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Short Communication

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Detection and Distribution of V1016I^{kdr} Mutation in the Voltage-Gated Sodium Channel Gene in *Aedes aegypti* (Diptera: Culicidae) Populations From Sergipe State, Northeast Brazil

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Abstract

Aedes aegypti (L.) resistance to pyrethroids was recorded in Brazil few years after its introduction as the adulticide in the National Dengue Control Program campaigns. Altered susceptibility to pyrethroids had been reported in the state of Sergipe, northeast Brazil, through biological assays, even before its use against *Ae. aegypti* in the state. Metabolic and target-site resistance mechanisms were also revealed in samples from Aracaju, the capital of Sergipe. Herein, we investigated the presence and distribution of the *kdr* mutation V1016I^{kdr} in *Ae. aegypti* populations from different municipalities of the state. *Aedes aegypti* eggs were collected from seven municipalities located in areas showing different climatic types and infestation levels. Approximately 20 *Ae. aegypti* females from each municipality (total of 135 subjects) were individually submitted to allele-specific polymerase chain reaction (AS-PCR) for the 1016 site of the voltage-gated sodium channel (Na_V). The V1016I^{kdr} mutation was found in subjects from all the municipalities under study with a high frequency of heterozygotes in several locations. Homozygous recessive subjects (resistant *kdr* genotype) were found only in one municipality. The results suggest a wide distribution of the V1016I^{kdr} mutation in the northeast Brazil, which indicates urgent need for monitoring the effectiveness of the pyrethroids currently used for vector control.

Key words: *Aedes aegypti*, kdr mutation, pyrethroid resistance, sodium channel

Organophosphate and pyrethroid insecticides are widely used in Brazil for the control of *Aedes aegypti* (L.), followed by biolarvicides and insect growth regulators to a lesser extent (Maciel-de-Freitas et al. 2012). Pyrethroid use was introduced in Brazil in 1989 in Sao Paulo state, replacing organophosphates for mosquito control. Its use in the northeast region of the country began only 10 yr later (Macoris et al. 2007) and remains the first choice for mosquito control. Biological assays with *Ae. aegypti* populations from Sergipe state (northeast region) showed altered susceptibility to pyrethroids even before the introduction and use of cypermethrin and deltamethrin via ultralow volume application in governmental campaigns (Da-Cunha et al. 2005, Macoris et al. 2007). The widespread, frequent use of these compounds has caused the establishment of resistant mosquito populations and contributed to the failure of the vector control programs (Montella et al. 2007, Maciel-de-Freitas et al. 2014).

Pyrethroids (deltamethrin, alpha-cypermethrin, permethrin, and cypermethrin) are among the most widely used chemical compounds for the control of adult insects. These insecticides target the voltage-dependent sodium channel (Na_v), in the central nervous system of the insects, increasing the influx of Na+ in neurons. Consequently, the insect suffers repetitive muscle contractions, followed by paralysis, and eventually death, an effect known as knockdown (Dong 2007). Resistance to this effect is thus called knockdown resistance (*kdr*), described in several insect species, including mosquitoes (Knipple et al. 1994, Liu et al. 2000, Davies et al. 2007, Martins and Valle 2012, Linss et al. 2014).

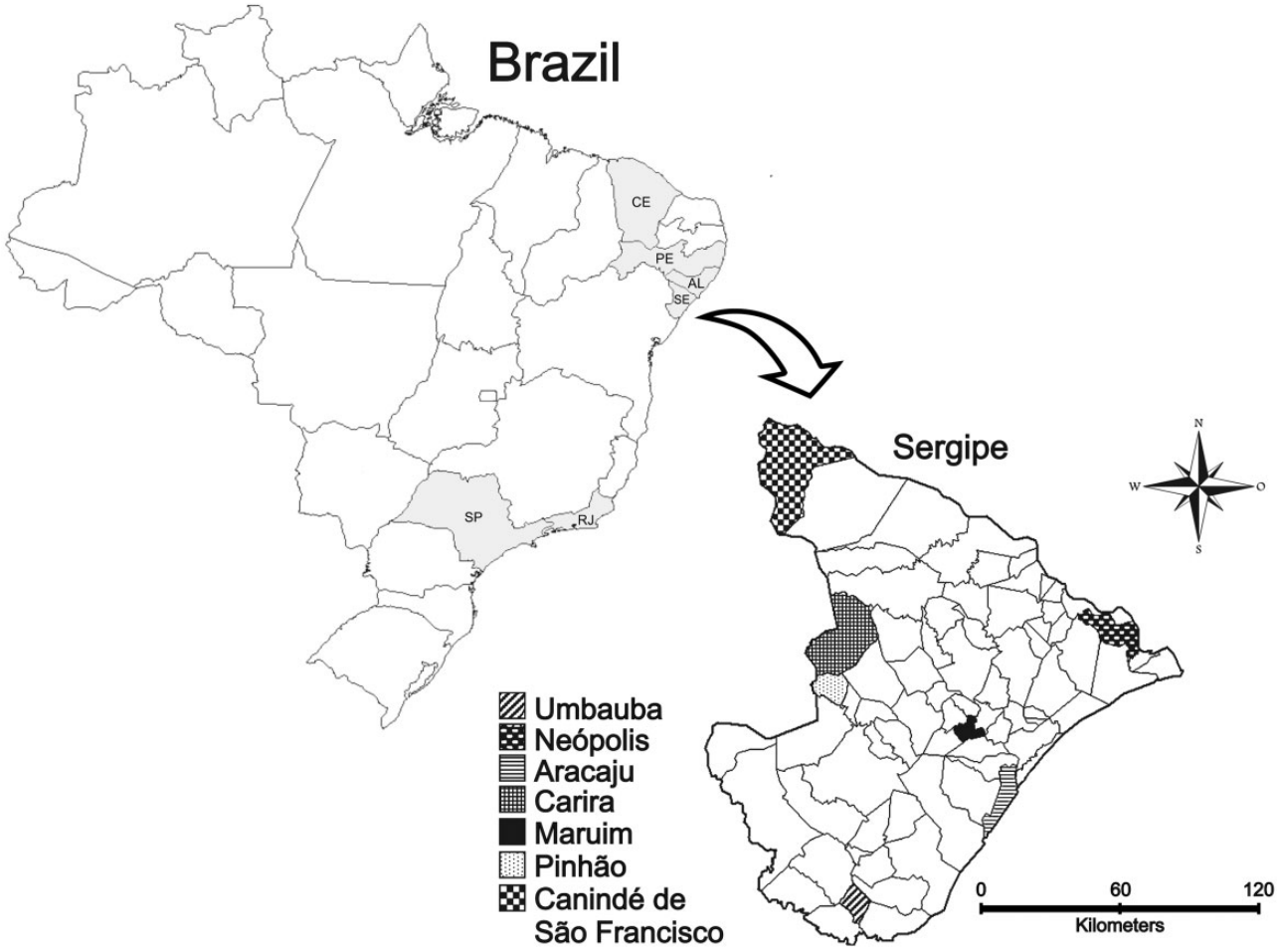

Fig. 1. Map of Brazil showing the states with reported pyrethroid resistance cited in this study and the municipalities in Sergipe state selected for sample collection. SP: São Paulo, RJ: Rio de Janeiro, SE: Sergipe, AL: Alagoas, PE: Pernambuco, CE: Ceará.

Different point mutations that generate amino acid substitutions in the Na_v have been linked to pyrethroid resistance in many mosquito species such as *Culex quinquefasciatus* Say (Chen et al. 2010), *Aedes albopictus* (Skuse) (Kasai et al. 2011), and at least 13 different anophelines species (Silva et al. 2014). In *Ae. aegypti*, point mutations in several sites have been found in pyrethroid-resistant populations (Brengues et al. 2003). However, substitutions in two sites: 1016 Val/Ile (Latin America) or Val/Gly (southeast Asia) and 1534 Phe/Cys (Americas and Asia) stand out with strong evidence for a correlation with *kdr*-based pyrethroid resistance in field populations (Saavedra-Rodriguez et al. 2007, Yanola et al. 2011, Linss et al. 2014). Altered susceptibility to pyrethroids was recorded in Sao Paulo, Rio de Janeiro, Pernambuco, Alagoas, and Sergipe states (as shown in Fig. 1) just a few years after its introduction for the control of *Ae. aegypti* in the National Dengue Control Program (Da-Cunha et al. 2005, Macoris et al. 2007). The $V1016I^{kdr}$ mutation was recorded in populations collected since 2006 throughout Brazil, especially in southeastern and central-western regions (Martins et al. 2009). In the northeastern states of Brazil, the presence of this mutation was first recorded in 2009 in State of Ceará, although only in heterozygous form (Lima et al. 2011), and a few years later in three other states, including Sergipe. By 2012, the variant double mutant ($1016I^{kdr} + 1534C^{kdr}$) was found to be the most frequent Na_V allele in the *Ae. aegypti* population from Aracaju, the capital of Sergipe (Linss et al. 2014), where biological assays had already indicated low susceptibility to pyrethroids (Da-Cunha et al. 2005). In this context, we evaluated the occurrence of $1016I^{kdr}$ mutation in a more extended panel, from seven municipalities of Sergipe state.

Materials and Methods

Aedes aegypti eggs were collected from the following seven municipalities of the state of Sergipe, Brazil: Umbaúba, Neópolis, Aracaju, Carira, Maruim, Pinhão, and Canindé de São Francisco (Fig. 1). All sampling was carried out between 2011 and 2012. In total, 100 ovitraps were installed in urban areas in each city. In Aracaju, the study was focused on a specific district, and 30 ovitraps were installed. The selection of the municipalities was designed to represent most of the state and reach different climatic types (Atlantic Rainforest, Caatinga, and Agreste representing the transition area) and different infestation levels (Table 1). Infestation levels are estimated using a Larval Index Rapid Assay used for Dengue surveillance in Brazilian cities since 2003. The index is calculated using a random sampling technique with a sample unit of 9,000 to 12,000 dwellings, from which a maximum of 450 houses are randomly selected for inspection for the presence of containers with *Ae. aegypti* larvae (Coelho et al. 2008).

Mosquitoes were reared in the insectary of the Laboratory of Entomology and Tropical Parasitology of the Federal University of

Table 1. Characterization of the municipalities in Sergipe state used for sample collection

Municipality	Climatic type	Infestation level[a]	Ovitrap positivity (%)[b]
Umbaúba	Atlantic rainforest	2.2	76
Neópolis	Agreste	0.2	28
Aracaju	Atlantic rainforest	1.0	56
Carira	Agreste	5.4	80
Maruim	Atlantic rainforest	3.8	91
Pinhão	Agreste	NR	39
Canindé de São Francisco	Caatinga	0.6	51

NR,—Not reported.

[a]*Aedes aegypti* infestation results from sampling carried out in 2012. Infestation levels correspond to the number of houses (per 100 houses) that tested positive for the presence of breeding sites that contain *Ae. aegypti* larvae. Numbers <1 are considered satisfactory, 1–3.9 denote a condition of alert, and numbers >4 indicate risk of an outbreak. Source: Ministry of Health, available at: www.dengue.org.br/tabela_municipios_dengue_191_11_2013.pdf

[b]The accumulated number of positive ovitraps in two weeks per 100 ovitraps installed.

Sergipe in an acclimatized environment with controlled temperature and humidity (temperature of 26 ± 2°C, relative humidity 70% ± 20°C, and a photoperiod of 12:12 [L:D] h).

Approximately 20 wild *Ae. aegypti* females were analyzed from each municipality (total of 135 subjects). Genomic DNA was extracted according to the protocol described by Ayres et al. (2004) with modifications. Briefly, individual insects were macerated in 500 µl lysis buffer (5 M NaCl, 0.5 M Tris-HCl, pH 8.0, 0.5 M ethylenediaminetetraacetic acid [EDTA], pH 8.0), 3.6 µl proteinase K (0.5 mg/ml), and 60 µl of 10% sodium dodecyl sulfate. The macerate was incubated at 60°C for 5 h. Subsequently, 67 µl of 5 M NaCl was added, and the mixture was vortexed for 30 s and centrifuged at 14,000 rpm for 20 min. The supernatant was removed and transferred to a new microtube. DNA was precipitated by adding an equal volume of isopropanol, incubated at −20°C overnight, and centrifuged at 14,000 rpm for 20 min. The pellet was washed with 70% ethanol, centrifuged at 14,000 rpm for 10 min, dried, and resuspended in 30 µl TE (Tris-EDTA, 10:1 mM). After extraction, the DNA samples were stored at −20°C until their use in PCR analysis.

To detect the V1016I^{kdr} mutation, PCR was performed using the following allele-specific primers developed by Saavedra-Rodriguez et al. (2007): mutant allele primer (5′-GCG GGC ACA AAT TGT TTC CCA CCC GCA CTG A-3′), wild allele primer (5′-GCG GGC AGG GCG GGG GCG GGG CCA CAA ATT GTT TCC CAC CCG CAC CGG-3′), and common allele primer (5′-GGA TGA ACC GAA ATT GGA CAA AAG C-3′). The PCR was performed in a final volume of 15 µl that contained 50 ng of genomic DNA, 10× RB buffer (10 mM Tris-HCl pH 8.5, 50 mM KCl, 1.5 mM $MgCl_2$), 0.3 units of Taq polymerase (Phoneutria Biotechnology and Services, São Paulo, Brazil), 200 µM deoxynucleotide triphosphates (dNTP), 0.30 µM of the common primer, and 0.15 µM of each specific primer in a Veriti thermocycler (Applied Biosystems, Foster City, CA) for 5 min at 95°C for initial denaturation, followed by 35 cycles of 30 s at 95°C for denaturation, 40 s at 60°C for annealing, and 45 s at 72°C for extension. Three positive controls (wild type homozygous, mutant heterozygous, and mutant recessive genotypes), and a negative control (without any DNA) were included. The separation of the PCR products was performed by electrophoresis on 10% polyacrylamide gel. According to the authors (Saavedra-Rodriguez et al. 2007), presence of a single band of 98 bp indicates dominant homozygous subject (susceptible genotype), the presence of two bands of 98 and 78 bp represents a heterozygous subject (susceptible genotype), whereas the presence of a single band of 78 bp indicates a homozygous recessive subject (resistant genotype). Based on these observations, allelic and genotypic frequencies were calculated and tested for Hardy–Weinberg equilibrium.

Results and Discussion

Monitoring insecticide resistance is essential to prevent the establishment of resistant populations and guide decision-making for vector control. However, lack of resources and government monitoring initiatives often leave small states, like Sergipe, with little information to implement insecticide resistance management programs. So far, there are two *kdr* alleles described in Brazilian *Ae. aegypti* populations (Linss et al. 2014): R1 (mutant only in the 1534 Na_V site—1016V$^+$ + 1534C^{kdr}) and R2 (mutant in both 1016 and 1534 Na_V sites—1016I^{kdr} + 1534C^{kdr}). Here, we investigated the variation in the 1016 Na_V site in mosquitoes from seven municipalities of Sergipe state. The *kdr* mutation 1016I^{kdr}, corresponding to the R2 allele, was evident in all localities under study (Fig. 2), with a high frequency of heterozygotes. As the knockdown resistance driven by the *kdr* mutation is a recessive trait, the 1016I^{kdr} allele can spread quickly departing from a small frequency of homozygous individuals if selection pressure is maintained (Saavedra-Rodriguez et al. 2007, Linss et al. 2014).

The 1016I^{kdr} allelic frequency varied from 2.5% in Umbaúba up to 40% in Pinhão. Although these localities are distant from each other (~91 km), not only they present distinct urban characteristics but also have different environmental characteristics. This may favor the mosquito populations to become well structured, with reduced gene flow among them, and consequently subject the gene pool to very local selection pressures. High variation in the 1016I^{kdr} allelic frequency (similar to Umbaúba and Pinhão) was also observed between two municipalities of Ceará state in northeast Brazil (44% in Crato and 8% in Juazeiro do Norte; Lima et al. 2011) and among closely located Mexican cities (Garcia et al. 2009).

Although the *kdr* allele was found in all localities sampled from Sergipe, only Neopolis showed homozygous individuals for the mutation (Table 2). As a matter of fact, a recent study from our group (data not shown), using nine single-nucleotide polymorphism markers previously used for a population of *Ae. aegypti* in Brazil (see Paduan and Ribolla 2009), indicated that the vector population from Neópolis had a significant deviation ($P > 0.05$) between the observed (Ho) and expected (He) heterozygosity for three out of the nine loci analyzed, including Na/K (sodium/potassium channel), CYP9J2 (cytochrome P450), and Chym (chymotrypsin). These results together suggest that the mosquito population of this municipality is subjected to a distinct selection pressure, compared with the other populations, although Neopolis presented the lowest infestation level at the time of the study.

Pyrethroids have been used in the northeast of Brazil for past 15 yr for *Ae. aegypti* control, and their use is intensified during Dengue epidemics, which could result in the rapid rise of resistant populations as well as the increase in resistance levels (Maciel-de-Freitas 2014). This factor may have influenced the fast spread of the *kdr* mutation in Sergipe, along with control programs for visceral leishmaniasis, which also use pyrethroids. The presence of different

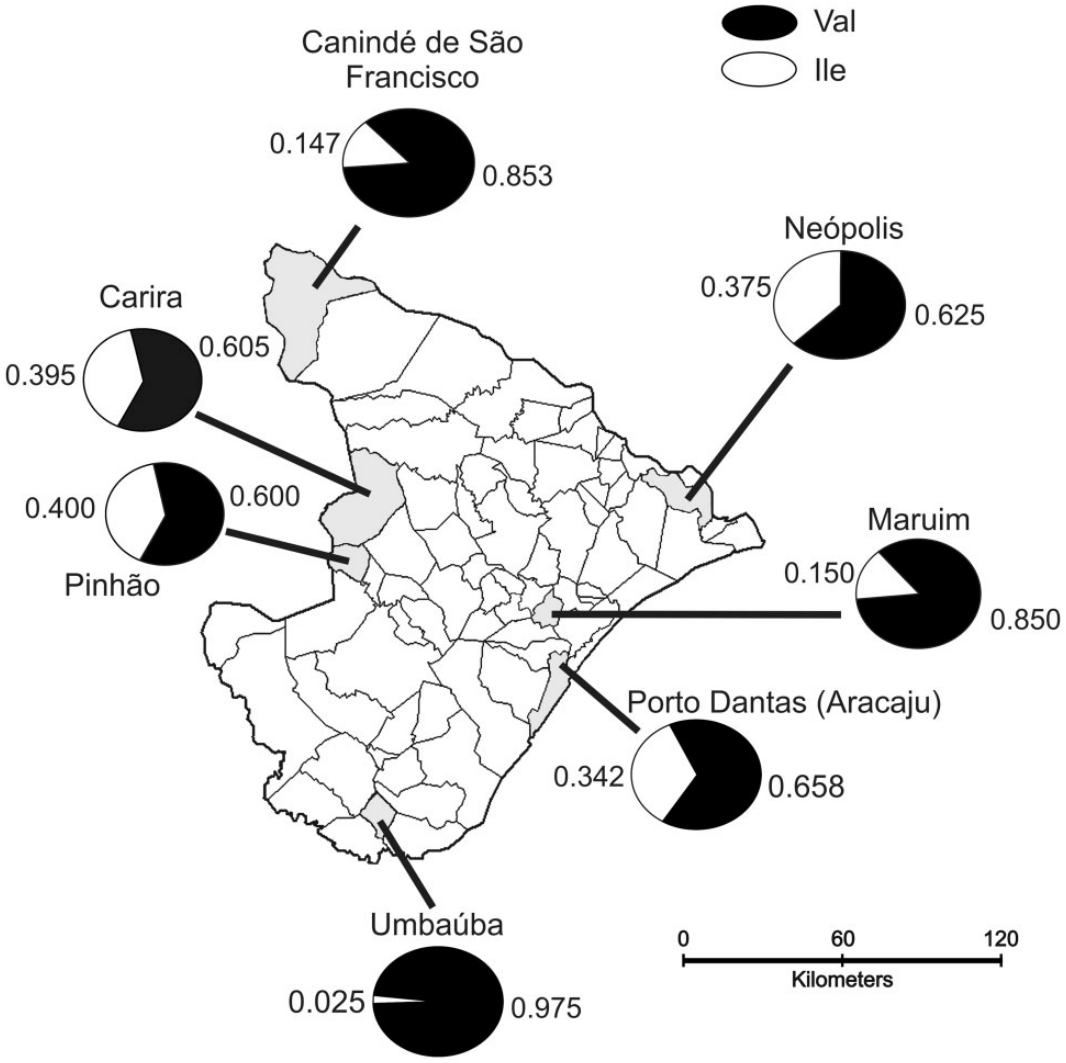

Fig. 2. Wild type (1016Val) and mutant alleles (1016Ile) frequencies for the mutation at codon 1016 of the voltage-dependent sodium channel in *Ae. aegypti* collected from seven locations in Sergipe state, Northeast Brazil.

Table 2. Genotypic frequencies of *Ae. aegypti* populations collected in 2012

Genotypic frequency						
Municipality	*n* total	Val/Val (*n*)	Val/Ile (*n*)	Ile/Ile (*n*)	c2	*P*
Umbaúba	20	0.950 (19)	0.050 (1)	0 (0)	0.01	0.9087
Neópolis	20	0.500 (10)	0.250 (5)	0.250 (5)	4.36	0.0369
Aracaju	19	0.316 (6)	0.684 (13)	0 (0)	5.14	0.0234
Carira	19	0.211 (4)	0.789 (15)	0 (0)	8.08	0.0045
Maruim	20	0.700 (14)	0.300 (6)	0 (0)	0.62	0.4300
Pinhão	20	0.200 (4)	0.800 (16)	0 (0)	8.89	0.0029
Canindé	17	0.706 (12)	0.294 (5)	0 (0)	0.51	0.4772

P = probability based on distribution c2, with one degree of freedom.

allelic frequencies among relatively near municipalities may be partially explained by the multifactorial nature of insecticide resistance and its dependency on the environment (availability and types of breeding sites), operational issues (frequency, amount of insecticide application, and exposure time), and genetic factors (changes in metabolic genes and target sites). Furthermore, it was recently shown that *Ae. aegypti* populations from neighborhoods in Rio de Janeiro have high molecular diversity, including in genes related to insecticide detoxification and xenobiotics (Rasic et al. 2015).

Besides mutations in target site of the insecticide, alteration in the activity of detoxifying enzymes is another important process selected for resistance. This characteristic is generally referred to as metabolic resistance mechanism and is commonly associated with cross-resistance between different classes of insecticides (Hemingway and Ranson 2000). Increased activity of GST and esterases had been previously found in *Ae. aegypti* from Aracaju (Montella et al. 2007), suggesting that other mechanisms besides *kdr* mutations may be under selection for pyrethroid resistance. Here, we aimed to explore the distribution of an important molecular marker for pyrethroid resistance in localities across Sergipe state, revealing that the $1016I^{kdr}$ is well disseminated, but at variable frequencies. However, the mechanisms at play for insecticide resistance can be multiple and very dynamic over space and time. Therefore, in order to access a better panorama of insecticide susceptibility in *Ae. aegypti* populations from Sergipe, it will be necessary to have a consistent monitoring based on functional bioassays, complemented with the investigation of molecular markers for selected mechanisms.

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