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Research

Identification of Molecular Determinants of Resistance to Pyrethroid Insecticides in *Aedes aegypti* (Diptera: Culicidae) Populations in California, USA

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Abstract

The first breeding populations of *Aedes aegypti* (Linnaeus) were identified in California in 2013, and have since been detected in 13 counties. Recent studies suggest two introductions likely occurred, with genetically distinct populations in the central and southern regions of the state. Given the threat of dengue, chikungunya, and Zika virus transmission, it is imperative to understand if these populations harbor genes that could confer resistance to pyrethrin-based insecticides, known as pyrethroids, the most commonly used class of adulticides in the state. In 2017, the California Department of Public Health initiated a pesticide resistance screening program for *Ae. aegypti* to assess the presence of specific mutations on the sodium channel gene (V1016I and F1534C) associated with knockdown resistance to pyrethroids. Mosquitoes collected between 2015 and 2017 from 11 counties were screened for mutations using real-time polymerase chain reaction assays. Results revealed distinctly different resistance profiles between the central and southern regions. The central population displayed nearly fixed resistant mutations at both loci, whereas the southern population was more variable. The relative proportion of resistant alleles observed in sampled mosquitoes collected in southern California increased each year from 2015 through 2017, indicating potential increases in resistance across this region. The presence of these mutations indicates that these mosquitoes may be predisposed to surviving pyrethroid treatments. Additional biological and biochemical assays will help better elucidate the mechanisms underlying insecticide resistance in California *Ae. aegypti* and prompt the use of pesticides that are most effective at controlling these mosquitoes.

Key words: *Aedes aegypti*, insecticide resistance, California, knockdown resistance

The yellow fever mosquito, *Aedes aegypti*, is the primary vector of arthropod-borne viruses that include dengue, yellow fever, chikungunya, and Zika (Pialoux et al. 2007, Bhatt et al. 2013, Petersen et al. 2016). These pathogens are a persistent threat to people in regions where *Ae. aegypti* is established, and with the recent and projected range expansion of *Ae. aegypti* (CDC 2018), risk of transmission of these viruses is also spreading. During the 2015–2016 Zika virus outbreak in the Americas, many urban areas in the southern and southeastern United States became acutely aware of this new threat (Grubaugh et al. 2017). Due to the lack of vaccine for Zika, chikungunya, and dengue viruses, mosquito control is the primary method utilized to minimize mosquito bite encounters, thereby decreasing the risk of infection (Morrison et al. 2008, Webster et al. 2009).

In California, *Ae. aegypti* were first detected in 2013, and enhanced surveillance revealed populations were well established in the surrounding urban area (Yoshimizu et al. 2016, Metzger et al. 2017). By the end of 2018, detections had been made in 222 cities and census designated areas in 13 counties, spanning a large portion of the urbanized central and southern regions of the state (CDPH 2018). Studies on the population genetics of *Ae. aegypti* in California have identified two genetically distinct populations: the 'central' population (San Mateo, Fresno, Madera, and Tulare counties) and the 'southern' population (Orange, San Diego, and Los Angeles

counties), having likely originated in the South Central United States and Southwest United States/northern Mexico regions, respectively (Gloria-Soria et al. 2014, Pless et al. 2017). Though introduction and establishment of this invasive species was cause for concern given the number of travel-related cases of dengue and chikungunya in California each year (Porse et al. 2015), the outbreak of Zika virus beginning in 2015 brought the issue to the forefront.

In November 2015, the first cases of Zika virus were reported in California (Porse et al. 2018). The California Department of Public Health (CDPH) and local health departments investigated each case and determined that all cases were travel-associated. While no local *Aedes* mosquito-borne disease transmission has occurred in California to date, the possibility does exist. Small Zika virus outbreaks occurred in two other states in the continental United States with established *Ae. aegypti* populations: Florida and Texas (Grubaugh et al. 2017). Given the significant outcomes of Zika virus infection on pregnant women and their fetuses (World Health Organization 2016), it is imperative to develop preparedness plans in which adult mosquito control plays a primary role to halt local transmission.

Insecticide resistance in *Ae. aegypti* has been well documented in many parts of the world (Montella et al. 2007, Lima et al. 2011, Marcombe et al. 2012, Vontas et al. 2012, Smith et al. 2016). One of the most common chemical classes of pesticides used to control adult *Ae. aeygpti* are pyrethrin-based (e.g., pyrethroids) because they are relatively low in cost and toxicity to mammals (WHO 2014). Knockdown resistance (*kdr*) results from a nonsynonymous mutation occurring on the voltage-sensitive sodium channel (*Vssc*) transmembrane protein that prevents pyrethroid insecticides from attaching properly and causing mortality (Soderlund and Knipple 2003). There are several single nucleotide polymorphism mutations along the *Vssc* known to confer *kdr*-type resistance in *Ae. aegypti*. In the Americas, two of the most common mutations are substitutions occurring at codon 1016, resulting in an amino acid change of valine (V) to isoleucine (I) (V1016I), and at codon 1534, resulting in an amino acid change of phenylalanine (F) to cysteine (C) (F1534C) (Saavedra-Rodriguez et al. 2007). Analyses of adult *Ae. aegypti* from Fresno County, California, collected in 2013 indicated high levels of resistance to certain pyrethroid adulticides, as well as fixed resistant mutations at the 1016 locus (Cornel et al. 2016). Biological assays of the CLOVIS strain also indicated decreased susceptibility to sumithrin, pyrethrum, and permethrin (Cornel et al. 2016).

In 2017, CDPH initiated an *Ae. aegypti* pesticide resistance testing program designed to screen for the V1016I and F1534C mutations. Herein, we describe the *kdr*-type genetic profiles of *Ae. aegypti* collected from the central and southern infestation zones in California from 2015 through 2017. Using samples collected by multiple local vector control agencies, we determined the proportion of resistant alleles at the 1016 and 1534 loci. Due to the focal nature of *Ae. aegypti* (Harrington et al. 2005), the resistance frequencies were analyzed at both county and regional levels, and where possible, temporally. These results will help vector control agencies develop a plan to combat the spread of *Ae. aegypti* and effectively reduce the risk of local disease transmission.

Materials and Methods

Mosquito Samples 2015

Adult *Ae. aegypti* mosquitoes collected by local California vector control agencies were submitted to Yale University for genetic analysis and DNA was extracted as described in Pless et al. (2017). Aliquots of 80 µl of extracted DNA maintained in a cold chain to prevent degradation were provided to CDPH for pesticide resistance analysis.

Mosquito Samples 2016–2017

Adult *Ae. aegypti* mosquitoes were collected by local California vector control agencies from October 2016 through December 2017 using multiple sampling schemes, including CDC Autocidal Gravid Ovitraps (CDC-AGOs), BioGents Sentinel (BGS) traps, Encephalitis Vector Survey (EVS) traps, and backpack aspirators. Larval and pupal samples were also collected through source surveys, and raised to adults in the laboratory. Agencies stored adult mosquitoes individually in empty and dry 1.5 ml Eppendorf tubes at −80°C or in a dry ice chest. Where cold storage access was limited, mosquitoes were preserved in 70% ethanol. Mosquitoes were shipped to CDPH, maintaining the cold chain for all dry specimens.

Upon receipt, all adult mosquito samples were stored at −80°C prior to processing. Abdomens were removed from all dry, female samples that were collected in a live-trap or reared in a laboratory. These abdomens and all remaining mosquitoes were then stored in 70% ethanol at −20°C in preparation for extraction. The head and thoraces of viable mosquitoes were stored at −80°C for future analyses.

Genotyping Assays

DNA extractions of abdomens or whole mosquitoes were conducted using Qiagen DNeasy Blood and Tissue Kit per the manufacturer's protocol (Qiagen, Hercules, CA). DNA samples were eluted to a final volume of 200 µl.

Identification of *kdr*-type *Vssc* mutations was conducted using melt-curve assays. For the V1016I mutation, the protocol described by Saavedra-Rodriguez et al. (2007) was slightly modified. The 21 µl reaction mixture contained 10 µl of iQ Syber Green Supermix (Bio-Rad, Hercules, CA), 2.5 µl of the valine forward primer (5′-GCG GGCAGGGCGGCGGGGGCGGGGCCACAAATTGTTTCCCACC CGCACCGG-3′), 2 µl of the isoleucine forward primer (5′-GCGG GCACAAATTGTTTCCCACCCGCACTGA-3′), 2 µl of the reverse primer (5′-TGATGAACCSGAATTGGACAAAAGC-3′), 3.5 µl PCR-grade water, and 1 µl of DNA template. All primer concentrations were 10 µM. Amplification consisted of 95°C for 3 min, followed by 35 cycles of 95°C for 10 s, 60°C for 10 s, and 72°C for 30 s. The melt-curve protocol followed with 10 s each at 0.2°C increments between 65 and 95°C. Melt curves were generated by the CFX Manager Software Version 3.1 (Bio-Rad) in which homozygous susceptible individuals had a single peak at 86°C (V/V), heterozygous individuals had two peaks at 79°C and 86°C (V/I), and homozygous resistant individuals had a single peak at 79°C (I/I).

The F1534C mutation was identified using a slightly modified protocol described by Yanola et al. (2011). The 20 µl reaction mixture contained 10 µl of iQ Syber Green Supermix (Bio-Rad), 0.33 µl of the phenylalanine forward primer (5′-GCGGGCTCTACTTTGTGTTCT TCATCATATT-3′), 1 µl of the forward cysteine primer (5′-GCGGG CAGGGCGGCGGGGGCGGGGCCTCTACTTTGTGTTCTTCATC ATGTG-3′), 1 µl of the reverse primer (5′-TCTGCTCGTTGAAGTT GTCGAT-3′), 5.67 µl PCR-grade water, and 2 µl of DNA template. All primer concentrations were 10 µM. Amplification consisted of 95°C for 3 min, followed by 35–40 cycles of 95°C for 10 s, 57°C for 10 s, and 72°C for 30 s. The melt-curve protocol followed with 5 s each at 0.5°C increments between 65 and 95°C. Melt curves were generated by the CFX Manager Software Version 3.1 (Bio-Rad) in which homozygous susceptible individuals had a single peak at 80°C (F/F), heterozygous individuals had two peaks at 80°C and 85°C (F/C), and homozygous resistant individuals had a single peak at 85°C (C/C).

Analysis

Analyses of the resistant and susceptible alleles for each mutation locus were conducted at two geographic levels: by region (central and southern) and by county. The frequency of each mutation in a given population was calculated using the following allelic frequency formula:

$$F_p = \frac{2\,(\mathrm{pp}) + pq}{2(n)} * 100$$

where F_p= the frequency of the resistant allele, p = resistant allele, q = susceptible allele, and n = total number of samples.

Geographic maps of allelic frequencies were created using the ggplot2, ggmap, maps, and mapdata libraries for the R statistical software package (Team and R Development Core Team 2016).

Results

Mosquito Samples

From 2015 through 2017, a total of 4,968 mosquitoes were submitted for testing from 11 California counties. Of these, 4,076 whole mosquitoes and 892 mosquito abdomens were tested. Conclusive results for the V1016I and F1534C assays were obtained from 4,852 and 4,870 samples, respectively (Table 1).

Analysis

California counties were divided into two populations: central (Fresno, Kern, Madera, Merced, and Tulare counties) and southern (Imperial, Los Angeles, Orange, Riverside, San Bernardino, and San Diego counties) based on the analysis by Pless et al. (2017). The frequency of the resistant genotypes for the V1016I and F1534C mutations in the central population is nearly fixed at 100% (Figs. 1 and 2). Of the 1,249 mosquitoes tested from 2015 through 2017, only 14 contained a susceptible allele for the 1016, and no samples were homozygous susceptible. For the 1534 mutation, no susceptible alleles were identified in the 1,252 samples tested (Table 1).

The resistance allele profile in the southern population of the state differed from the central population. In 2015, the frequency of the resistant allele was less than 50% in the southern population for both V1016I and F1534C (36.71 and 43.10%, respectively; Table 1, Figs. 1 and 2). The regional resistant allele percentages increased each year, reaching as high as 62.32 and 86.64%, respectively, in 2017 (Table 1, Figs. 1 and 2). Of the counties included in the southern region population, Orange County was the only one with three consecutive years of data, showing that even on a smaller county level, the frequency of the resistant alleles increased over time. From 2015 through 2017, the percentage of resistant alleles increased by nearly 2-fold for the 1016 mutation locus, and 2.5-fold for the 1534 mutation locus (Table 2, Figs. 3 and 4).

Discussion

The introduction and establishment of *Ae. aegypti* in California have led to an increased risk of local transmission of arboviruses in the state (Metzger et al. 2017). Travel-associated cases of chikungunya, dengue, and Zika viruses have resulted in local outbreaks of disease in other states

Table 1. Allelic frequencies of the V1016I and F1534C mutations by region of the state and year (central—Fresno, Kern, Madera, Merced, and Tulare counties; southern—Imperial, Los Angeles, Orange, Riverside, San Bernardino, and San Diego counties), California, 2015 through 2017

Region	Year	V1016I					F1534C				
		Total	SS	SR	RR	Resistance frequency	Total	SS	SR	RR	Resistance frequency
Central	2015	42	0	0	42	100.0%	44	0	0	44	100.0%
	2016	160	0	0	160	100.0%	161	0	0	161	100.0%
	2017	1,047	0	14	1,027	98.8%	1,047	0	0	1,047	100.0%
	2015–2017	1,249	0	14	1,229	99.0%	1,252	0	0	1,252	100.0%
Southern	2015	143	58	65	20	36.7%	145	56	53	36	43.1%
	2016	212	43	97	72	56.8%	214	27	66	121	72.0%
	2017	3,248	538	1,370	1,339	62.3%	3,259	197	477	2,585	86.6%
	2015–2017	3,603	639	1,532	1,431	61.0%	3,618	280	596	2,742	84.0%

SS, indicates homozygous susceptible; SR, heterozygous; RR, homozygous resistant individuals

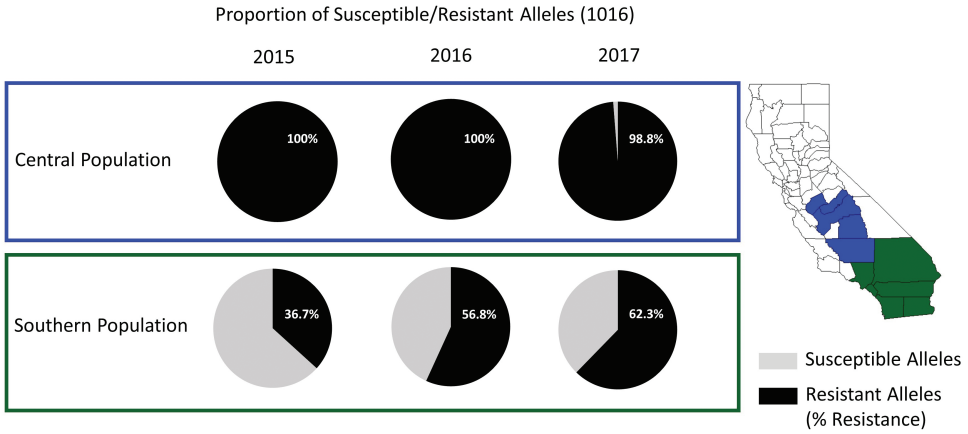

Fig. 1. Proportions of the susceptible and resistant alleles of the 1016 locus mutation at a regional-level by year.

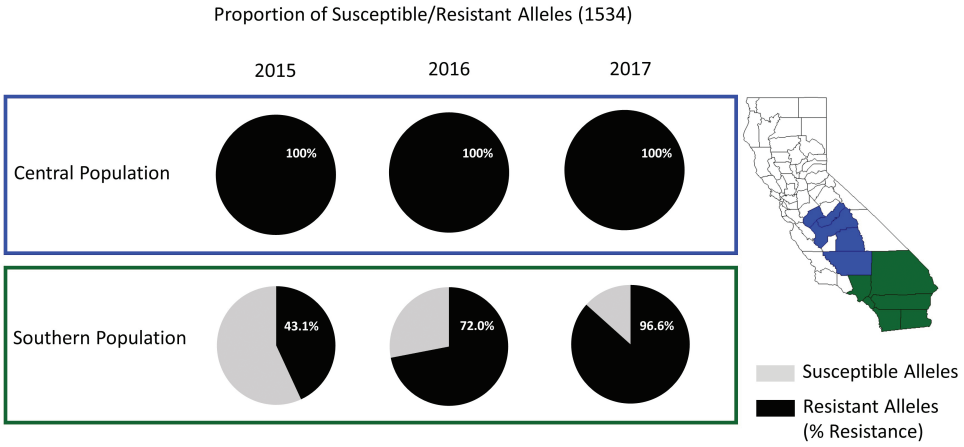

Fig. 2. Proportions of the susceptible and resistant alleles of the 1534 locus mutation at a regional-level by year.

Table 2. Allelic frequencies of the V1016I and F1534C mutations by region (central and southern), year, and county, California, 2015 through 2017

	Region	County	V1016I					F1534C				
			Total	SS	SR	RR	Resistance Frequency	Total	SS	SR	RR	Resistance Frequency
2015	Central	Fresno	20	0	0	20	100.0%	20	0	0	20	100.0%
	Central	Madera	22	0	0	22	100.0%	24	0	0	24	100.0%
	Southern	Orange	89	45	30	14	32.6%	91	45	32	14	33.0%
	Southern	San Diego	54	13	35	6	43.5%	54	11	21	22	60.2%
2016	Central	Madera	160	0	0	160	100.0%	161	0	0	161	100.0%
	Southern	Imperial	40	8	16	16	60.0%	42	1	1	40	96.4%
	Southern	Orange	127	18	64	45	60.6%	127	9	51	67	72.8%
	Southern	Riverside	45	17	17	11	43.3%	45	17	14	14	46.7%
2017	Central	Fresno	511	0	7	504	99.3%	513	0	0	513	100.0%
	Central	Kern	5	0	1	4	90.0%	5	0	0	5	100.0%
	Central	Madera	403	0	0	403	100.0%	401	0	0	401	100.0%
	Central	Merced	57	0	0	51	100.0%	57	0	0	57	100.0%
	Central	Tulare	71	0	6	65	95.8%	71	0	0	71	100.0%
	Southern	Imperial	33	0	15	18	77.3%	37	1	0	36	97.3%
	Southern	Los Angeles	1,123	207	513	403	58.7%	1,119	27	139	953	91.4%
	Southern	Orange	141	23	65	52	60.6%	141	11	31	99	81.2%
	Southern	Riverside	1,093	199	453	441	61.1%	1,101	113	169	819	82.1%
	Southern	San Bernardino	719	82	270	367	69.8%	722	28	101	593	89.1%
	Southern	San Diego	139	27	54	58	61.2%	139	17	37	85	74.5%

and U.S. territories (Florida, Texas, and Puerto Rico) with established populations of this mosquito species (Ramos et al. 2008, Kendrick et al. 2014, Rey 2014, Baud et al. 2017, Grubaugh et al. 2017). In response to this new threat, CDPH and local vector control agencies have developed invasive *Aedes* mosquito surveillance and response plans that recommend the use of adulticides in the event of local disease transmission.

Understanding the resistance profiles of *Ae. aegypti* populations in the state is imperative for selecting and deploying appropriate pesticides in the event of local disease transmission. If, for instance, a population is highly resistant to most pyrethroid insecticides, effective control will require the use of an alternative class of insecticide such as organophosphates. The results of these two *kdr* assays indicate that such measures may need to be taken, particularly in the Central Valley region of the state. The extremely high and fixed resistant mutations observed in the central population could result in control failure if pyrethroid insecticides were to be used to control adult mosquitoes during a local transmission event. Although the *kdr* resistance profiles for the southern population show a high percentage of susceptible alleles remaining, the proportion of resistant alleles for both the V1016I and F1534C loci have increased steadily since 2015 and will need to be continually monitored. At the county level, Orange County clearly demonstrates that these resistance profiles can change rapidly over time. This knowledge supports the ongoing implementation by local vector control agencies of integrated vector management methods, including pesticide rotation and source reduction, to prevent further resistance from developing.

Additional research is essential to evaluate the efficacy of commonly used adulticides against *Ae. aegypti* in California. Biological assays, such as bottle bioassays and outdoor cage trials that challenge live mosquitoes against diagnostic and label doses of insecticides, are needed to determine whether functional resistance is present in local mosquito populations. Unfortunately, it can be difficult to collect the large numbers of mosquitoes required for these types of assays. Biochemical assays focusing on enzymatic activity in adult female mosquitoes could reveal metabolic mechanisms behind resistance in local *Ae. aegypti* populations. The data obtained from these molecular assays, in conjunction with data

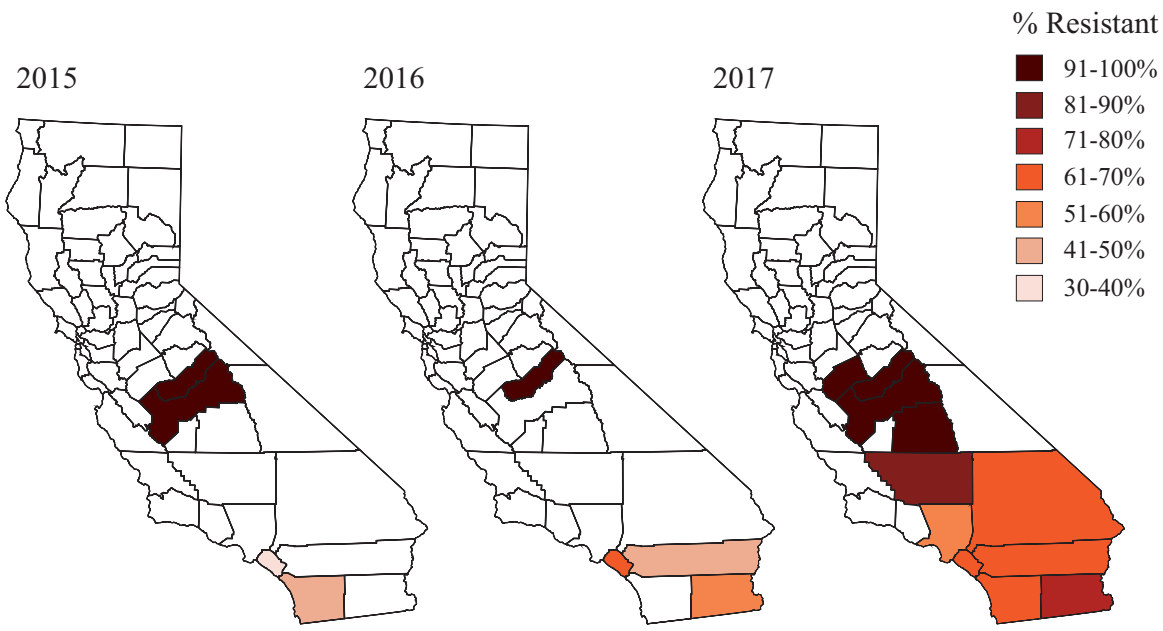

Fig. 3. County-level frequencies of the resistant allele of the 1016 mutation locus by year.

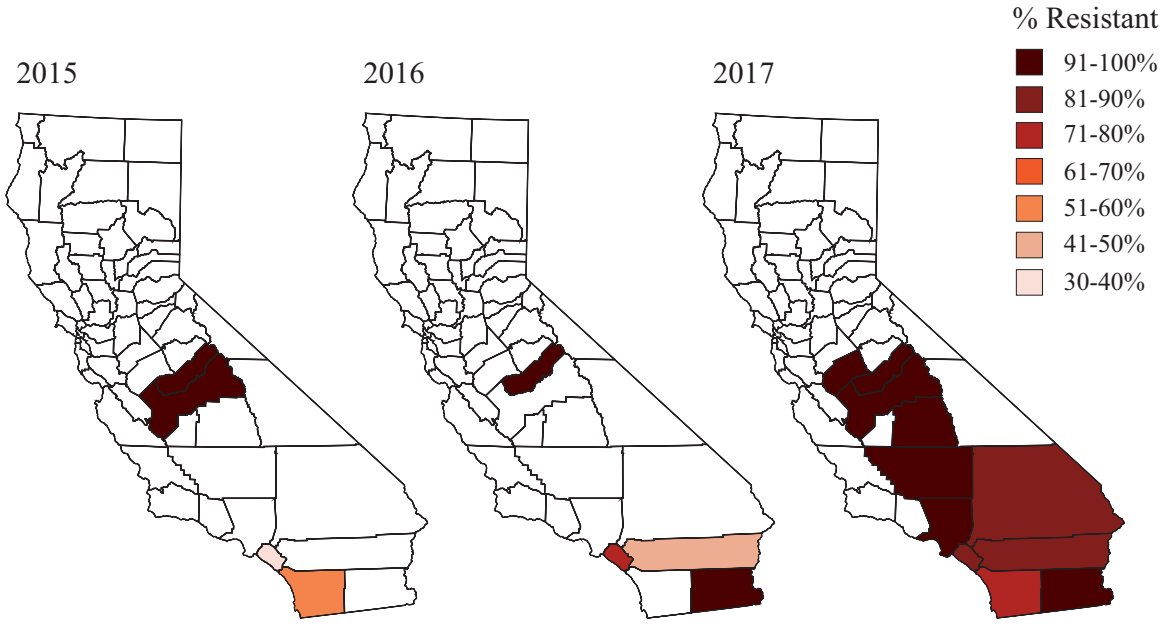

Fig. 4. County-level frequencies of the resistant allele of the 1534 mutation locus by year.

from the field, biochemical assays, and bottle bioassays, will provide vector control agencies with comprehensive information on the pesticide resistance profile of local *Ae. aegypti* populations. Such information will help ensure that the pesticides selected for adult mosquito control are effective in preventing or interrupting local transmission of dengue, chikungunya, or Zika viruses in California.

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