Voltage-sensitive sodium channel mutations S989P + V1016G in Aedes aegypti confer variable resistance to pyrethroids, DDT and oxadiazines

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Abstract

BACKGROUND: Aedes aegypti is a vector of several important human pathogens. Control efforts rely primarily on pyrethroid insecticides for adult mosquito control, especially during disease outbreaks. A. aegypti has developed resistance nearly everywhere it occurs and insecticides are used. An important mechanism of resistance is due to mutations in the voltage-sensitive sodium channel (Vssc) gene. Two mutations, in particular, S989P + V1016G, commonly occur together in parts of Asia.

RESULTS: We have created a strain (KDR:ROCK) that contains the Vssc mutations S989P + V1016G as the only mechanism of pyrethroid resistance within the genetic background of Rockefeller (ROCK), a susceptible lab strain. We created KDR:ROCK by crossing the pyrethroid-resistant strain Singapore with ROCK followed by four backcrosses with ROCK and Vssc S989P + V1016G genotype selections. We determined the levels of resistance conferred to 17 structurally diverse pyrethroids, the organochloride DDT, and oxadiazines (VSSC blockers) indoxacarb (proinsecticide) and DCJW (the active metabolite of indoxacarb). Levels of resistance to the pyrethroids were variable, ranging from 21- to 107-fold, but no clear pattern between resistance and chemical structure was observed. Resistance is inherited as an incompletely recessive trait. KDR:ROCK had a >2000-fold resistance to DDT, 37.5-fold cross-resistance to indoxacarb and 13.4-fold cross-resistance to DCJW.

CONCLUSION: Etofenprox (and DDT) should be avoided in areas where Vssc mutations S989P + V1016G exist at high frequencies. We found that pyrethroid structure cannot be used to predict the level of resistance conferred by kdr. These results provide useful information for resistance management and for better understanding pyrethroid interactions with VSSC.

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

Keywords: Vssc; pyrethroid resistance; oxadiazine insecticides; sodium channel; DDT

1 INTRODUCTION

Aedes aegypti is an anthropophilic mosquito and vector of four important human disease viruses: dengue, yellow fever, chikungunya, and Zika. It is estimated that dengue is a risk to >40% of the world’s population. There are an estimated 50 to 100 million dengue virus infections a year worldwide.1–3 Yellow fever, like dengue, is a viral hemorrhagic fever. This can be a lethal disease if not properly treated and despite the existence of a preventative vaccine, there are still ~ 200 000 cases of yellow fever each year, resulting in 30 000 deaths worldwide.4 Chikungunya and Zika viruses are new to the Americas as of 20135 and 2015,6 respectively. Chikungunya is no longer a disease of developing and low-income countries, and can be endemic even in the wealthy and developed countries of temperate regions.7 Zika is an emerging disease that has existed in Africa and Asia with little apparent impact on human health prior to 2007.8 This disease has generated a great deal of human health concern since it reached the Americas in 2014 because of its rapid spread and associations with microcephaly and Guillain-Barré syndrome.9 Given that A. aegypti has a wide global distribution and thrives in urban environments, it poses a serious risk to human health.

Insecticide resistance is a problem affecting the control of a wide range of pests and almost every aspect of human life including health, agriculture and recreation. As of 2012, over 450 insecticide-resistant arthropods species have been reported, with at least 198 of these being of medical importance.10,11 In regions where A. aegypti-transmitted diseases are most prevalent, mosquitoes have high levels of resistance to insecticides due to
intensive selection pressure. This compromises our ability to control these mosquitoes and the diseases they spread.

Pyrethroid insecticides, such as cypermethrin, deltamethrin and permethrin, are widely used for control of mosquitoes, including *A. aegypti*. Pyrethroids are one of the most commonly used insecticide classes because of their high toxicity to insects and low risk to mammals. Insecticides are still the primary means by which to control *A. aegypti* in endemic areas, and this has led to extensive use of pyrethroids for the last three decades.

*A. aegypti* has developed two major resistance mechanisms against pyrethroids, and resistance is found worldwide. The two major mechanisms are mutations in the voltage-sensitive sodium channel (Vssc) gene (generally known as knockdown resistance or kdr) and detoxification by cytochrome P450 monooxygenases (CYPs). Pyrethroids act directly on the insect VSSC and certain mutations in Vssc (such as S989P + V1016G) make the channel less sensitive to pyrethroids. The S989P + V1016G Vssc mutations (herein referred to as kdr) are common in Asia, yet the level of resistance these mutations confer to pyrethroids is not known.

In this study, we determined the levels of resistance conferred to *A. aegypti* by Vssc mutations S989P + V1016G (kdr) for 20 insecticides that target the VSSC: 17 structurally diverse pyrethroids, the organochloride DDT and two oxadiazines (VSSC blockers indoxacarb [proinsecticide] and DCJW [active metabolite of indoxacarb]). Our goal was to discover if there are any relationships between pyrethroid structure and the resistance conferred by these mutations, and to identify insecticides that should be avoided in areas where Vssc mutations S989P + V1016G exist at high frequencies. Knowing the levels of resistance to structurally diverse pyrethroids facilitates a greater understanding of how pyrethroids interact with VSSC and will provide insight into what pyrethroid structures are most affected by the S989P + V1016G mutations. Information from this study has direct application for resistance management practices because it will identify the chemicals that may be the most, or least, effective against a population harboring these resistance mutations.

### 2 MATERIALS AND METHODS

#### 2.1 Strains

In order to study the effects of Vssc mutations S989P + V1016G (kdr) without the influence of other resistance mechanisms or strain variations, we needed a strain of *A. aegypti* that was closely related to a susceptible strain but containing kdr. To accomplish this, we used the following parental strains: Rockefeller (ROCK), an insecticide-susceptible strain that has been reared in laboratories without exposure to insecticides for decades, which originated from the Caribbean, and Singapore (SP), a pyrethroid-resistant strain in which the mechanisms of resistance have been well studied. Adult SP are 579-fold resistant to permethrin (relative strain in which the mechanisms of resistance have been well studied). Adult SP are 579-fold resistant to permethrin (relative strain in which the mechanisms of resistance have been well studied). Adult SP are 579-fold resistant to permethrin (relative strain in which the mechanisms of resistance have been well studied).

Mosquitoes were reared at 27 ± 1 °C and 70–80% relative humidity (RH). Females were blood-fed using membrane-covered water-jacketed glass feeders with cow blood obtained locally. 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) (ground pellets for first instar and medium size pellets for second to fourth instar). Food pellets were given daily, as needed depending on larval density (~ 400–600) and instar.

297 Singapore (SP) × 236 ROCK
297 Singapore (SP) × 236 ROCK
~ 300 F1(kdr+) × ROCK(kdr+ + )
239 BC1(kdr+) × ROCK(kdr+)
190 BC2(kdr+) × ROCK(kdr+)
202 BC3(kdr+) × ROCK(kdr+)
263 BC4(kdr+) × 137 BC5(kdr+)
186 BC6F1(kdr+ kdr) × 107 BC6F1(kdr+ kdr)

**Figure 1.** Protocol for isolating the congenic strain containing Vssc mutations V1016G + S989P (KDR:ROCK). Number of ROCK males included in each backcross varied depending on the number of females added to the cage (approximately half the number of females).

The strain KDR:ROCK was isolated from crossing ROCK with SP followed by four backcrosses and genotype selections (see Section 2.2). KDR:ROCK is congenic to ROCK, but resistant to pyrethroids due to the Vssc mutations S989P + V1016G while containing no CYP-mediated resistance. The procedure for isolating KDR:ROCK is illustrated in Fig. 1. In short, unmated SP females were crossed en masse with ROCK males. Unmated F1 females were backcrossed with ROCK and unmated BC1 females were genotyped for the presence of Vssc mutations S989P + V1016G (see Section 2.2). BC1 females that were heterozygous for P989 + G1016 (kdr) were backcrossed to ROCK males. This process was repeated for the BC2 and BC3 generations. At BC3, both males and unmated females were genotyped and individuals that were heterozygous for P989 + G1016 were reared en masse. Males and unmated females from the next generation were genotyped and individuals that were homozygous for P989 + G1016 were reared en masse. The resultant strain was named KDR:ROCK (Fig. 1).

#### 2.2 Genotype selections

Genomic DNA was extracted from the hind tarsus of unmated adults using an alkali extraction method as follows: legs were placed in individual wells of a 96-well PCR plate (Bio-Rad, Hercules, CA, USA) with approximately three 2.3-mm diameter zirconia/silica beads (BioSpec Products, Bartlesville, OK, USA) and 10 μL of 0.2 M NaOH per tube. The samples were pulverized for 1–2 min on a vortex mixer at maximum speed and then incubated for 10 min at 70 °C. Ten microliters of neutralization buffer (360 mM Tris–HCl, pH 8.0 and 10 mM EDTA) and 80 μL ddH2O were then added to each well. PCR was carried out using 1 μL gDNA, 12.5 μL GoTaq® Green Master Mix 2x (Promega, Madison, WI, USA), 9.5 μL ddH2O, and 2 μL of 10 μM forward and reverse primer mix (see Table 1) using the following thermocycler conditions: 94 °C for 2 min, 35 × (94 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s) and 72 °C for 10 min.

The genotypes were determined using two methods: Sanger sequencing (BC1 to BC6) and allele-specific polymerase chain reaction (ASPCR) (BC4 and BC4F1). For Sanger sequencing genotyping, PCR products were treated with ExoSAP (5 μL PCR product/1 μL ExoSAP mix), and sequenced (6 μL ExoSAP treated PCR product, 1 μL primer, 11 μL ddH2O) at the Cornell University Biotechnology Resource Center. For ASPCR, forward and reverse primers (see
with distilled water and held at 25 °C. A minimum of 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 indoxacarb, mortality was recorded at 72 h due to the slow action of this insecticide, and mosquitoes were given sugar water-saturated cotton balls that were rehydrated at 24 and 48 h during the bioassay. Probit analysis, as adapted to personal computer use using Abbott’s correction for control mortality, was used to calculate the LD₅₀ and the 95% confidence intervals (CI). All the bioassay data fit a line (chi-square test). Resistance ratios (RR) were calculated by dividing the LD₅₀ of KDR:ROCK by the LD₅₀ of ROCK. Significant differences were determined by calculating the RR for the minimum and maximum LD₅₀ values based on the 95% CI. If the minimum and maximum RR values did not overlap, they were deemed significantly different.

Bioassays using a synergist (PBO or DEM) were performed as described above, except that 2.5 μg PBO or 2.5 μg DEM was applied to each mosquito 2 h prior to the insecticide application. For this, the mosquitoes were anesthetized on ice twice, once for PBO or DEM and once for permethrin or DDT application. Controls included a double acetone and an acetone plus synergist application.

### 2.5 Larval bioassays

Bioassays using mosquito larvae were performed with permethrin to allow comparison of the levels of resistance conferred by Vssc mutations S989P + V1016G between life stages. Bioassays were carried out on fourth instar larvae reared as described above. Twenty larvae were placed in a 177-mL frozen yogurt cup (Solo, Lake Forest, IL, USA) containing 99 mL deionized water and 1.0 mL permethrin in acetone. Controls received 1 mL acetone without insecticide. At least five concentrations were used per bioassay with at least three giving mortality values between 0% and 100%. A total of six replicates from two rearing batches were done for each strain. Larvae that were floating, ataxic or unable to surface were counted as dead.

### 2.6 Degree of dominance

Unmated ROCK females (~ 400) were crossed with KDR:ROCK males (~ 200) and the F₁s were tested (as described in Section 2.4) to determine the degree of dominance (D) of the Vssc mutations S989P + V1016G. Reciprocal crosses were not needed because resistance is not sex-linked nor due to maternal factors. Permethrin was used to determine D because it was the insecticide used for selecting the parent resistant strain SP. D was calculated using the formula given by Stone 1968.

### Table 1. Vssc primers used for genotype selections.

| Primer         | Sequence                        | Purpose                                      |
|---------------|---------------------------------|----------------------------------------------|
| AaSFCF20      | GACAATGTTGATGCGTTCCG           | PCR amplification (Forward)*                 |
| AaSCR21       | GCAATCGCTGGTAACTTG             | PCR amplification (Reverse)*                 |
| AaSCR22       | TTCAACGAATTCGGCCCGTTG          | Sequencing                                  |
| Ser 1F        | GCGGCGATGGATGCGATG             | Allele-specific susceptible genotype (Forward)† |
| Val 1R        | GCAAGGCTAAGAAAAGTTAGTA         | Allele-specific susceptible genotype (Reverse)† |
| Pro 1F        | GCGGCGATGGATGCGAAC             | Allele-specific resistant genotype (Forward)† |
| Gly 1R        | GCAAGGCTAAGAAAAGTTAGTC         | Allele-specific resistant genotype (Reverse)† |

*PCR product = 614 bp  
†ASPCR product = 357 bp
3 RESULTS

To confirm that KDR:ROCK did not have CYP-mediated resistance, we performed permethrin bioassays with and without PBO. As expected, there was no significant difference between the RR values for permethrin and for PBO + permethrin (Fig. 3). We conclude that KDR:ROCK does not have CYP-mediated resistance.

We tested 17 pyrethroids against KDR:ROCK and ROCK (Fig. 2). RR values varied by up to fivefold for the different pyrethroids (Fig. 3) ranging from 21 (bioallethrin) to 107 (etofenprox). Permethrin, the insecticide used to select for resistance in the SP strain had a RR of 40. There was a significantly higher RR (1.5-fold) for \textit{trans}-permethrin compared with \textit{cis}-permethrin. Interestingly, the RR for permethrin, which is a mix of isomers containing 75.8% of the \textit{trans} isomer, was not significantly different from \textit{cis}-permethrin. Comparing insecticides with a single structural change in the acid moiety revealed some interesting differences and similarities. Cyfluthrin, which differs from flumethrin by a chlorobenzene substitution in the acid moiety (see Fig. 2), had a 2.5-fold lower resistance relative to flumethrin. Cyhalothrin and cypermethrin differ only due to a trifluoro- rather than a chloro- substituent in the alkene, yet the RR value for cyhalothrin was approximately twofold higher than cypermethrin. However, we found no significant difference between cypermethrin and deltamethrin, which differ only by chlorines (cypermethrin) versus bromines (deltamethrin) on the alkene substituent. No broad generalization can be made concerning larger structural changes in the acid moiety and resistance levels, despite the variable RRs. Cyhalothrin, for example, which has an identical structure to acrinathrin, except for the substituent on the alkene group, has a nearly twofold greater RR than acrinathrin. However, there was no significant difference between RRs for acrinathrin, deltamethrin and cypermethrin, which also have different alkene substituents. Comparing insecticides with a single structural change in the alcohol moiety revealed no differences in RR values. Transfluthrin and \textit{1-R} trans flumethrin, for example, vary only by a single fluorine (on the benzene), but do not differ significantly in RR values. There was also no trend between the resistance levels of pyrethroids with and without \textit{α}-CN groups. Comparing pyrethroids with large structural differences in the alcohol portion of the molecule, but with identical acid moieties, also showed no general trends. In one case, a change in the alcohol caused a threefold increase in resistance (bioresmethrin versus bioallethrin), whereas in others (transfluthrin versus \textit{1-R} trans fenfluthrin), there was no significant difference in resistance. Therefore, despite the variable RR values, we observed no general relationships between the RR values and chemical structures.

We determined the resistance to DDT because \textit{Vssc} mutations are known to confer cross-resistance between pyrethroids and DDT. The RR for DDT was >2000-fold with only 27% mortality observed at the maximum concentration that was feasible to use (Fig. 3). CYPs and glutathione-S transferases (GSTs) can both metabolize DDT. To determine whether KDR:ROCK had metabolism-mediated resistance to DDT (in addition to \textit{kdr}), we used the CYP and GST inhibitors PBO and DEM, respectively. Neither PBO nor DEM measurably increased the toxicity of DDT to the KDR:ROCK strain (Table 2), suggesting that neither CYPs nor GSTs played a major role in the observed DDT resistance.

We tested the VSSC blocker (oxadiazine), indoxacarb, to see if \textit{kdr} conferred any cross-resistance to this class of insecticide, which targets the VSSC, albeit with a different mode of action from pyrethroids or DDT. The RR for indoxacarb was 37.5-fold (Fig. 3). Because of the high cross-resistance, we tested DCJW to determine if the resistance was due to the \textit{Vssc} mutations or the bioactivation of indoxacarb. Based on neurophysiological studies, indoxacarb itself has little toxicity towards VSSC, whereas its metabolite, DCJW, has high insecticidal potency (> 25-fold increased toxicity). The RR to DCJW was 13.4; significantly less (2.8-fold) than indoxacarb, but still significantly cross-resistant compared to ROCK mosquitoes. This suggests that indoxacarb is bioactivated less in KDR:ROCK than in ROCK and may be a hint that the gene coding for the bioactivation enzyme is close to \textit{Vssc} (i.e. at the resistance locus).

The inherent toxicity of the 20 insecticides, as judged by the ROCK LD50 varied over nearly three orders of magnitude (Table 2). There was even a 460-fold variation in toxicity of the pyrethroids. Deltamethrin (LD50 = 0.01 ng/mosquito), acrinathrin (0.02 ng/mosquito), cyhalothrin (0.02 ng/mosquito), cyfluthrin (0.02 ng/mosquito) and cypermethrin (0.06 ng/mosquito) had highest toxicity (Fig. S2). The pyrethroids with the lowest toxicity were tefluthrin (4.64 ng/mosquito), bioallethrin (3.00 ng/mosquito), etofenprox (2.06 ng/mosquito) and \textit{1-R} trans fenfluthrin (1.16 ng/mosquito). Generally, the pyrethroids with an \textit{α}-CN group were the most toxic. DDT was the least toxic insecticide tested, with a ROCK LD50 value of 10.1 ng/mosquito. Indoxacarb was the second least toxic compound (7.84 ng/mosquito), however DCJW had toxicity similar to that of some pyrethroids (1.30 ng/mosquito). We found no relationship between the pyrethroid LD50 to ROCK and the levels or resistance in KDR:ROCK (data not shown).

Fourteen of the pyrethroids tested in this study were also tested against a housefly strain, ALkdr, which is resistant to pyrethroids due to the classic L1014F mutation in an otherwise susceptible genetic background. This provided a unique opportunity to look at how these different \textit{Vssc} mutations, L1014F and S989P + V1016G, affect pyrethroid resistance. Here, when we compared the RR values for both housefly and mosquito \textit{Vssc} mutations, we found no relationship (R2 = 0.546) between them (Fig. 4). Four pyrethroids (etofenprox, permethrin, cyfluthrin and bioresmethrin) had higher resistance in ALkdr, whereas 10 (fenpropatrin, tau-fluvalinate, flumethrin, \textit{1-R} trans fenfluthrin, transfluthrin, acrinathrin, cyhalothrin, cypermethrin, tefluthrin and deltamethrin) were higher in KDR:ROCK (Fig. S3). However, there was no pattern between pyrethroid structure and resistance levels for either one.

We performed additional bioassays to compare resistance between the different life stages and to determine the inheritance of \textit{Vssc} mutations S989P + V1016G. The KDR:ROCK larvae LD50 to permethrin was 48.3 ng mL\(^{-1}\) (95% CI = 42.8 – 54.5; n = 836), and the ROCK LD50 was 1.66 ng mL\(^{-1}\) (95% CI = 1.48 – 1.94; n = 573). Thus, the permethrin resistance of the adults (40-fold) was not significantly different from that in larvae (29-fold). The degree of dominance (D) for the \textit{F}_{1} hybrid was −0.4, indicating an incompletely recessive inheritance of permethrin resistance.

4 DISCUSSION

There was a fivefold overall difference in RR levels between the structurally diverse pyrethroids. We found no general relationship between pyrethroid structure and RR values, but did observe a few differences and similarities due to single changes in chemical structures. These differences were, however, no more than 2.5-fold. Similarly, we observed no general resistance patterns for larger alcohol or acid moiety changes in the pyrethroid structures. The major challenge in making a comparison such as this one is that...
The commercial availability of bioactive compounds with single, specific changes in their structures is limited. The problem is that even small changes in the chemical structure of a pyrethroid may make it lose its toxicity to insects, or fail to increase the toxicity enough to warrant developing it into a new commercial product.

The >2000-fold DDT resistance appears to be due entirely to the Vssc mutations S989P + V1016G, because PBO and DEM were not able to measurably reduce the level of resistance. This high level of resistance raises the question of whether this pair of mutations evolved in response to DDT use (and subsequently conferred cross-resistance to pyrethroids), or whether one or both mutations evolved in response to pyrethroid use. A common mechanism of resistance to DDT was initially linked to the gene knockdown resistance ($kdr$).$^{30}$ It was demonstrated that $kdr$ could confer cross-resistance to pyrethrins and pyrethroids in several insects,$^{31–33}$ including $A. aegypti$,$^{34}$ and that this was due to insensitivity of the nervous system.$^{33,35,36}$ This was subsequently found to be due to a L1014F mutation in the Vssc gene in houseflies and
Figure 3. Resistance ratios (RRs) for the 17 pyrethroids, DDT, DCJW and indoxacarb. RRs were calculated as the LD$_{50}$ of KDR:ROCK divided by the LD$_{50}$ of ROCK. Error bars indicate the minimum and maximum 95% confidence interval range for the RRs of each insecticide.

Figure 4. Comparison of the fold difference in resistance ratios (RRs) for 14 pyrethroids between a strain with the S989P + V1016G mutations (KDR:ROCK; *Aedes aegypti*) and one with the L1014F mutation (ALkdr; housefly). Both strains were congeneric with the susceptible strains to which they were being compared. Insecticides with higher resistance due to S989P + V1016G are in light gray and those with higher resistance conferred by L1014F are in black.

German cockroaches. In *Culex*, the L1014F mutations was found to confer cross-resistance between permethrin and DDT. In *A. aegypti*, the classic L1014F mutation in Vssc is absent, although other mutations have been found. In fact, over two decades the number of Vssc mutations that confer resistance has expanded greatly, but the timelines over which these mutations occurred is unclear, particularly because sequencing Vssc only became a reality more than a decade after pyrethroids were introduced. Thus, it is unclear whether DDT or pyrethroids (the two most commonly used pyrethroids in Singapore have been permethrin and cypermethrin) or both, are responsible for the evolution of the S989P + V1016G mutations in *A. aegypti*.

The 37.5-fold cross-resistance to indoxacarb was partially (2.8-fold) lost when we tested the bioactivated form of this insecticide (DCJW), suggesting that the gene for the indoxacarb bioactivating enzyme was linked to the resistance locus. This led us to examine the genome sequence of *A. aegypti* for candidate genes. The enzyme that catalyzes the conversion of indoxacarb to DCJW is thought to belong to the esterase/amidase subclass of hydrolases. Using NCBI’s most current *A. aegypti* genome assembly Aaeq5.0 (www.ncbi.nlm.nih.gov) we looked for ~ 2 megabases in both upstream (5’) and downstream (3’) directions from Vssc. We found three genes (AAEL006034, AAEL006024 and AAEL006023) with hydrolase activity (according to gene ontology annotations GO:0016810 and GO:0016811 in VectorBase [www.VectorBase.org]) between ~ 740 and 860 kb downstream from Vssc (Table S1). These genes are reasonable candidates for the indoxacarb bioactivation enzyme, and worthy of further study. There were no genes in the upstream region that were annotated as having esterase/amidase activity.

The mechanism responsible for the 13.4-fold cross-resistance to DCJW is unclear. This could be due to the S989P + V1016G Vssc...
Table 2. Toxicity of insecticides that target the VSSC against susceptible (ROCK) and resistant (KDR:ROCK) strains of Aedes aegypti

| Insecticide   | Strain       | LD$_{50}$ (95% CI) | Slope (SE) | n   |
|---------------|--------------|--------------------|------------|-----|
| Acrinathrin   | ROCK         | 0.021 (0.019–0.024) | 3.9 (0.4)  | 480 |
| Acrinathrin   | KDR:ROCK     | 0.62 (0.54–0.70)   | 2.7 (0.2)  | 580 |
| Bioallethrin  | ROCK         | 3.00 (2.72–3.31)   | 4.5 (0.4)  | 480 |
| Bioallethrin  | KDR:ROCK     | 63.2 (56.1–70.9)   | 4.0 (0.5)  | 520 |
| Bioresemethrin| ROCK         | 0.68 (0.61–0.75)   | 5.5 (0.7)  | 383 |
| Bioresemethrin| KDR:ROCK     | 41.5 (39.0–44.3)   | 4.1 (0.2)  | 1456|
| Cis-permethrin| ROCK         | 0.55 (0.50–0.62)   | 4.4 (0.5)  | 540 |
| Cis-permethrin| KDR:ROCK     | 24.4 (21.3–28.0)   | 2.8 (0.3)  | 520 |
| Cyfluthrin    | ROCK         | 0.023 (0.021–0.026) | 3.5 (0.3)  | 700 |
| Cyfluthrin    | KDR:ROCK     | 0.54 (0.49–0.60)   | 3.9 (0.4)  | 680 |
| Cyhalothrin   | ROCK         | 0.21 (0.02–0.03)   | 3.5 (0.2)  | 959 |
| Cyhalothrin   | KDR:ROCK     | 1.24 (1.05–1.49)   | 1.9 (0.2)  | 560 |
| Cypermethrin  | ROCK         | 0.65 (0.56–0.75)   | 5.5 (0.7)  | 383 |
| Cypermethrin  | KDR:ROCK     | 41.5 (39.0–44.3)   | 4.1 (0.2)  | 1456|
| Deltamethrin  | ROCK         | 0.39 (0.33–0.44)   | 2.4 (0.1)  | 1269|
| Deltamethrin  | KDR:ROCK     | 2.06 (1.18–2.26)   | 4.0 (0.4)  | 620 |
| Etofenprox    | ROCK         | 221 (198–246)      | 2.6 (0.2)  | 1030|
| Etofenprox    | KDR:ROCK     | 1.16 (1.00–1.32)   | 2.7 (0.2)  | 858 |
| Fenpropathrin | ROCK         | 54.8 (48.6–61.6)   | 3.1 (0.2)  | 660 |
| Fenpropathrin | KDR:ROCK     | 0.53 (0.46–0.63)   | 2.7 (0.3)  | 671 |
| Flumethrin    | ROCK         | 28.7 (26.4–31.2)   | 2.9 (0.2)  | 1162|
| Flumethrin    | KDR:ROCK     | 0.50 (0.45–0.55)   | 3.6 (0.3)  | 647 |
| Permethrin    | ROCK         | 27.7 (24.4–31.3)   | 2.5 (0.2)  | 877 |
| Permethrin    | KDR:ROCK     | 0.56 (0.52–0.60)   | 4.7 (0.3)  | 1040|
| Permethrin    | KDR:ROCK     | 22.5 (20.8–24.4)   | 4.4 (0.3)  | 980 |
| Permethrin    | KDR:ROCK     | 1.80 (1.76–1.84)   | 3.8 (0.1)  | 500 |
| Permethrin+PBO | ROCK        | 0.22 (0.20–0.24)   | 3.1 (0.2)  | 1361|
| Permethrin+PBO| KDR:ROCK     | 9.14 (7.00–14.2)   | 1.7 (0.3)  | 504 |
| Tau-fluvalinate| ROCK        | 0.32 (0.29–0.36)   | 4.1 (0.4)  | 680 |
| Tau-fluvalinate| KDR:ROCK    | 22.5 (18.8–27.0)   | 1.7 (0.2)  | 660 |
| Tefluthrin    | ROCK         | 4.64 (4.14–5.25)   | 3.3 (0.3)  | 830 |
| Tefluthrin    | KDR:ROCK     | 134 (120–153)      | 2.3 (0.2)  | 1040|
| Transfluthrin | ROCK         | 0.66 (0.60–0.72)   | 3.2 (0.2)  | 840 |
| Transfluthrin | KDR:ROCK     | 38.0 (35.1–41.5)   | 4.0 (0.3)  | 840 |
| Trans-permethrin| ROCK        | 0.46 (0.42–0.50)   | 4.1 (0.4)  | 600 |
| Trans-permethrin| KDR:ROCK   | 31.3 (28.5–34.3)   | 3.8 (0.3)  | 820 |
| DCJW          | ROCK         | 1.30 (1.20–1.41)   | 5.5 (0.6)  | 480 |
| DCJW          | KDR:ROCK     | 17.4 (15.1–19.9)   | 2.4 (0.2)  | 620 |
| Indoxacarb    | ROCK         | 7.84 (6.84–8.87)   | 2.7 (0.3)  | 500 |
| Indocarbar    | KDR:ROCK     | 293 (256–340)      | 2.0 (0.1)  | 770 |
| DDT           | ROCK         | 10.1 (9.31–11.0)   | 5.8 (0.6)  | 500 |
| DDT           | KDR:ROCK     | > 22 000*          | –          | 650 |
| DDT+PBO       | KDR:ROCK     | > 22 000           | –          | 360 |
| DDT+DEM       | ROCK         | 8.61 (7.58–9.68)   | 3.5 (0.4)  | 540 |
| DDT+DEM       | KDR:ROCK     | > 22 000           | –          | 358 |

*Less than 50% kill at 22 μg/mosquito.
LD$_{50}$, ng/mosquito.

mutations or to some other factor at the pyrethroid resistance locus. Few other studies have investigated resistance to oxadiazines in pyrethroid resistant strains.24–47 Of those, only one had a known Vssc mutation (L1014F) in a resistant strain of housefly, and no cross-resistance was observed.47 However, this is not the same mutation found in A. aegypti, so whether S989P + V1016G confers cross-resistance is still unclear. One study done in field collected A. albopictus in Pakistan found resistance to multiple classes of insecticides including pyrethroids and indoxacarb;48 however, they did not evaluate the mechanisms of resistance present. Identification of the factor responsible for the DCJW cross-resistance in KDR:ROCK will require further study.

When comparing the resistance levels in the KDR:ROCK strain to a housefly strain (ALkdr) with the L1014F mutation,29 we found no relationship between the resistance levels for the different pyrethroids. This suggests that L1014F and S989P + V1016G confer
pyrethroid protection somewhat differently. In contrast to the difference in pyrethroid protection given by the different Vssc mutations, the inheritance of this trait seems very consistent. Our results indicate the inheritance of resistance conferred by S989P + V1016G is incompletely recessive. This has commonly been observed for the inheritance of resistance for other Vssc mutations such as A1215D + F1538I, V1023G + D1794Y and L1014F, although some variation has been observed between pyrethroids for the L1014F mutation in houseflies. The inheritance of a resistance allele could have important implications for resistance management. For example, control problems are likely to be manifest more slowly in a population if the resistance allele is recessive.

It would be expected that kdr would confer resistance in all life stages, due to the nature of this resistance (mutation in the target site). However, to our knowledge, the levels of resistance conferred by kdr have not been compared between life stages. We found no significant difference in the levels of permethrin resistance in larvae and adults. This was somewhat surprising because different bioassay methods were used, and different methods can sometimes manifest different RRs.

5 CONCLUSION

In summary, we report for the first time the levels of pyrethroid, DDT and oxadiazine resistance conferred by Vssc mutations S989P + V1016G in vivo. Etofenprox (and DDT) should be avoided in areas where Vssc mutations S989P + V1016G exist at high frequencies. We also found that pyrethroid structure cannot be used to predict the level of resistance conferred by kdr. Investigations of the type we carried out are hampered because small changes in the structure of a pyrethroid can sometimes lead to dramatic loss of toxicity. These results provide essential background data for future investigations of pyrethroid interactions with VSSC and for resistance management of this important disease vector.

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SUPPORTING INFORMATION

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

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