

25 Hamid PH, Prastowo J, Widiyasa I, Taubert A and Hermosilla C, Knockdown resistance (kdr) of the voltage-gated sodium channel gene of Aedes aegypti population in Denpasar, Bali, Indonesia. Parasit Vectors 10:283 (2017).

26 Estep AS, Sanscraintre ND, Waits CM, Bernard SJ, Lloyd AM, Lucas KJ et al., Quantification of permethrin resistance and kdr alleles in Florida strains of Aedes aegypti (L) and Aedes albopictus (Skuse). PLoS Negl Trop Dis 12:e0006544 (2018).

27 Badolo A, Sombié A, Pignatelli PM, Sanon A, Yameogo F, Wangrawa DW et al., Insecticide resistance levels and mechanisms in Aedes aegypti populations in and around Ouagadougou, Burkina Faso. PLoS Negl Trop Dis 13:e0007439 (2019).

28 Harris AF, Rajatikela S and Ranson H, Pyrethroid resistance in Aedes aegypti from Grand Cayman. Am J Trop Med Hyg 83:277–284 (2010).

29 Hamid PH, Prastowo J, Ghifari A, Taubert A and Hermosilla C, Aedes aegypti resistance development to commonly used insecticides in Jakarta, Indonesia. PLoS ONE 12:e0189680 (2017).

30 Stenhause SA, Plensm S, Yanola J, Lumjua N, Dantrakool A, Chochoch W et al., Detection of the V1016G mutation in the voltage-gated sodium channel gene of Aedes aegypti (Diptera: Culicidae) by allele-specific PCR assay, and its distribution and effect on deltamethrin resistance in Thailand. Parasit Vectors 6:253 (2013).

31 Li C-X, Kaufman PE, Xue R-D, Zhao M-H, Wang G, Yan T et al., Relationship between insecticide resistance and kdr mutations in the dengue vector Aedes aegypti in Southern China. Parasit Vectors 8:325 (2015).

32 Finney DJ, Probit Analysis. Cambridge University Press, Cambridge (1971).

33 Abbott WS, A method of computing the effectiveness of an insecticide. J Econ Entomol 18:265–267 (1925).

34 Smith LB, Sears C, Sun H, Mertz RW, Kasai K and Scott JG, CYP-mediated resistance and cross-resistance to pyrethroids and organophosphates in Aedes aegypti in the presence and absence of kdr. Pestic Biochem Physiol 160:119–126 (2019).

35 Kasai S, Komagata O, Itokawa K, Shono T, Ng LC, Kobayashi M et al., Mechanisms of pyrethroid resistance in the dengue mosquito vector, Aedes aegypti: target site insensitivity, penetration, and metabolism. PLoS Negl Trop Dis 8:e2948 (2014).

36 Stone BF, A formula for determining degree of dominance in cases of monofactorial inheritance of resistance to chemicals. Bull World Health Organ 38:325–326 (1968).

37 Dong K, Du Y, Rinkevich F, Nomura Y, Xu P, Wang L et al., Molecular biology of insect sodium channels and pyrethroid resistance. Insect Biochem Mol Biol 50:1–17 (2014).

38 Loughney K, Kreber R and Ganetzky B, Molecular analysis of the para locus, a sodium channel gene in Drosophila. Cell 58:1143–1154 (1989).

39 Plensmb S, Saingamsook J, Yanola J, Lumjuan N, Tippawangkposol P, Sukontason K et al., Additive effect of knockdown resistance mutations, S989P, V1016I and F1534C, in a heterozygous genotype conferring pyrethroid resistance in Aedes aegypti in Thailand. Parasit Vectors 9:417 (2016).

40 Kasai S, Caputo B, Tsonuda T, Cuong TC, Maekawa Y, Lamphua SG et al., First detection of a Vssc allele V1016G conferring a high level of insecticide resistance in Aedes albopictus collected from Europe (Italy) and Asia (Vietnam), 2016: a new emerging threat to controlling arboviral diseases. Eurosurveillance 24:1–12 (2019).

41 Burton MJ, Mellor IR, Duce IR, Davies TGE, Field LM and Williamson MS, Differential resistance of insect sodium channels with kdr mutations to deltamethrin, permethrin and DDT. Insect Biochem Mol Biol 41: 723–732 (2011).

42 Sun H, Tong KP, Kasai S and Scott JG, Overcoming super-kdr mediated resistance: multi-halogenated benzyl pyrethroids are more toxic to super-kdr than kdr house flies. Insect Mol Biol 25:126–137 (2016).

43 Shono T, Pyrethroid resistance: importance of the kdr-type mechanism. J Pestic Sci 10:141–146 (1985).