Research Article

Levels of Resistance to Pyrethroid among Distinct kdr Alleles in Aedes aegypti Laboratory Lines and Frequency of kdr Alleles in 27 Natural Populations from Rio de Janeiro, Brazil

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Background. Several mutations in voltage gated sodium channel (NaV) have been identified in Aedes aegypti populations worldwide. However, only few are related to knockdown resistance to pyrethroids, most of which with variations in the 1016 and 1534 NaV sites. In Brazil, at least two NaV alleles are known: NaVR1, with a substitution in the 1534 (1016 Val + 1 5 3 4 I l e kdr) and NaVR2, with substitutions in both 1016 and sites (1016Ile kdr + 1534Cys kdr). There is also the duplication in the NaV gene, with one copy carrying the substitution Ile1011Met, although its effects on pyrethroid resistance remain to be clarified. Our goals in this study were (1) to determine the role of each kdr NaV allele and the duplication on pyrethroid resistance and (2) to screen the frequency of the kdr alleles in 27 natural Aedes aegypti populations from the metropolitan region of Rio de Janeiro.

Methods. Pyrethroid resistance was evaluated by a knockdown time (KdT) assay, an adaptation of the WHO test tubes with paper impregnated with deltamethrin. We used laboratory-selected Ae. aegypti lineages: R1R1 and R2R2 (homozygous for the kdr NaVR1 and NaVR2 alleles, respectively), Dup (with duplication in the NaV gene), Rockefeller (the susceptibility reference control), and F1 hybrids among them. Genotyping of both 1016 and 1534 NaV sites was performed in 811 Aedes aegypti sampled from 27 localities from Rio de Janeiro (17), Niterói (6) and Nova Iguaçu (4) cities, Rio de Janeiro State, Brazil, with a TaqMan real time PCR approach. Results. The laboratory lineages R1R1, R2R2, and R1R2 were the only ones that needed more than 60 minutes to knock down all the insects exposed to the pyrethroid, being the KdT R2R2 > R1R2 > R1R1, corroborating the recessive nature of the kdr mutations. Frequency of kdr alleles NaVR1 and NaVR2 in field-caught mosquitoes varied from 0 to 52% and 43 to 86%, respectively, evidencing high levels of “resistant genotypes” (R1R1, R1R2, and R2R2), which together summed 60 to 100% in Aedes aegypti populations from Rio de Janeiro. Conclusions. The NaVR1 and NaVR2 kdr alleles confer resistance to the pyrethroid deltamethrin in homozygotes and R1R2 heterozygotes, being the R2R2 most resistant genotype. The allele containing duplication in the NaV gene, with a mutation in the 1011 site, did not confer resistance under the tested conditions. The frequencies of the “resistant genotypes” are elevated in Aedes aegypti natural populations from Rio de Janeiro.

1. Introduction

Aedes aegypti is the primary vector of dengue, chikungunya, and Zika virus in tropical and subtropical regions of the globe. The incidence of dengue cases has dramatically increased in the last decade, with an estimation of 390 million dengue infections per year [1], summed with the recent re-emergence of chikungunya and Zika. New-born malformations and neurological complications associated with Zika led the World Health Organization (WHO) to declare the “Public Health Emergency of International Concern” in 2016 [2]. This situation worsened with a strong concern regarding a potential reurbanization of yellow fever virus in Brazil, which have killed more than 200 people in rural municipalities from...
July 2017 to March 2018, i.e., 8 months [3]. This scenario reinforces the need to strengthen vector control measures to mitigate disease transmission.

New vector control strategies to reduce Ae. aegypti population density below a critical threshold have been proposed, which are expanding to vast open field application tests, with support of local communities, governments, and stimulation by WHO [4]. The dissemination of transmission blocking mosquitoes carrying Wolbachia and the release of transgenic-based sterile mosquitoes (RIDL) are among the most promising approaches developed so far [5, 6]. On the other hand, the employment of insecticides will persist for a long time as a prime strategy for rapidly reduction on mosquito density, especially during an outbreak. In this sense, it is important that these compounds are efficient over target populations. Among the classes of insecticides recommended by the World Health Organization Pesticide Scheme (WHO), pyrethroids are the most employed against Aedes mosquitoes since they provoke the fast-acting knockdown effect, are cheaper, and cause less nuisance to householders indoor. However, the excessive and uncontrolled employment of insecticides has been selecting Ae. aegypti resistant populations worldwide [7, 8]. The principal physiological mechanisms selected for pyrethroid resistance are related to increase in the expression profile of metabolic enzymes, especially cyp450 genes of the multifunction oxidases class, and point mutation in its target site, the voltage gated sodium channel (Nav) [8, 9].

There are several mutations in insect Nav genes conferring resistance to pyrethroid, with the L1014F kdr substitution being the most common, conserved among distinct insect orders. This happens since there are few modifications permitted in the highly conserved Nav gene, which responds for a central role in the neuron physiology of animals [10]. Additionally, the same kdr mutations may have multiple origins in a species, as evidenced for insects such as M. domestica [11] and An. gambiae [12]. In Ae. aegypti, however, the L1014F kdr mutation is not found due to codon constraint, to which the simultaneous selection of two mutations in the same codon would be necessary, which is unlikely to happen [13]. Besides the fact that some kdr mutations are specific for some species, in Ae. aegypti this number is uniquely high [14]. Other mutations are found in several positions of the Ae. aegypti Nav, where the relationship with resistance to pyrethroid is better described to 1016 (Val to Ile in America and Africa or Gly in Asia and Middle Eastern) and 1534 (Phe to Cys) Nav sites [15–18]. In Ae. aegypti, however, the L1014F kdr mutation is not found due to codon constraint, to which the simultaneous selection of two mutations in the same codon would be necessary, which is unlikely to happen [13]. Considering these two sites, in Brazil there is evidence of three alleles, here called Nav-S (1016 Val+ 1534 Phe+), Nav-R1 (1016 val– 1534 Cyskdr), and Nav-R2 (1016 Ilekdr + 1534 Cyskdr) [19, 20]. The I1011M is another substitution found in Ae. aegypti populations from Latin American and is involved in a gene duplication event [21]. Although it was proved to alter the sodium channel sensitivity to pyrethroids [22], its actual role in resistance is controversial in natural populations where other kdr mutations occur [23, 24]. The S989P substitution (together with V1016G and F1534C) also plays an important part for pyrethroid resistance, but its distribution seems to be restricted to Middle East/Asia [15]. Genotyping of known kdr single nucleotide polymorphisms (SNPs) in susceptible and resistant individuals from laboratory-selected lines as well as natural populations and electrophysiological studies evidencing altered Nav sensibility to pyrethroids have been corroborating the hypothesis of such SNPs with knockdown resistance [14]. Electrophysiological assays are important to determine the role of only and combined mutations in the sensibility to pyrethroids. Not less important is to evaluate the whole organism, making use of laboratory lines with homogeneous genetic backgrounds.

Here we evaluated the role of kdr mutations occurring in Brazilian Ae. aegypti populations in response to the pyrethroid deltamethrin, based on bioassays with laboratory-selected lines without interference of other known mechanisms. In addition, the frequency of kdr mutations was established for Ae. aegypti natural populations from 27 localities in Rio de Janeiro State, the most touristic city from South America and likely the port of entry of dengue virus, serotypes 1, 2, and 3 in Brazil [25, 26].

2. Methods

2.1. Laboratory Lineages. Rockefeller is an Ae. aegypti lineage reference for physiology experiments and constantly employed as a baseline of insecticide susceptibility [27]. In our laboratory, it has been continuously maintained since 1999 [28]. Rockefeller is homozygous for the NaV S allele (1016 Val+ 1534 Phe+). The lineage here called R2R2 is the same Rock-kdr previously described in [29], homozygous for the allele NaV R2 (1016 Ilekdr + 1534 Cyskdr) and maintained in the laboratory since 2012. The lineage Dup does not harbour the kdr mutations in the 1016 and 1534 NaV sites but a duplication in the NaV gene and a substitution at the 1011 site, keeping both variations 1011 Ile and 1011 Met [21].

For obtaining the RIRI lineage, we used insects maintained in the laboratory, originally collected at Santarem, PA, Brazil, a city with high frequency of the NavR1 allele and no register of NavV2R2 [19, 30]. We set up groups of one male with three virgin females, maintained together for three days in 50 mL conical plastic tubes, under the insectary conditions. Afterwards, males were removed and genotyped for the Nav 1534 site (see below); meanwhile females were offered to blood meal on anesthetized mice and three days after were individually induced to egg-laying in 6 cm Petri dishes covered with a wet filter paper, as described elsewhere [29]. After egg-laying, females were also genotyped. Eggs from both parents revealed as RIRI were induced to hatch, resulting in a total of 86 larvae that gave origin to this first RIRI lineage.

In order to homogenize the genetic background of the kdr lineages, we further backcrossed that RIRI new lineage with the previous established R2R2 [29]. To accomplish that, we first mixed RIRI males with R2R2 females, obtaining an RIR2 offspring (F1). Males from this F1 (RIR2) were then
backcrossed with R2R2 females, resulting in a F2 with the genotypes R1R2 and R2R2, in an expected 1:1 proportion. Groups of one F2 male with two R2R2 females were set up in conic tubes and carried out similarly as above. Males were genotyped and females were induced to lay eggs (F3). The F3 eggs resulting from RIR2 males were used for producing the next generation. This procedure was repeated for two more generations until F5. Then, similar groups were formed, now with both males and females from this F5. The F6 resulting eggs used for moving forward belonged to the offspring of male and female both genotyped as RIR2, among which 25% was expected to be RIR1. Finally, new groups were set up among the F6 adults. From the F7 resulting eggs, those originated from RIR1 parental were used to finally establish the new RIR1 lineage (supplementary figure S1).

Therefore, a part of the NaV, locus, the original homozygote colonies (Rockefeller, RIR1 and R2R2) had more similar genetic background, with exception of the Dup lineage with duplication in the NaV, which was not backcrossed with any of these lineages.

2.2. Bioassays. An adaptation of WHO test tubes bioassays [31] was performed with Ae. aegypti females exposed to papers impregnated in the laboratory with the pyrethroid deltamethrin at 1.7 g/cm² (0.034 % solution). Deltamethrin (Sigma-Aldrich) was dissolved to a stock concentration at 10% in acetone and then diluted to an intermediate solution at 1% in silicone (Dow Corning), following a new dilution to a work solution at 0.034 % (340 mg/L) also in the silicone. A total of 840 µL of this work solution was then pipetted over a 12x14 cm² filter paper sheet (Whatman Grade 1), with the help of an electronic multichannel pipette (Eppendorf) and a frame, which oriented the dispersion of 5 µL at each 96 equidistant spots. At all batches of impregnation, some papers were filled only with silicone as negative control. The paper sheets were air-dried for two days before their use in the bioassays. The bioassays proceeded as indicated [31]. Around 20 3-5 days old Ae. aegypti females were transferred to the resting tube and then gently blown into the respective test tube, where the knockdown was followed for up to 2 h, in intervals of 2 or 5 minutes. Each lineage was assayed with four tubes, in at least two independent times (females resulted from distinct batches of eggs and papers from different impregnation lots). Probit analysis [32] was performed in order to infer the time necessary to knockdown 95% of the individuals of each lineage (KdT 95%). The resistance ratio (RR 95) was taken by the quotient between the KdT 95 of a given lineage with the Rockefeller’s.

2.3. Ae. aegypti from Rio de Janeiro State (RJ). We took advantage of a wide sampling previously performed in 2012 in 27 localities in RJ metropolitan area, comprising three municipalities: Rio de Janeiro city (Tubiacanga, Valqueire, Urca, Olaria, Gamboa, Cajú, Pavuna, Méier, Grajaú, Paquetá, Vaz Lobo, Jardim Guanabara, São Cristóvão, Humaitá, Rio Comprido, Rio das Pedras, and Taquara neighbourhoods), Niterói City (Jurujuba, Itacoatiara, São Francisco, Fonseca, Ponta D’Areia, and Piratininga neighbourhoods), Nova Iguacu City (Cabeçú, Cerâmica, and Moquetá neighbourhoods), and Belford Roxo City (Heliópolis neighbourhood) [33]. Briefly, mosquito eggs were collected during three consecutive weeks using 60 ovitraps in a grid of 500 x 500 m² per neighbourhood. Thus, we believe our sample was representative of the genetic variation presented in each neighbourhood. Ovitraps’ paddles were brought to the lab, eggs were hatched, and larvae were reared until the adult stages, when the DNA was extracted.

2.4. kdr Genotyping. DNA was extracted from individual insects tittered in 200 µL squishing buffer (10 mM Tris-HCl pH 8.2, 2 mM EDTA, and 0.2 % Triton X-100) and 0.2 mg/L Proteinase K (Promega), as described elsewhere [34]. A customized TaqMan genotyping assay (Thermo Fisher Scientific) was employed for 1016 (Val and Ile 1016F) and 1534 (Phe and Cys 1534F), independently for each NaV site [20]. Reactions were performed in 10 µL containing 1 µL DNA, IX TaqMan Genotyping Master Mix (Thermo Fisher Scientific), the mix of primers and probes Custom TaqMan SNP Genotyping Assay (IX for Val1016Ile, AHSIDL6 and 0.5X for Phe1534Cys, AHUADFSA, Thermo Fisher Scientific), and H2O q.s. 10 µL, with the thermocycling program in accordance with the manufacturer’s instructions in QuantStudio 6 qPCR equipment (Thermo Fisher Scientific).

For genotyping variations in the 101I site (Ile or Met) we employed an allelic-PCR approach, in which the specific products were detected through a dissociation curve analysis after the amplification reaction, as described elsewhere [13, 21]. Reactions contained IX Sybr Green Master mix (ThermoFischer), 20 ng DNA, 0.24 µM primer 101I_forward 5’-GTCTCTGTATCTCGGGCTTTT-3’ common to both sequences, and 0.12 µM of each two specific primers: 101I_Ile_reverse 5’-TACCTACTAGATTGCC-3’ and 101I_Met_reverse 5’-TACCTACTAGATTACTAGAC-3’ and H2O q.s. 12.5 µL. The specificity lay on the 3’-end of the specific primers and the discrimination of the amplicons was possible due to a GC tail at the 5’-end of both specific primers, however with distinct sizes: short [GGGGGC] and long [GGGGGCAGGTTTCCGGGG] (0.7 cm), providing ‘M’ of 77 °C and 82 °C, respectively, in a dissociation curve analysis. For more details about this method, please check Saavedra-Rodriguez et al 2007 [13]. The thermocycling program consisted of 35 cycles (denaturation 94°C / 30s, annealing 57°C / 1’ and polymerase extension 72°C / 45s), followed by the standard melt curve analysis in a QuantStudio 6 qPCR equipment (Thermo Fisher Scientific).

The 95% Confidence Intervals (CI95%) of the allelic frequencies were calculated using the exact binomial approximation (http://www.biostathandbook.com/confidence.html). Comparisons among genotypic frequencies pairs were performed with exact G test and Fisher’s method, with default Markov chain parameters by Genepop version 4.2, online version (http://genepop.curtin.edu.au).

3. Results

3.1. Ae. aegypti Lineages and Bioassays. The results of 1016 and 1534 SNPs were merged to constitute the genotypes as
Table 1: List of genotypes based on SNP reactions for 1011, 1016, and 1534 Na_{v} sites of the *Aedes aegypti* laboratory lineages here evaluated.

| 1011 | 1016 | 1534 | Genotype* |
|------|------|------|-----------|
| Ile/Ile | Val/Val | Phe/Phe | SS |
| Ile/Ile | Val/Ile | Phe/Phe | SR1 |
| Ile/Ile | Val/Ile | Phe/Cys | SR2 |
| Ile/Ile | Val/Ile | Cys/Cys | SR3 |
| Ile/Ile | Val/Ile | R1R1 | R1R1 |
| Ile/Ile | Val/Ile | R1R2 | R1R2 |
| Ile/Ile | Val/Ile | R2R2 | R2R2 |
| Ile/Met | Val/Val | Phe/Phe | DD |

* The genotypes likely to occur based on the SNP genotype reactions are evidenced in bold.

Figure 1: *Voltage gated sodium channel (Na_{v}) kdr alleles of *Aedes aegypti* populations from Brazil*. Schematic representation of the Na_{v}, with its four domains, each with six hydrophobic segments. The kdr sites 1011 and 1016 are in the IIS6 segment, while the 1534 site lies in the IIIS6. Each haplotype is represented by means of the variation at each kdr site, where wild-type and kdr amino acids are indicated in black and red, respectively. The colours of the alleles are the same in the following figures.

Figure 2: The knockdown time (KdT) profile of *Aedes aegypti* laboratory lineages with distinct Na_{v} genotypes. The abscissa and ordinate in the graph indicate respectively the time in minutes for knockdown 95% of the insects (KdT_{95}) from the respective lineages and their resistant ratio (RR_{95}), considering Rockefeller (SS) as reference.

3.2. Kdr Genotyping of *Ae. aegypti* Natural Populations from Rio de Janeiro State (RJ). The three Na_{v} alleles Na_{v}S, Na_{v}R1, and Na_{v}R2 were observed among the total of 811 genotyped insects (Figure 3). Number of samples evaluated ranged from 12 (Jurujuba, Niterói) to 73 (Cerâmica, Nova Iguaçu). In total average, the Na_{v}R2 kdr allele was the most frequent (65.4%), followed by Na_{v}R1 (27.5%) and Na_{v}S (7.2%). Out of the 27 localities, only Urca presented the Na_{v}R1 allele with the highest frequency (52.5%) (Supp Table S1). The wild-type Na_{v}S was far the less frequent, ranging from 0 (Humaitá, Rio das Pedras and São Cristóvão, Rio de Janeiro) to 22.5% (Paquetá island, Rio de Janeiro), except Fonseca (Niterói) where the Na_{v}S frequency (20.6%) was higher than the Na_{v}R1 allele.
Interestingly, when pooling neighbourhood’s data to their respective regions, Na\textsubscript{V}S frequency was higher in Niterói (14.2%) than in Rio (4.0%), a significant difference, by considering that there is no overlapping among theirs IC95% (supplementary material S2). Paquetá was removed from this analysis, since it is an island distant from both Rio and Niteroi offshores. These cities are separated by the Guanabara Bay, connected by a 13 Km bridge and ferry boats.

The R2R2 genotype, which would potentially account for higher levels of resistance to pyrethroids, was the most frequent genotype (median 50.0%), and the “resistant genotypes” (R1R1, R2R2, and R1R2) together reached a median of 88.4% among Ae. aegypti from the sites evaluated (Table 2). The localities with the lowest frequency of the “resistant genotypes” were Paquetá island (60%), followed by five out of the six neighbourhoods evaluated from Niterói (64.7% in Fonseca-75.7% in Piratinha).

The genotypic frequencies among regions (Baixada, Rio, Niterói, and Paquetá island) did not differ significantly between Rio and Baixada, as well as between Niteroi and Paquetá (both exact G test P>0.05). In their turn, Rio/Baixada highly differed from Niteroi/Paquetá (see Table 3).

4. Discussion

Here we evaluated the difference in insecticide resistance levels to the pyrethroid deltamethrin in Ae. aegypti laboratory lines with distinct kdr mutations introgressed from the field and free of other known resistance mechanism. Several records of increased levels of resistance to pyrethroid, in parallel with dissemination of kdr alleles in natural vector populations, have been released in the last decade, as an indirect correlation between kdr mutation and pyrethroid resistance in Ae. aegypti [8, 35]. Laboratory selection pressure experiments with insecticide have also corroborated this correlation, when kdr frequencies have increased toward fixation [13]. In addition, electrophysiological tests based on natural populations as well as samples that undergone site
Table 2: Kdr genotypic frequencies in field populations of Aedes aegypti from Rio de Janeiro State, considering the 1016 and 1534 Na+ sites.

| locality     | n | SS  | SR1 | SR2 | R1R1 | R1R2 | R2R2 | Σχ² | p  |
|--------------|---|-----|-----|-----|------|------|------|-----|----|
| Cabucu       | 52| 0   | 0.019 | 0.115 | 0.135 | 0.462 | 0.269 | 8.8 | 0.032 | 0.865 |
| Ceramica     | 73| 0.014 | 0.041 | 0.041 | 0.110 | 0.411 | 0.384 | 0.0 | 1.000 | 0.904 |
| Moquetá      | 65| 0.015 | 0.015 | 0.077 | 0.077 | 0.215 | 0.600 | 31.5 | 0.000 | 0.892 |
| Helioápolis  | 36| 0   | 0.028 | 0.111 | 0.056 | 0.111 | 0.694 | 58.4 | 0.000 | 0.861 |
| Jurujuba     | 12| 0   | 0.250 | 0.083 | 0.583 | 0.083 | 8.5  | 0.036 | 0.750 |
| Itacoatiara  | 24| 0   | 0.167 | 0.208 | 0.292 | 0.333 | 16.3 | 0.001 | 0.833 |
| Sao Francisco| 16| 0   | 0.250 | 0.125 | 0.625 | 0.34  | 3.4  | 0.338 | 0.750 |
| Fonseca      | 17| 0.059 | 0   | 0.294 | 0.118 | 0.118 | 0.412 | 85.5 | 0.000 | 0.647 |
| Ponta D'Areia| 14| 0   | 0.286 | 0   | 0.714 | 3.4   | 0.064 | 0.714 |
| Piratininga  | 37| 0.081 | 0.054 | 0.108 | 0.270 | 0.189 | 0.297 | 27.8 | 0.000 | 0.757 |
| Tubiacanga   | 43| 0   | 0.070 | 0.047 | 0.186 | 0.163 | 0.535 | 21.3 | 0.000 | 0.884 |
| Valqueire    | 31| 0   | 0.032 | 0.097 | 0.032 | 0.290 | 0.548 | 10.0 | 0.019 | 0.871 |
| Urca         | 20| 0   | 0.050 | 0.050 | 0.300 | 0.400 | 0.200 | 1.4  | 0.708 | 0.900 |
| Olaria       | 35| 0   | 0.000 | 0.029 | 0.086 | 0.314 | 0.571 | 2.8  | 0.425 | 0.971 |
| Gamboa       | 17| 0   | 0.059 | 0.059 | 0.529 | 0.353 | 1.3  | 0.718 | 0.941 |
| Cajú         | 23| 0.043 | 0   | 0.043 | 0.087 | 0.087 | 0.739 | 4.4  | 0.224 | 0.913 |
| Pavuna       | 34| 0   | 0.118 | 0.118 | 0.265 | 0.500 | 20.7 | 0.000 | 0.882 |
| Meier        | 37| 0   | 0.054 | 0   | 0.135 | 0.297 | 0.514 | 6.6  | 0.086 | 0.946 |
| Grajaú       | 38| 0   | 0.053 | 0.053 | 0.316 | 0.579 | 4.7  | 0.194 | 0.947 |
| Paquetá      | 20| 0.050 | 0   | 0.350 | 0.250 | 0.150 | 0.200 | 54.6 | 0.000 | 0.600 |
| Vaz Lobo     | 35| 0   | 0.143 | 0.057 | 0.143 | 0.229 | 0.429 | 13.0 | 0.005 | 0.800 |
| Jardim Guanab| 32| 0   | 0.031 | 0   | 0.094 | 0.375 | 0.500 | 0.8  | 0.841 | 0.969 |
| Sao Cirstovao| 23| 0   | 0   | 0.043 | 0.522 | 0.435 | 1.2  | 0.266 | 1.000 |
| Humaitá      | 18| 0   | 0.056 | 0.111 | 0.167 | 0.500 | 0.167 | 2.6  | 0.454 | 0.833 |
| Rio Comprido | 16| 0   | 0   | 0.125 | 0.313 | 0.563 | 0.8  | 0.364 | 1.000 |
| Rio das Pedras| 17| 0   | 0   | 0.118 | 0.529 | 0.353 | 0.2  | 0.618 | 1.000 |
| Taquara      | 26| 0   | 0   | 0.038 | 0.077 | 0.192 | 0.692 | 7.9  | 0.048 | 0.962 |
| median       | 0 | 0   | 0.059 | 0.110 | 0.292 | 0.500 |       | 0.884 |    |

*Hardy-Weinberg Equilibrium test.
**Probability considering the chi-squared distribution, for one or three degrees of freedom, respectively, for three or six genotypes.

Table 3: Comparisons among kdr genotypic frequencies of Aedes aegypti from distinct regions of Rio de Janeiro State.

| Region pair         | χ² | df | P-value |
|---------------------|----|----|---------|
| Baixada x Niterói   | 10.08 | 2 | 0.006  |
| Baixada x Rio       | 1.79  | 2 | 0.409  |
| Niterói x Rio       | infinity | 2 |          |
| Baixada x Paquetá   | 13.30 | 2 | 0.001  |
| Niterói x Paquetá   | 2.09  | 2 | 0.351  |
| Rio x Paquetá       | infinity | 2 |          |

Baixada (Cabucu, Ceramica, and Moquetá ans Heliopolis), Niterói (Jurujuba, Itacoatiara, São Francisco, Fonseca, Ponta D'Areia, and Piratininga), Rio (Tubiacanga, Valqueire, Urca, Olaria, Gamboa, Cajú, Pavuna, Meier, Grajaú, Vaz Lobo, Jardim Guanabara, São Cristóvao, Humaitá, Rio das Pedras, and Taquara), and Paquetá (Paquetá island).

directed mutagenesis confirmed that kdr mutations found in Ae. aegypti alter sensibility to pyrethroids [36, 37]. However, to our knowledge, this is the first study in vivo that evinces the importance of such mutations in homogeneous kdr laboratory lines of Ae. aegypti, except for the kdr locus, i.e., with minimum interference of other possibly selected mechanisms, as well as of pleiotropic effects that might respond differently to each distinct genetic background.

WHO Pesticide Evaluation Scheme (WHOPES) recommends discriminating concentrations of insecticides for the
impregnated paper tests [38]. The most recent WHOPES plan for detecting and monitoring IR in *Aedes aegypti* recommended 0.03% as a discriminating concentration of deltamethrin [39], to where mortality is recorded 24 h after 1 h of exposition to the insecticide. Mortality under 90% indicates resistance of the evaluated population, as long as at least 100 mosquitoes were tested. This is a qualitative dose-diagnostic test, good for determining the susceptibility status of a given population although not suitable for comparing levels of resistance among populations. For this matter, a quantitative dose-response test is indicated, in which the mortality rates over a range of insecticide concentrations generate the lethal concentrations (LC) of each population. In turn, LC produces the resistance rations (RR), based on a reference lineage [40]. However, this sort of test requires a large number of insects compared to the dose-response approach. Here we applied a WHO-like dose-diagnostic test with papers impregnated on our own. Nevertheless, instead of evaluating mortality 24 h after 1h of exposition, we followed the knockdown rate for up to 2 h. With this record over the time, a semiquantitative analysis was performed by extracting the RR of the populations, in this case based on their time of knockdown. It is worth noting that this knockdown time RR displays a different scale, generally shorter than those produced by truly qualitative dose-response tests.

We employed a very efficient TaqMan assay for the rapid genotyping of the *kdr* alleles present in *Ae. aegypti* Latin American populations. Our previous allele specific PCR, although useful, generally needed constant adjustments in the number of cycles and/or the concentration of specific primers according to different thermal-cycle machinery or PCR kit employed [19, 21]. Sometimes, unspecific shallow amplification also occurred, pointing to the need of additional confirmatory reactions. The present TaqMan assay renders the genotyping process more clear and straightforward. However, one must be aware that any genotyping assay will only reveal the specific alleles available in that assay. For instance, instead of a Val/Ile mutation found in *Ae. aegypti* from Latin American, the *kdr* mutation in the 1016 Na site of Asian populations is a Val/Gly [14], to which the assay employed here is unable to detect. This is also true for other potential mutations initially under low frequencies. Therefore, these allele specific methods should only be used to evaluate genotypic frequencies of populations with a previously well-explored genetic background of the target genes.

The physiological importance of the *kdr* mutations and altered sensitivity to pyrethroids have been evaluated with mutant insect *Na* 
<sup>V</sup> gene presenting punctual or combined mutations in *Xenopus* oocytes heterologous expression system, followed by electrophysiological assays [22]. Such assays, employing direct mutagenesis of *AaNa* 
<sup>V</sup> cDNA, demonstrated that the V1016I mutation alone (which we would call Na 
<sup>V</sup>R3 allele) did not alter the channel sensitivity to pyrethroids, while F1534C *kdr* mutation (Na 
<sup>V</sup>R1 allele) reduced the channel affinity of pyrethroid type I (permethrin), but not type II (deltamethrin) [37]. In agreement, this same mutation reduced the affinity to type I but not to type II pyrethroids in the cockroach *Blatella germanica*

channel [41]. However, herein we found that although Na 
<sup>V</sup>R1 conferred lower level of resistance than Na 
<sup>V</sup>R2, RIRI insects were resistant to deltamethrin, a type II pyrethroid. The Na 
<sup>V</sup>R2 allele used to be absent in some deltamethrin resistant populations from the Northeast of Brazil, where the Na 
<sup>V</sup>R1 was found in high frequencies [19]. More recent samplings evidenced that the Na 
<sup>V</sup>R2 is disseminating and increasing in frequency also through those areas [20, 30, 42]. Vera-Malof et al. [17] proposed that the Na 
<sup>V</sup>R1 had emerged first, conferring low levels of resistance to pyrethroids, and then the V1016I arose from that allele, originating the Na 
<sup>V</sup>R2. Na 
<sup>V</sup>R2 would have been rapidly selected and dispersed, by conferring higher levels of resistance to pyrethroids. The possible Na 
<sup>V</sup>R3 (1016Ile
<sup>kd</sup>r + 1534Phe
<sup>kd</sup>r) has not ever been evidenced in Brazilian *Ae. aegypti* populations and therefore was not considered in our analysis. Indeed, we did not find any SR3, RR3, and RR3 individual.

In the house fly *Musca domestica*, the relationship of three Na 
<sup>V</sup> alleles with resistance to pyrethroid was investigated, by evaluating the susceptibility of congenic strains and their hybrids to a range of several pyrethroid compounds. The double mutant super-
<sup>kd</sup>r allele (M918T + L1014F) conferred more resistance than the classical *kdr* (L1014F), which in its turn conferred more resistance than the *kdr-his* (L1014H). The heterozygotes kdr/super-kdr and super-kdr/kdr-his presented intermediate resistance between the homozygous, characterizing an incomplete recessive inheritance partner for these *M. domestica* Na 
<sup>V</sup> alleles [43]. *Ae. aegypti* field populations from Malaysia showed increased resistance when presenting *kdr* mutation in both 1016 and 1534 Na 
<sup>V</sup> sites [44]. In that case, however, substitution in the 1016 site was Val to Gly, as common in Middle Eastern and Asian populations [15, 45, 46]. Here a similar partner was observed in *Ae. aegypti*. The double mutant kdr allele Na 
<sup>V</sup>R2 conferred more resistance to deltamethrin than Na 
<sup>V</sup>R1, evidenced by the *KD<sub>50</sub>* of the homozygote R2R2 (97.8 min), higher than the homozygote R1R1 (67.4 min), corroborating the hypothesis that the 1016 Ile
<sup>kd</sup>r mutation synergises with 1534 Cys
<sup>kd</sup>r, providing higher levels of resistance.

The heterozygote RIR2 was intermediate (79.0 min), suggesting a synergistic effect of these two alleles. The heterozygotes with the wild-type Na 
<sup>V</sup>S or the "duplicated" Na 
<sup>V</sup>D alleles were all knocked down before 60 minutes, however with higher *KD<sub>50</sub>* than the homozygotes SS and DD, characterizing an incomplete recessive inheritance of the *kdr* alleles.

The II01M mutation is frequent in *Ae. aegypti* Brazilian natural populations resistant to pyrethroids, especially on those where the Na 
<sup>V</sup>R2 allele is absent [30]. A selection pressure with pyrethroid in the laboratory increased the frequency of II01M, where 100% of the insects had the mutation, but only heterozygotes were found [24] likewise in field populations [21]. This finding raised the hypothesis that II01M mutation was part of a duplication event, which was evidenced by DNA sequencing and copy number variation qPCR assays [21]. It is of note that this Dup lineage had been originally selected from a field population and was not backcrossed with Rockefeller, differently from the process that originated RIR1 and RIR2 colonies. Although, we cannot
assume the inexistence of any other resistance mechanism in the Dup lineage, its $K_d T_{50}$ to deltamethrin (30 min) was only twice the Rockefeller's (14.5 min), not representing an expressive tolerance. It is possible to conclude that the 1011 Ile/Met mutation in this "heterozygous" conformation is not important for knockdown resistance compared to those in 1016 and 1534 Na$_V$ sites. In agreement, the same aforementioned electrophysiological assays demonstrated that I101IM reduced the sensitivity of the channel to permethrin but not to deltamethrin [37].

We took advantage of a large sampling of *Ae. aegypti* in neighbourhoods from Rio de Janeiro city and surroundings [33] for exploring the frequency of the *kdr* alleles of 27 localities. The predominance of the "resistant genotypes" (RIR1, RIR2, and R2R2) ranged from 65 to 100% among the sampled localities. Additionally, the high frequency of "resistant genotypes" matches the higher levels of resistance to pyrethroids observed from South-eastern Brazilian localities [8, 30, 47]. When pooling the samples in Rio, Baixada, and Niterói, we do not find significant difference in the genotypic frequencies between Rio and Baixada, but between Niterói and both Rio and Baixada sites. The distinct intensity of insecticide application and an unlikely active migration of *Ae. aegypti* among these localities may reflect the observed differences in the *kdr* genetic background. A population genetic analysis based on nuclear single nucleotide polymorphisms (SNP) and microsatellites revealed low overall spatial structuring among 15 out of the same 27 *Ae. aegypti* populations from Rio herein evaluated for *kdr* genotyping. The exception was the population from Paquetá island, the only which significantly differed from the other localities [33], likely due to the limited gene flow island-continent. Accordingly, samples from Paquetá presented the most divergent *kdr* frequencies and presented the highest level of the wild-type Na$_V$S allele. In this island there is a preoccupation about natural and cultural heritage conservation, in a way that the employment of insecticides is supposedly better planned and controlled than in the continent. Even though still very high, the lower frequency of *kdr* alleles was therefore expected in Paquetá island. In a recent study with *Ae. aegypti* from five neighbour towns around Merida, Yucatan, and Mexico, *kdr* frequencies, in both 1016 and 1534 Na$_V$ sites, significantly varied among towns. These differences were also significant in a finer scale at the block levels in two of the evaluated towns [48].

5. Conclusion

Several mutations in the Na$_V$ gene are likely to confer pyrethroid resistance; however due to significant fitness cost they remain at very low frequencies. Other mutations conferring similar or higher levels of resistance, nevertheless with lower fitness cost, are expected to evolve and disseminate in the population, under pyrethroid selection pressure [10]. As the selection exerted by governmental campaigns and by household insecticide applications has been continuous over *Ae. aegypti*, new other mutations are likely to be emerging from the current known wild-type and *kdr* alleles and possibly conferring even higher resistance levels, as recently evidenced in the house fly *M. domestica* [49]. This highlights the importance of monitoring not only the currently known *kdr* sites by direct genotyping technics but also the strategies of whole Na$_V$ gene sequencing associated with bioassays. Here we evidenced that the Na$_V$R2 *kdr* allele confers higher level of resistance to pyrethroid than this counterpart Na$_V$RI in *Ae. aegypti* laboratory lines with similar genetic backgrounds, also corroborating with the hypotheses of recessive inherent pattern of these *kdr* mutations. Therefore, the homozygous *kdr* genotypes, as well as the heterozygous RIR2, are likely to be the ones selected for pyrethroid resistance. Additionally, the mutation in the 1011 Na$_V$ site was not important for resistance in the lineage with a duplication in the Na$_V$ gene, conferring a heterozygous-like aspect to this mutation. A *kdr* genotyping survey of *Ae. aegypti* from 27 distinct localities from Rio de Janeiro city and surroundings detected high frequencies of "resistant genotypes," probably reflecting the high selection pressure exerted principally by household insecticide applications. This kind of molecular monitoring is of relevance, yet studies for unrevealing new markers related to resistance to other classes of insecticides are necessary.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Supplementary Materials

Supplementary Figure S1. Scheme of backcrosses toward selection of *Aedes aegypti* *kdr* lineages, highlighting the process of background homogenization between R2R2 and RIR1. *Please follow detailed explanation in the Material and Methods.* Table Spp S1. Time of knockdown profile under exposition to the pyrethroid deltamethrin (0.03%) in *Aedes aegypti* laboratory lineages with distinct genotypes for *kdr* alleles. Table Spp S2. *Kdr* allelic frequencies, considering 1016 and 1534 Na$_V$ sites, in *Aedes aegypti* populations from Rio de Janeiro State. *Supp. Material S2.* *Kdr* allelic frequencies in *Aedes aegypti* populations from Rio de Janeiro State. Each dot represents the frequency of the respective allele, with the confidence interval 95% amplitude indicated by the vertical bars. A = Cabuçu, B = Carioca, C = Moquetá, D = Heliópolis, E = Jurujuba, F = Itacoatiara, G = São Francisco, H = Fonseca, I = Ponta D’Areia, J = Piratininga, K = Tubiacanha, L = Vaiqueire, M = Urca, N = Olaria, O = Gamboa,
P = Cajú, Q = Pavuna, R = Méier, S = Grajaú, T = Paquetá, U = Vaz Lobo, V = Jardim Guanabara, W = São Cristóvão, X = Rio Comprido, Y = Humaitá, Z = Rio das Pedras, and 2 = Taquara. (Supplementary Materials)

References

[1] S. Bhatt, P. W. Gething, O. J. Brady et al., “The global distribution and burden of dengue,” Nature, vol. 496, no. 7446, pp. 504–507, 2013.

[2] WHO. WHO statement on the first meeting of the International Health Regulations (2005) (IHR 2005) Emergency Committee on Zika virus and observed increase in neurological disorders and neonatal malformations 2016 Available from: http://www.who.int/mediacentre/news/statements/2016/1st-emergency-committee-zika/en/.

[3] D. Couto-Lima, Y. Madec, M. I. Bersot et al., “Potential risk of re-emergence of urban transmission of Yellow Fever virus in Brazil facilitated by competent Aedes populations,” Scientific Reports, vol. 7, no. 1, 2017.

[4] WHO. Mosquito (vector) control emergency response and preparedness for Zika virus 2016 Available from: http://www.who.int/mediacentre/news/statements/2016/1st-emergency-committee-zika/en/.

[5] A. Malavasi, “Project Aedes transgenic population control in Juazeiro and Jacobina Bahia, Brazil,” BMC Proceedings, vol. 8, no. Suppl 4, p. O11, 2014.

[6] L. B. Carrington, B. C. N. Tran, N. T. H. Le et al., “Field- and clinically derived estimates of Wolbachia-mediated blocking of dengue virus transmission potential in Aedes aegypti mosquitoes,” Proceedings of the National Academy of Sciences of the United States of America, vol. 115, no. 2, pp. 361–366, 2018.

[7] H. Ransan, J. Burhani, N. Lumjuan, and I. V. W. C. Black, “Insecticide resistance in dengue vectors,” TropIKAnet, no. 1, 2010.

[8] C. L. Moyes, J. Vontas, A. J. Martins et al., “Contemporary status of insecticide resistance in the major Aedes vectors of arboviruses infecting humans,” PLOS Neglected Tropical Diseases, vol. II, no. 7, Article ID e0005625, 2017.

[9] S. Kasai, O. Komagata, K. Itokawa et al., “Mechanisms of Pyrethroid Resistance in the Dengue Mosquito Vector, Aedes aegypti: Target Site Insensitivity, Penetration, and Metabolism,” PLOS Neglected Tropical Diseases, vol. 8, no. 6, Article ID e2948, 2014.

[10] R. H. Ffrench-Constant, B. Pittendrigh, A. Vaughan, and N. Anthony, “Why are there so few resistance-associated mutations in insecticide target genes?” Philosophical Transactions of the Royal Society B: Biological Sciences, vol. 353, no. 1376, pp. 1685–1693, 1998.

[11] F. D. Rinkevich, S. M. Hedtke, C. A. Leichter et al., “Multiple Origins of kdr-type Resistance in the House Fly, Musca domestica,” PLoS ONE, vol. 7, no. 12, Article ID e52761, 2012.

[12] J. Pinto, A. Lynd, J. L. Vicente et al., “Multiple origins of knockdown resistance mutations in the afrotropical mosquito vector Anopheles gambiae,” PLoS ONE, vol. 2, no. II, Article ID e1243, 2007.

[13] K. Saavedra-Rodriguez, L. Urdaneta-Marquez, S. Rajatileka et al., “A mutation in the voltage-gated sodium channel gene associated with pyrethroid resistance in Latin American Aedes aegypti,” Insect Molecular Biology, vol. 16, no. 6, pp. 785–798, 2007.

[14] Y. Du, Y. Nomura, B. S. Zhorov, and K. Dong, “Sodium channel mutations and pyrethroid resistance in Aedes aegypti,” Insects, vol. 7, no. 4, 2016.

[15] A. M. Al Nazawi, J. Aqlli, M. Alzahrani, P. J. McCall, and D. Weetman, “Combined target site (kdr) mutations play a primary role in highly pyrethroid resistant phenotypes of Aedes aegypti from Saudi Arabia,” Parasites & Vectors, vol. 10, no. 1, pp. 1–10, 2017.

[16] H. Kawada, Y. Higa, K. Futami et al., “Discovery of Point Mutations in the Voltage-Gated Sodium Channel from African Aedes aegypti Populations: Potential Phylogenetic Reasons for Gene Intronversion,” PLOS Neglected Tropical Diseases, vol. 10, no. 6, Article ID e0004780, 2016.

[17] F. Z. Vera-Maloof, K. Saavedra-Rodriguez, A. E. Elizondo-Quiroga, S. Lozano-Fuentes, and W. C. Black IV, “Coevolution of the Ile1,016 and Cys1,534 Mutations in the Voltage Gated Sodium Channel Gene of Aedes aegypti in Mexico,” PLOS Neglected Tropical Diseases, vol. 9, no. 12, Article ID e0004263, 2015.

[18] G. Seixas, L. Grigoraki, D. Weetman et al., “Insecticide resistance is mediated by multiple mechanisms in recently introduced Aedes aegypti from Madeira Island (Portugal),” PLOS Neglected Tropical Diseases, vol. 11, no. 7, Article ID e0005799, 2017.

[19] J. G. B. Linss, L. P. Brito, G. A. Garcia et al., “Distribution and dissemination of the Val1016Ile and Phe1534Cys Kdr mutations in Aedes aegypti Brazilian natural populations,” Parasites & Vectors, vol. 7, no. 1, article 25, 2014.

[20] D. F. Bellinato, P. F. Viana-Medeiros, S. C. Araújo et al., “Resistance Status to the Insecticides Temephos, Deltamethrin, and Diflubenzuron in Brazilian Aedes aegypti Populations,” BioMed Research International, vol. 2016, Article ID 8603263, pp. 1–12, 2016.

[21] A. J. Martins, L. P. Brito, J. G. Linss et al., “Evidence for gene duplication in the voltage-gated sodium channel gene of Aedes aegypti,” Evolution, Medicine, and Public Health, vol. 2013, no. 1, pp. 148–160, 2013.

[22] K. Dong, Y. Du, F. Rinkevich et al., “Molecular biology of insect sodium channels and pyrethroid resistance,” Insect Biochemistry and Molecular Biology, vol. 50, no. 1, pp. 1–17, 2014.

[23] A. J. Martins, R. M. Lins, J. G. Linss et al., “Voltage-gated sodium channel polymorphism and metabolic resistance in pyrethroid-resistant Aedes aegypti from Brazil,” The American Journal of Tropical Medicine and Hygiene, vol. 81, no. 1, pp. 108–115, 2009.

[24] A. J. Martins, C. D. M. Ribeiro, D. F. Bellinato, A. A. Peixoto, D. Valle, and J. B. P. Lima, “Effect of insecticide resistance on development, longevity and reproduction of field or laboratory selected Aedes aegypti populations,” PLoS ONE, vol. 7, no. 5, Article ID e31889, 2012.

[25] R. M. R. Nogueira, M. P. Miagostovich, E. Lampe, R. W. Souza, M. A. S. Pereira, and H. G. Schatzmayr, “Dengue Virus Type 3 and dengue 2 serotypes,” Memórias do Instituto Oswaldo Cruz, vol. 96, no. 7, pp. 925–926, 2001.

[26] R. M. R. Nogueira, P. M. Miagostovich, E. Lampe, R. W. Souza, S. M. O. Zagne, and H. G. Schatzmayr, “Dengue epidemic in the state of Rio de Janeiro, Brazil, 1990–1: Co-circulation of dengue 1 and dengue 2 serotypes,” Epidemiology and Infection, vol. 111, no. 1, pp. 163–170, 1993.

[27] G. Kuno, “Early history of laboratory breeding of Aedes aegypti (Diptera: Culicidae) focusing on the origins and use of selected strains,” Journal of Medical Entomology, vol. 47, no. 6, pp. 957–971, 2010.
[28] J. B. Lima, M. P. Da-Cunha, R. C. Da Silva et al., “Resistance of Aedes aegypti to organophosphates in several municipalities in the State of Rio de Janeiro and Espírito Santo,” The American Journal of Tropical Medicine and Hygiene, vol. 68, no. 3, pp. 329–333, 2003.

[29] L. P. Brito, J. G. B. Linss, T. N. Lima-Camara et al., “Assessing the effects of Aedes aegypti kdr mutations on pyrethroid resistance and its fitness cost,” PLoS ONE, vol. 8, no. 4, Article ID e60878, 2013.

[30] G. d. Garcia, M. R. David, A. d. Martins et al., “The impact of insecticide applications on the dynamics of resistance: The case of four Aedes aegypti populations from different Brazilian regions,” PLOS Neglected Tropical Diseases, vol. 12, no. 2, p. e0066227, 2018.

[31] WHO. Monitoring and managing insecticide resistance in Aedes mosquito populations - Interim guidance for entomologists. Geneva: World Health Organization; 2016.

[32] M. Raymond, “Presentation d’une programme d’analyse log-probit pour microordinateur cahiers Orstrom,” Sérr Ent Med Parasitol, vol. 22, pp. 117–121, 1985.

[33] G. Rašić, R. Schama, R. Powell et al., “Contrasting genetic structure between mitochondrial and nuclear markers in the dengue fever mosquito from Rio de Janeiro: Implications for vector control,” Evolutionary Applications, vol. 8, no. 9, pp. 901–915, 2015.

[34] A. J. Martins, J. B. P. Lima, A. A. Peixoto, and D. Valle, “Frequency of Val1016Ile mutation in the voltage-gated sodium channel gene of Aedes aegypti Brazilian populations,” Tropical Medicine & International Health, vol. 14, no. 11, pp. 1351–1355, 2009.

[35] G. P. García, A. E. Flores, I. Fernández-Salas et al., “Recent rapid rise of a permethrin knock down resistance allele in Aedes aegypti in México,” PLOS Neglected Tropical Diseases, vol. 3, no. 10, article e531, 2009.

[36] C. Brengues, N. J. Hawkes, F. Chandre et al., “Pyrethroid and DDT cross-resistance in Aedes aegypti is correlated with novel mutations in the voltage-gated sodium channel gene,” Medical and Veterinary Entomology, vol. 17, no. 1, pp. 87–94, 2003.

[37] Y. Du, Y. Nomura, G. Satar et al., “Molecular evidence for dual pyrethroid-receptor sites on a mosquito sodium channel,” Proceedings of the National Academy of Sciences of the United States of America, vol. 110, no. 29, pp. 11785–11790, 2013.

[38] WHO. Discriminating concentrations of insecticides for adult mosquitoes. In: Organization WH, editor. WHO Pesticide Evaluation Scheme 2016.

[39] WHO. Monitoring and managing insecticide resistance in Aedes mosquito populations. Interi, guidance for entomologists.: World Health Organization; 2016. page 11.

[40] W. R. Halliday and K. P. Burnham, “Choosing the optimal diagnostic dose for monitoring insecticide resistance,” Journal of Economic Entomology, vol. 83, no. 4, pp. 1151–1159, 1990.

[41] Z. Hu, Y. Du, Y. Nomura, and K. Dong, “A sodium channel mutation identified in Aedes aegypti selectively reduces cockroach sodium channel sensitivity to type I, but not type II pyrethroids,” Insect Biochemistry and Molecular Biology, vol. 41, no. 1, pp. 9–13, 2011.

[42] P. F. Viana-Medeiros, D. F. Bellinato, A. J. Martins, and D. Valle, “Insecticide resistance, associated mechanisms and fitness aspects in two Brazilian Stegomyia aegypti (= Aedes aegyptii) populations,” Medical and Veterinary Entomology, vol. 31, no. 4, pp. 340–350, 2017.

[43] H. Sun, K. P. Tong, S. Kasai, and J. G. Scott, “Overcoming super-knock down resistance (super-kdr) mediated resistance: multi-halogenated benzyl pyrethroids are more toxic to super-kdr than kdr house flies,” Insect Molecular Biology, vol. 25, no. 2, pp. 126–137, 2016.

[44] I. H. Ishak, Z. Jaal, H. Ranson, and C. S. Wondji, “Contrasting patterns of insecticide resistance and knockdown resistance (kdr) in the dengue vectors Aedes aegypti and Aedes albopictus from Malaysia,” Parasites & Vectors, vol. 8, no. 1, article no. 181, 2015.

[45] S. A. Stenhose, S. Plernsub, J. Yanola 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,” Parasites & Vectors, vol. 6, no. 1, article no. 253, 2013.

[46] C. Chang, W.-K. Shen, T.-T. Wang, Y.-H. Lin, E.-L. Hsu, and S.-M. Dai, “A novel amino acid substitution in a voltage-gated sodium channel is associated with knockdown resistance to permethrin in Aedes aegypti,” Insect Biochemistry and Molecular Biology, vol. 39, no. 4, pp. 272–278, 2009.

[47] M. L. G. Macoris, M. T. M. Andrighetti, D. M. V. Wanderley, and P. E. M. Ribolla, “Impact of insecticide resistance on the field control of Aedes aegypti in the State of São Paulo,” Journal of the Brazilian Society of Tropical Medicine, vol. 47, no. 5, pp. 573–578, 2014.

[48] R. Deming, P. Manrique-Saide, A. Medina Barreiro et al., “Spatial variation of insecticide resistance in the dengue vector Aedes aegypti presents unique vector control challenges,” Parasites & Vectors, vol. 9, no. 1, article no. 67, 2016.

[49] S. Kasai, H. Sun, and J. G. Scott, “Diversity of knockdown resistance alleles in a single house fly population facilitates adaptation to pyrethroid insecticides,” Insect Molecular Biology, vol. 26, no. 1, pp. 13–24, 2017.