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RESISTENCIA A INSECTICIDAS: MECANISMOS METABÓLICOS Y MUTACIONES PUNTUALES DEL SITIO BLANCO DE LOS PIRETROIDES, EN POBLACIONES DE *Aedes aegypti* DEL ESTADO DE GUERRERO

REQUISITO PARCIAL PARA OBTENER EL TITULO DE MAESTRO EN CIENCIAS DE LA SALUD CON AREA DE CONCENTRACIÓN EN ENFERMEDADES TRANSMITIDAS POR VECTOR.

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INSECTICIDE RESISTANCE: METABOLIC MECHANISMS AND POINT MUTATIONS ON THE TARGET SITE OF PYRETHROIDS IN *Aedes aegypti* POPULATIONS FROM GUERRERO STATE IN MEXICO

Artículo sometido de acuerdo a los requerimientos del Instituto Nacional de Salud Pública para obtener el grado de Maestro en Ciencias de la Salud con Área de Concentración en Enfermedades Transmitidas por Vector.

Por

Angélica Aponte Hincapié

Noviembre 2011

Seguir los sueños y alcanzar las metas.

DEDICADA

Especialmente a mi madre (Olinde), por su apoyo incondicional durante los casi tres años de extancia en tierras lejanas. A mis hermanos, Fernando y Orlando, a sus esposas (Marytza y Patricia) y a mi sobrino Santiago.

A Juan Orozco S, porque durante siete años ha estado conmigo en las buenas y en las malas y con quien quiero seguir compartir mis exitos profesionales.

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Resumen

El dengue es una de las más importantes enfermedades transmitidas por vectores en todo el mundo y es un grave problema de salud pública en México. La mayoría de los programas de control en los países con enfermedades endémicas dependen de insecticidas para controlar los vectores del dengue. En México, los insecticidas piretroides y en menor medida organofosforados, han sido ampliamente utilizadas como adulticidas para el control del dengue desde hace más de una década. Esto ha llevado a la resistencia a los piretroides en varias partes del país. Los mecanismos responsables de esta resistencia fueron estudiados en poblaciones de *Aedes aegypti* de seis localidades en el estado de Guerrero, donde el control químico de los vectores históricamente ha sido muy intenso y existe un alto riesgo de transmisión del virus del dengue. Huevos de *Ae. aegypti* fueron recolectados desde octubre 2009 a enero 2010 con ovitrampas. La generación F1 de *Ae. aegypti* de estas localidades fueron expuestas a los piretroides y tanto la resistencia a la permetrina y deltametrina, fueron confirmados. Ensayos bioquímicos mostraron la actividad de las esterasas elevadas en cinco de las seis localidades, mientras que las actividad de la glutatión S-transferasa se encuentra elevada en todas las localidades. La mutación V1016I kdr estuvo presente en alta frecuencia (frecuencia de los alelos = 0,80) y la presencia de esta mutación se correlaciona con la supervivencia en los bioensayos (prueba exacta de Fisher P = 0,0002). Una segunda mutación, F1534C en el dominio IIIS6 del canal de sodio también fue detectada. Esto representa el primer informe en Guerrero de esta mutación. Nuestros resultados demuestran la presencia de resistencia metabólica y resistencia por alteraciones en los sitios blancos del insecticida en *Ae. aegypti* en el estado de Guerrero en México.

Palabras clave *Aedes aeypti*, resistencia a los piretroides, resistencia metabólica, mutación, resistencia Knockdown, canal de sodio.

ABSTRACT

Dengue is one of the most important vector-borne diseases worldwide and is a serious public health problem in Mexico. Most control programs in disease endemic countries rely on insecticides to control dengue vectors. In Mexico, pyrethroid insecticides, and to a lesser extent organophosphates have been extensively used as adulticides for dengue control for over a decade. This had lead to resistance to pyrethroids in several parts of the country. The mechanisms responsible for this resistance were studied in *Aedes aegypti* populations from six localities in Guerrero state, where the chemical control of vectors has historically been intense and there is a high risk of dengue virus transmission. *Ae*. *aegypti* eggs were collected from October 2009 to January 2010 using ovitraps. *Ae. aegypti* F_1 generation from these localities was exposed to pyrethroids and resistance to both permethrin and deltamethrin confirmed. Biochemical assays showed elevated esterase activity in five out of six localities, while elevated glutathione S-transferase was found in all localities. The V1016I kdr mutation was present at high frequency (allele frequency = 0.80) and the presence of this mutation correlated with bioassay survival (Fisher test P=0.0002). A second mutation, F1534C on the IIIS6 domain of the sodium channel was also detected. This represents the first report in Guerrero of this mutation. Our results demonstrate the presence of both metabolic and target site resistance in *Ae. aegypti* from Guerrero state in Mexico.

Key words *Aedes aegypti*, pyrethroid, metabolic resistance, knockdown resistance, sodium channel, target site, esterases, glutathione S-transferases.

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INTRODUCTION

Dengue fever (DF) and dengue haemorrhagic fever (DHF) are caused by flaviviruses and are the most important diseases in public health in terms of morbidity and mortality. The virus is transmitted by mosquitoes and *Aedes aegypti* is the primary vector. This disease affects tropical and subtropical areas and is endemic in many countries of Latin America. Data from the Pan American Health Organization [http://new.paho.org], indicate the incidence rate in Mexico ranged from 20 to 51 cases/100.000 inhabitants from 2009 – 2010, Historically, Guerrero state regularly reports about the 10% of cases in the country and in 2009, the dengue incidence reached 139 cases /100.000 hab.), Acapulco was the municipality most affected of Guerrero state with 2742 cases.

In Mexico the prevention and elimination of DF and DHF transmission focuses on the dengue vector, and insecticides still play a major role in disease control. The organophosphate (OP) malathion was extensively used as an adulticide prior to the introduction of pyrethroids and the OP temephos is still widely used as a larvicide. DDT was widely used for indoor residual spraying between 1950 to 1960 and was still used for malaria control in some regions of the country until 1998. By 1997 the major insecticide class used in mosquito control programs was pyrethroids (PYR). These continue to be used as adulticides, either in residual spraying, impregnated mosquito nets, or space spraying. Specifically for dengue control the mixture of permethrin + esbiol + piperonyl butoxide came in use after the OP malathion was suspended as adulticide for *Aedes* control [1]. During 2007, 60,944 kg of PYR active ingredient was used for vector borne diseases control [2], demonstrating the widespread use of this class of insecticide throughout the country. Resistance to organophosphate and pyrethroid insecticides is now widespread throughout the range of *Ae. aegypti* [3]; exhibiting that the prolonged and intense insecticide pressure allowed the resurging of resistance to the insecticides used by public health services [4].

Metabolic resistance and resistance by alteration in the target site of the insecticides, are two of the most important resistance mechanisms. Metabolic resistance is conferred by alterations in the levels or activities of detoxification enzymes, predominately esterases, glutathione transferases of cytochrome P450s. [5]. Structural changes in the insecticide target site, in the voltage-gated channel sodium channel can lower the affinity for the insecticide [5]. Cross-resistance between pyrethroids and DDT is frequently due to mutations in this protein and this mechanism is known as "knockdown resistance" or "kdr" [6, 4, 7, 8]. Most resistance associate mutations are found in segment 6 of domain II (IIS6) and domain III (IIIS6) of the sodium channel protein. In *Aedes aegypti* populations from Latin America, several mutations that correlate with resistance to DDT and PYR have been identified (V1016I I1011M, I1011V and F1534C [6, 7, 4, 9, 10]). The V1016I mutation, is widely distributed in Mexico and rapidly increased in frequency from 1996 to 2009 in populations of *Ae. aegypti*.

Here we report the analysis of resistance mechanisms in *Aedes. aegypti* from Guerrero state.

MATERIALS AND METHODS

Mosquito collection:

Aedes aegypti eggs were collected in the field between October 2009 and January 2010, from 4.500 ovitraps placed in six municipalities from Guerrero state (Fig 1). These municipalities have been considered by the health authorities as localities with high risk for the dengue virus transmission, due to its history of persistent transmission and high number of cases of dengue fever (Table 1).

Table 1. Number of cases and incidence rate of dengue fever in the years 2009 - 2011 in six localities from Guerrero state.

	Cases of dengue fever confirmed by laboratory (1-52 week epidemiology)				Incidence rate (incidence/100.000pop)			
	2008	2009	2010	2011*	2008	2009	2010	2011*
Mexico	31.154	44.565	22.352	4718	28.42	51.48	20.21	3.64
Guerrero	3.302	4.472	3.770	585	101.64	137.66	116.05	18.01
Iguala	63	242	1179	101	46.68	179.30	873.55	74.83
Acapulco	779	2742	1030	112	104.15	366.58	137.70	14.97
Chilpancingo	137	190	692	36	62.47	86.64	315.55	16.42
Zihuatanejo	26	51	167	17	23.96	46.99	153.88	15.66
Tlapa	10	260	5	9	17.12	445.19	8.59	15.41
Tecpan	7	15	39	2	11.09	23.76	61.78	3.17

* 39-epidemiology week Data from CENAVECE (http: www.cenavece.salud.gob.mx)

Eggs were hatched at the insectary of the Centro Regional de Investigación en Salud Pública (CRISP) and larvae were reared to adults at 25-27 ℃ and relative humidity 60-70% on a diet of 10% sucrose solution. One to three days old adults were used for the WHO bioassays, biochemical assays and molecular analysis. Two reference strains were used in this study: the New Orleans strain, originally colonized by the CDC (Centers for Disease Control and Prevention); and the pyrethroid resistant strain

(IMUS) [7], collected from Isla Mujeres, Quintana Roo, Mexico in January 2009, and used for this study in the F_{11} generation colonized in CRISP.

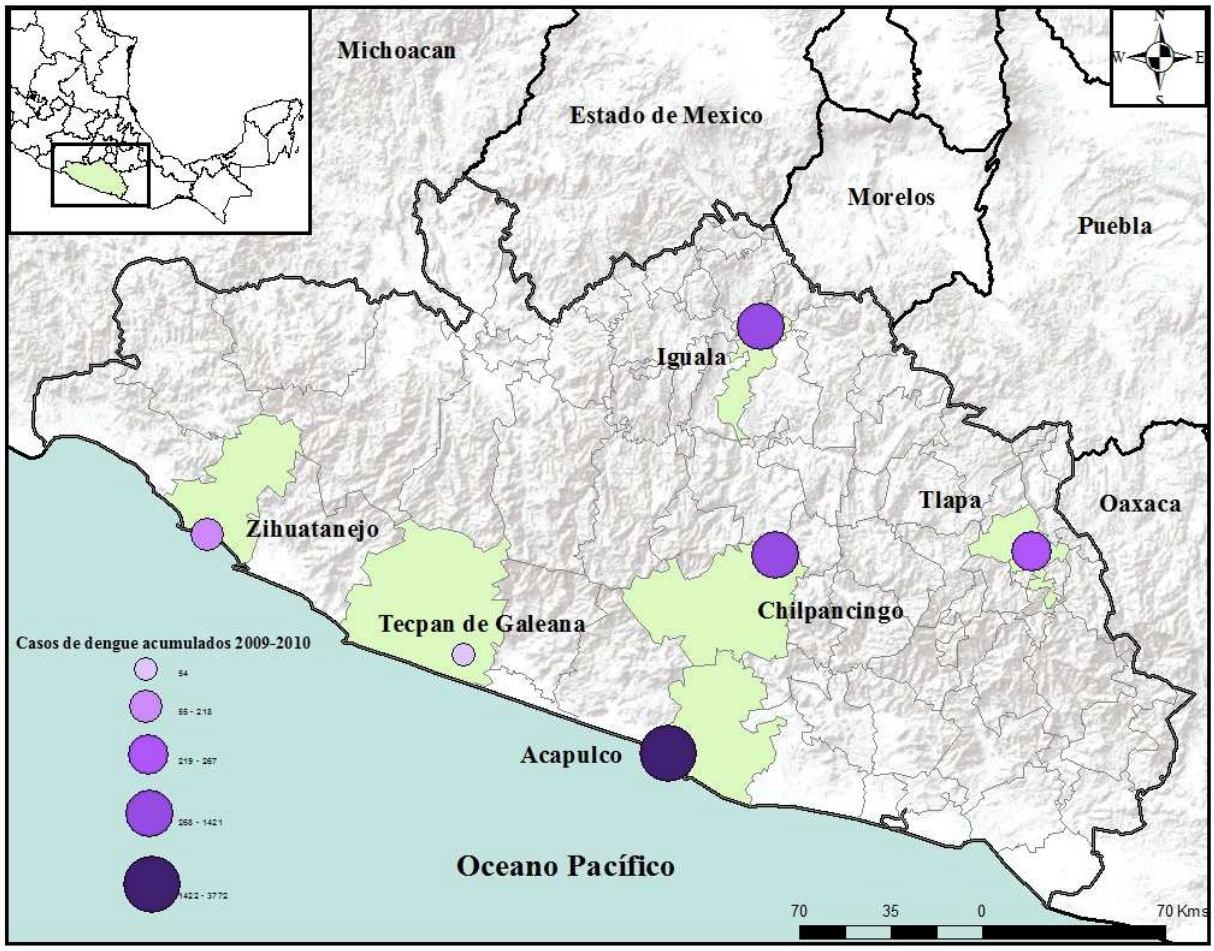


Fig 1. Municipalities of the Guerrero state selected for this study. Selection criteria based on the accumulated cases of dengue fever in years 2009-2010.

Determination of the diagnostic doses

The diagnostic concentration for pyrethroids published by WHO [11] resulted in low levels of mortality even for the susceptible New Orleans strain (80%). Hence, diagnostic concentrations (LC/EC values) were calculated from a base-line developed in our laboratory with mosquitoes of the New Orleans strain and data were analysed with the software EPA (Probit Analysis Program version 1.5). The resulting diagnostic

doses were: permethrin (0.816% concentration) and deltamethrin (0.034% concentration). Filter papers (Whatman # 1 of 12cm x 15cm) were then impregnated with these concentrations as described by WHO [11, 12].

Insecticide bioassays

Bioassays were carried out on one to three days old mosquitoes using insecticide susceptibility test kits of WHO [11, 12]. Over 100 mosquitoes were used in each bioassay and three repetitions were done for each insecticide and localities. After exposure, mosquitoes were transferred to the holding tube and provided with a 10% sugar solution soaked cotton pad. Mortality was recorded 24-hours after a one hour exposure, and dead and survivor mosquitoes were frozen for molecular assays. Control assays, in which mosquitoes were exposed to papers impregnated with carrier olive oil only, were conducted in parallel.

Enzymatic assays

Protein concentrations were calculated according to Bradford [13] and esterase, glutathione S-transferase (GST) activity and cytochrome P^{450} activities were measured as described by Penilla [14]. Batches of at least 48 females and 46 males of one-day-old mosquitoes, none exposed to insecticides, were used in the assay and were individually homogenized on ice in 200 µl of distilled water in a flat-bottomed micro titer [14].

Esterase activities were quantified using three distinct substrates: α and β- naphthyl acetate and para-nitrophenyl acetate (*p*NPA). Glutathione S-transferase activity was measured using the chlorodinitrobenzene (CDNB) substrate (Sigma catalog C-6396) and cytochrome P^{450} content was determined using the heme–peroxidase reactions. Absorbances at end points and kinetics, were read in a Multiskan® spectrum Microplate spectrophotometer reader (Thermo Labsystems catalog 15018860). Enzyme activities/mg proteins were calculated for all field samples and ANOVA and Dunnett ´t test were used to compare activity levels against those of the control strains.

Detection of mutations in *Ae. aegypti* voltage- gated sodium channel

Genomic DNA was extracted from Guerrero state mosquitoes, using the Livak method [5], The PCR primers Ae2021aF and Ae2021aR were used to amplify a 457bp fragment of exons 20 and 21, encoding the subunit 6 of the domain II of the sodium channel gene. Cycling conditions were 95℃ for 5 min followed by 35 cycles at 94℃ for 30 sec, 58℃ for 30 sec, and 72℃ for 30 sec followed by a final elongation stage at 72℃ for 10 min. To amplify the exon 31 encoding the subunit 6 of the domain III, we used the PCR primers AaEx31P and AaEx31Q, which amplified a 350 bp fragment [10, 16]. The conditions were 95℃ for 5 min followed by 35 cycles at 94℃ for 30 sec, 60℃ for 30 sec, and 72℃ for 30 sec followed by a final elongation stage at 72℃ for 10 min. PCR reactions were carried out according to the methods described by Harris [10]. For the primer set AaEx31, PCR products were visualized by gel electrophoresis and then purified using a Qiagen® PCR purification kit

Products of PCR of exons 20-21 and exon 31 from eight mosquitoes (seven permethrin survivor and one deltamethrin survivor), were sequenced directly by Macrogen.

Kdr genotyping:

Hot Oligonucleotide Ligation Assay (HOLA) for the detection of V1016I

For detection of the V1016I mutation in the exon 20-21 for a bigger number of samples and an allele frequency calculation in this population a HOLA assay procedure was used as described by Rajatileka [16]. Genomic DNA was amplified using the primers Ae2021F and Ae2021R under the PCR conditions described above. For each allele, a 20 µl ligation reaction containing 3 µl of PCR product, 1X Ampligase® buffer, 50 nM of each detector/reporter mix, and 0.05U/µl Ampligase® was set up. The reaction conditions for the hot ligation were 95 ˚C for 5 min followed by 25 cycles at 94 ˚C for 1 min, 62℃ for 2 min and 4 ˚C-hold. The sequence of the oligonucleotide reporter Ile1016rpt and detector Val1016dtc and Ile1016dtc are given in Table 2.

Streptavidin plates (Sigma®ScreenTM catalog M-5432) of 96-wells, were used for SNP detection. Colour change was scored visually after incubating with 100 µl TMB solution (Roche® BM Blue Pod Substrate) for 5 min. Plates were also read at 680 nm in a multiskan spectrum thermo labsystem.

Tetraplex Assay for the detection of F1534C

For detection of the mutation F1534C in a diagnostic method, a tetra primer PCR assay designed by Harris [10], using the primers AaEx31P, AaEx31Q, AaEx31wt and AaEx31mut, was used. In this PCR, the flanking primers amplify a control band about 350 pb. Two internal allele specific primers give products of either 231 bp (wild type, phenylalanine allele) or 163 bp (mutant, cysteine allele) by forming PCR primer pairs with the flanking primers. Each PCR reaction contained 2.5 mM McCl2, 0.4 mM each dNTPs, 0.5 µM each primer, 2.5 U taq polymerase, and 1% of the total genomic DNA extracted from three mosquitoes using the Livak method [15] for a total final volume of 25 µL. The cycling conditions were 95ºC for 5 min followed by 35 cycles at 94ºC for 30 sec, 60ºC for 30 sec, and 72ºC for 30 sec, and a final elongation at 72ºC for 10 min, PCR products were resolved on a 2% agarose gel.

Table 2. Sequences of primers used in the current study.

Primer name	Position	Oligonucleotide sequence (5´-3´)
Ae2021aF		ATTGTATGCTTGTGGGTG
Ae2021aR		GCGTTGGCGATGTTC
AaEx31P		(TCGCGGGAGGTAAGTTATTG)
AaEx31Q		GTTGATGTGCGATGGAAATG
AaEx31wt		CCTCTACTTTGTGTTCTTCATCATCTT
AaEx31mut		GCGTGAAGAACGACCCGC
Val1016dtc	1016-1st	GCAAGGCTAAGAAAAGGTTAAGTAC
Ile1016dtc	1016-1st	GCAAGGCTAAGAAAAGGTTAAGTAT
Ile1016rpt	1016-1st	CTGTGCGAGTGGGAAACAAT

rpt= reporter and dtc= detector.

Statistical analysis

Statistical analyses were performed using the SPSS 19.0 software [17]. Descriptive analyses of mortality were obtained from different exposures to insecticides. An analysis of variance (ANOVA) was conducted to compare the average mortality of all strains and the New Orleans control strain. The statistical analyses included, Levene´s test for homogeneity of variance and descriptive analysis (mean, standard deviation with a confidence interval for the mean of 95%).

The enzymatic activity data of the field mosquitoes were compared using ANOVA and the Levene´s and Dunnet´s tests were used to compare means against those activities of the control (α 0.05). The Fisher´s exact test was performed using the online GraphPad Software (http://www.graphpad.com). The allele frequency for the mutation V1016 was calculated from the formula or Hardy Weinberg through the program Hardy-Weinberg Equilibrium Calculator (http://www.changbioscien.com).

RESULTS

Insecticide bioassays

Results from the bioassays indicated that mosquito populations of the Guerrero state are highly resistant to both PYRs tested. Mortalities varied between 9.79%-16.02% and 8.70% - 45.28% for those exposed to permethrin and deltamethrin, respectively (Table 3) compared to > 99 % for the susceptible control strain.

Table 3. Bioassay results for *Ae. aegypti* adults collected in the Guerrero state and exposed to permethrin and deltamethrin.

Municipalities	0.816% permethrin				0.034% deltamethrin			
			Mortality %				Mortality %	
	N	Bioassays	Mean	SD	N	Bioassays	Mean	SD
Iguala	1847	18	12,3*	15,5	1853	18	20.1*	22.4
Acapulco	1135	11	9.7*	15.6	823	8	8.702*	8.7
Chilpancingo	517	5	14,2*	13,4	309	3	13,0*	13,4
Zihuatanejo	1225	12	11,3*	14,67	1025	10	22,2*	17,3
Tlapa	412	2	15,7*	7,710	402	2	45,2*	15,3
Tecpan	318	3	14,5*	23,783	312	3	28,3*	16,2
IMUS	307	3	28,8*	15,3	321	3	11,9*	11,7
New Orleans	311	3	99,4	1,5	307	3	99.0	3,1

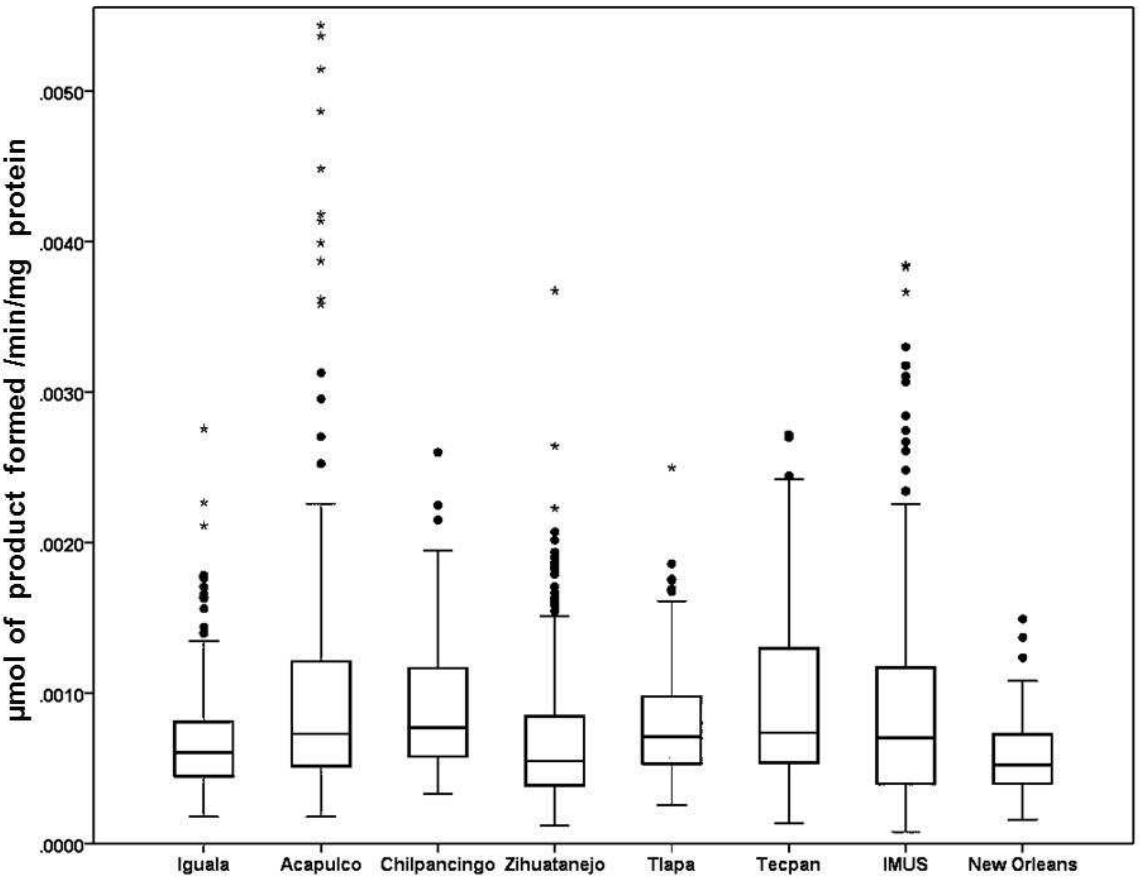
* Mean of mortality percentage significantly different to the New Orleans strain α=0.05, *P value = 0.000.* N: tested mosquitoes, Mean: mean of mortality percentage, SD: standard deviation.

Enzymatic assays

The enzyme activity median for each mosquito population is shown in the figures 2-5. Esterases with at least a substrate (Fig. 2), and GST activities (Fig. 3) were statistically elevated in all mosquito populations analysed when compared with the susceptible New Orleans strain. Being Acapulco the locality with the highest esterase activity mean obtained with the three substrates used: α and β naphthyl acetate and

*p*NPA (0.0011±0.0010; 0.0006±0.0005; 0.1968±0.1100, respectively $P < 0.001$). Whereas Tlapa was the locality with the highest GST activity mean (8.081±2.113), followed by Acapulco (4.469±2.175) and Tecpan (4.036±1.873) ($P < 0.001$). Mean of cytochrome P^{450} content was statistically higher only in Acapulco (0.0029±0.0061) compared with the mean of the New Orleans strain (0.0011±0.0008) ($P < 0.001$).

A)



B)

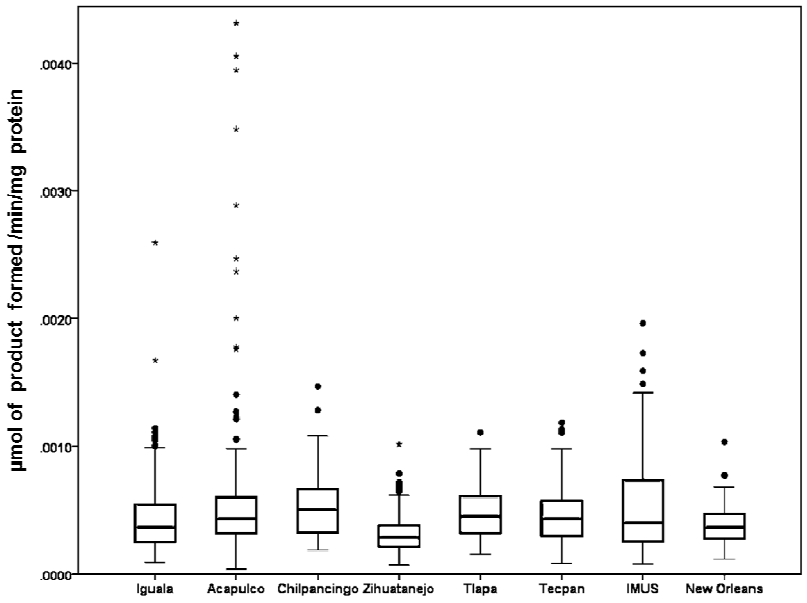


Fig 2. Box plots of results from biochemical assays using the substrates A) α naphthyl acetate and B) β naphthyl acetate.

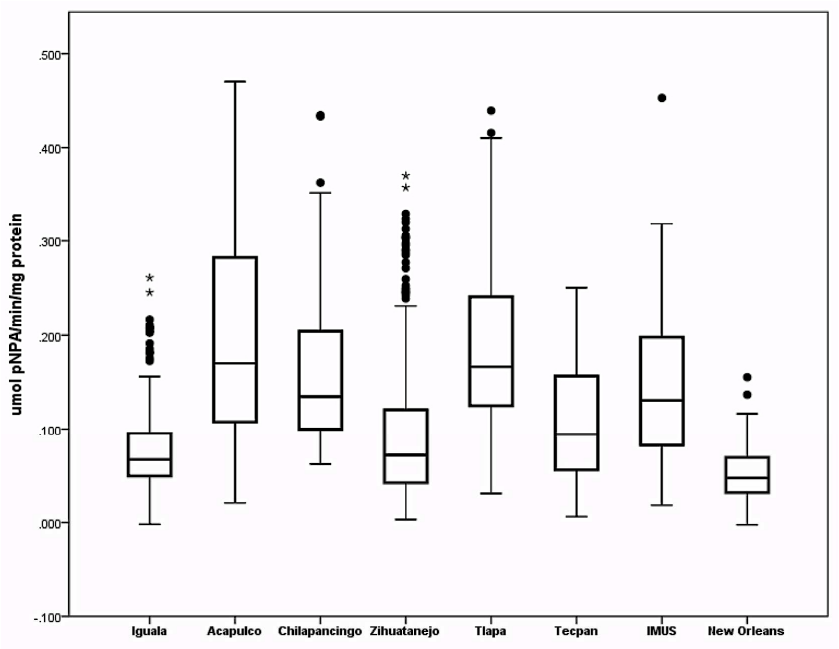


Fig 3. Box plots of results from biochemical assays using the substrate para-nitrophenyl acetate (*p*NPA).

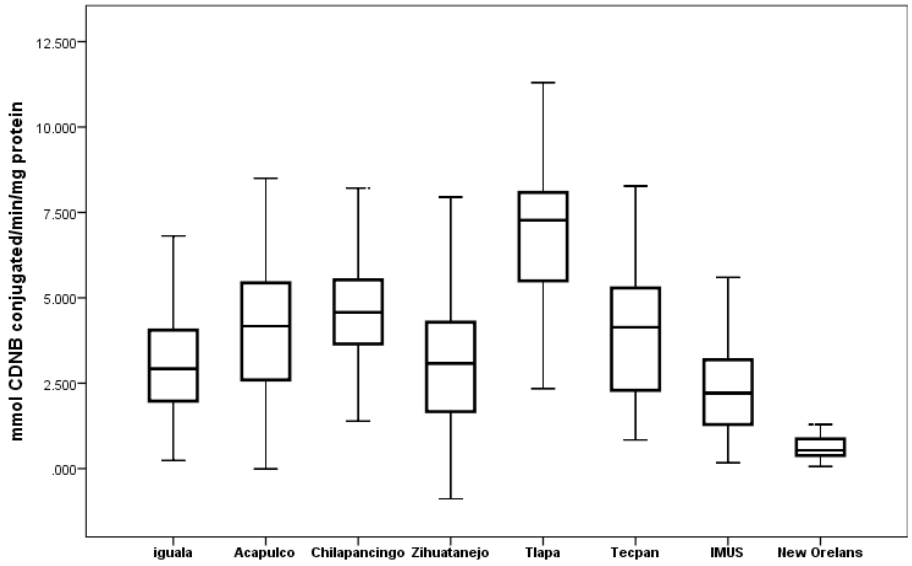


Fig 4. Box plots showing results from GST activity biochemical assays using the CDNB substrate.

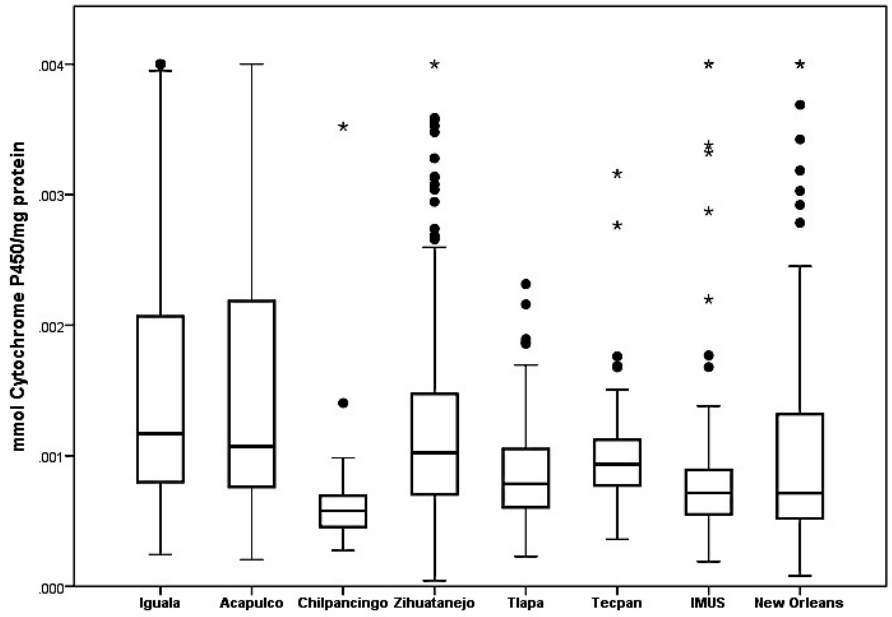


Fig 5. Box plot of biochemical assay results for cytochrome P^{450}. Those value points in 0.004 are ≥ 0.004.

Kdr genotyping

Six products of PCR were obtained from eight pyrethroid survivor mosquito samples. The products were sequenced and results showed two mutations, Val1016I and F1534C, from which two samples were homozygous resistant for both mutations. The first mutation is a result of a single nucleotide transition from GTA to ATA valine to isoleucine substitution in the IIS6 domain exon 20-21. The HOLA assay for V1016I mutation (Fig. 6) was conducted on 77 mosquitoes exposed to pyrethroids and randomly chosen (survivors and dead), from which 58 were positive to the colorimetric reaction. We calculated the frequencies for the pyrethroid resistance allele in 58 individuals analysed, using the formula of Hardy-Weinberg Equilibrium (Table 4).

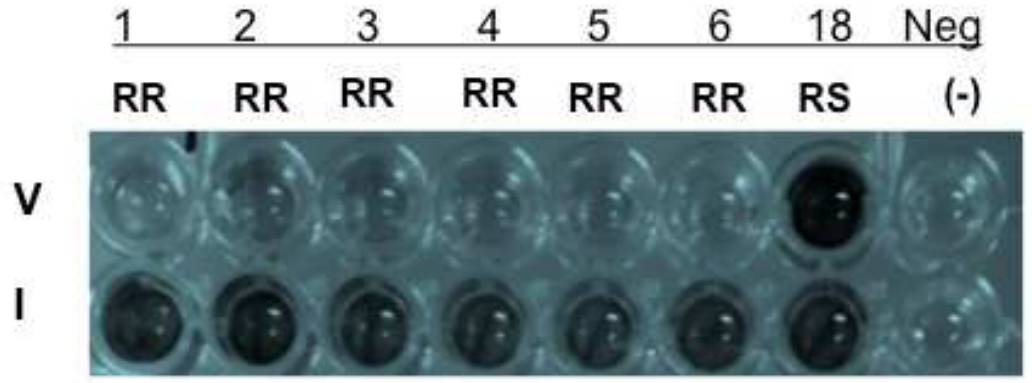


Fig 6. HOLA results: V=Valine, I=Isoleucine, RR=Homozygous Resistance RS= Heterozygous Susceptible, Acapulco mosquito samples: 1-6 Survivor mosquitoes, 18 Dead mosquito (susceptible control).

The V1016I mutation was found in all mosquitos analysed in the HOLA assay, with an allele frequency of 0.8 for sample studied. Whereas Acapulco had 1.0 allele frequency. The Fisher test showed a relation between the pyrethroid resistant phenotype and the homozygous genotype (I1016I) for the mutation V1016I (*P*=0.0002).

No homozygous wild type (V1016V) was identified in the mosquitoes from Guerrero.

Table 4. Phenotype and kdr allele frequencies of *Ae. aegypti* from the Guerrero state, for survivors and deads exposed to pyrethroids in WHO tests.

Municipality	Phenotype/ Bioassay	n sample	Genotype Kdr/ HOLA assay			F_1 Alleles	
			RR Ile/Ile	RS Val/Ile	SS Val/Val	R	S
Iguala	Resistant	18	13	5	0	0.86	0.14
	Susceptible	2	0	2	0	0.5	0.5
Acapulco	Resistant	12	12	0	0	1.0	0.0
	Susceptible	2	0	2	0	0.5	0.5
Chilpancingo	Resistant	4	2	2	0	0.75	0.25
	Susceptible	2	0	2	0	0.5	0.5
Zihuatanejo	Resistant	4	2	2	0	0.75	0.25
	Susceptible	7	2	5	0	0.64	0.36
Tecpan	Resistant	1	0	1	0	0.5	0.5
IMUS	Resistant	3	0	3	0	0.5	0.5
	Susceptible	1	0	1	0	0.5	0.5
New Orleans	Susceptible	2	0	2	0	0.5	0.5
Guerrero total		**52**	**31**	**21**		**0.8**	**0.2**
Sample Total		58 *					

* Only 58 of out 77 samples showed colorimetric reaction by HOLA.

The second mutation in the IIIS6 domain (exon 31) is a no-synonymous one, which is a single base pair substitution changes the code from TTC to TGC resulting in a phenylalanine to cysteine located in the 1534 codon. From the six mosquitoes sequenced all had the mutation, but five were homozygous and one was heterozygous.

Eighteen samples were tested in a PCR tetraplex to confirm the F1534C mutation. Results in agarose gel showed a band of 163 bp only for three samples (fig.7). The samples were from three different localities (Acapulco, Iguala and Zihuatanejo) and all of them were readily one homozygous genotype (RR).

This is the first time that the phenylalanine to cysteine mutation is found in this species in Guerrero, Mexico.

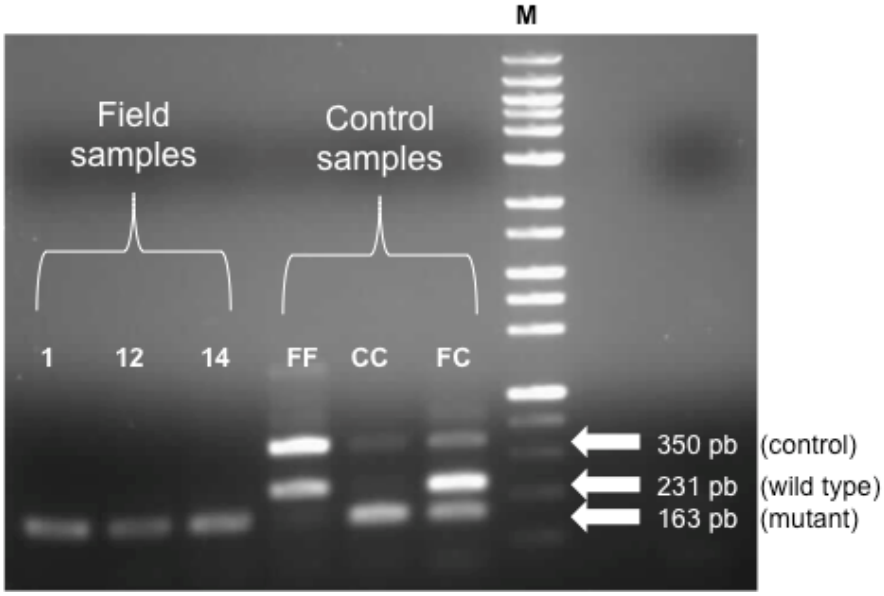


Fig 7. Agarose gel (2%) electrophoresis of tetraplex PCR products for F1534C mutation. Permethrin resistant mosquito samples: 1- Acapulco, 12- Iguala, 14 – Zihuatanejo, M Molecular marker. FF-Control – susceptible, CC – Control + resistant, FC- Heterozygous.

DISCUSSION

For decades chemical insecticides have been widely used as the major method for control of vector borne diseases. The use of pyrethroid insecticides applied as ultra-low volume (ULV) and indoor spraying is still widely recommended for dengue vector control and in Mexico is approved by the NOM-032 [1] (Norma Oficial Mexicana, Official Mexican Guiding for Control of Vector Borne Diseases) as adulticide during the high transmission peaks. Most formulations of pyrethroids on the Mexican market are synergised by PBO (piperonyl buthoxide), which enhances the efficacy of the active ingredient to reach the target site by suppressing the metabolic action of the enzymes [1]. Nevertheless, resistance to pyrethroid insecticides has been reported in several states from Mexico [18, 19], including the state of Guerrero (Rodriguez personal communication) from where the mosquitoes analysed in this study come. In this study we have found that populations of *Ae aegypti* from six selected municipalities of the Guerrero state were resistant to the pyrethroid evaluated, with an average of 12.3-16% mortality to permethrin and 8.7-28.3% to deltamethrin.

Our data suggest that resistance to pyrethroids may be mediated by metabolic or molecular mechanisms of resistance. Previous studies carried out in Latin America and the Caribbean demonstrated that pyrethroid insecticide resistance was correlated with high activities of at least one family of detoxification enzymes particularly the esterases family, in several *Ae. aegypti* populations; in addition it was also reported several non-synonymous mutations in the gene encoding the sodium channel, showing a relationship between the resistant phenotype and the state of susceptibility to pyrethroids and DDT [6,7,8]. In the *Ae. aegypti* populations from the Guerrero State, also both mechanisms were present. However biochemical assays results suggest that metabolism-based resistance in mosquito populations from Guerrero may be mediated by GST and cytochrome P^{450} too. Esterases and cytochrome P^{450} levels varied between *Ae. aegypti* populations from localities, whereas GST activities were elevated in all mosquito populations analysed. The esterase-based resistance are often involved in organophosphate, carbamate, and to a lesser extent pyrethroid

resistance, several Latin American studies have shown today that alpha and beta esterases play an important role in pyrethroids resistance; studies on *Ae. aegypti* from Cuba, Brazil and Mexico, reported high metabolic resistance, associated with α and β esterases [9,18,19, 20, 21, 22].

Spatial variation of resistance and mechanisms may be recorded depending on genetic background of the populations as well as on operational factors, basically the insecticide use patterns. Our biochemical tests showed that the activity of alpha and beta esterases were significantly higher with respect to those activities of the reference strain in several localities; four out of six localities studied while all localities had high activities of esterases with *p*-NPA substrate. However, the levels of cytochrome P^{450} were statistically higher to the susceptible strain only in three municipalities, where only Acapulco had multiresistance mosquitoes with both esterases and P^{450} based resistance mechanisms. Those levels were similar to the reference resistant strain (IMUS). In the north of Mexico was demonstrated that the mixed function oxidases were present as the primary mechanism of resistance to permethrin in mosquito populations from the Sonora state; mosquito populations of five locations in the state of Quintana Roo, showed that three major enzyme groups were responsible for metabolically based resistance, α and β esterases and cytochrome P^{450}, conferring resistance to organophosphates, carbamates and some insecticides pyrethroids in these populations [19, 22]. In Brazil the high esterase activity with *p*-NPA substrate in some populations could be construed as indirect evidence of the esterase role in pyrethroid resistance, because esterase activity increases with the introduction of PY in adult mosquito control [21]. Studies in Cuba in a deltamethrin selected laboratory strain, showed that esterase and GST enzymes were responsible for the resistance to pyrethroids [20].

Elevated expression of GST-2 has been associated with DDT resistance in *Ae aegypti* and recently RNAi experiments have demonstrated a role for Epsilon class GSTs in conferring resistance to pyrethroids [23].

Two non-synonymous mutations in the sodium channel, V1016I and F1534C, both involved in resistance to pyrethroids were detected in Guerrero.

The V1016I mutation has been previously reported in Mexico in the states of Chiapas, Quintana Roo, Yucatan, Veracruz and Nuevo Leon [4] and Guerrero [24]. In this study the presence of this mutation was correlated with the bioassays data (Fisher (P = 0.0002)) supporting the role for this mutation in conferring pyrethroid resistance shown in previous studies [7].

The mutation F1534C was found in all six mosquitoes sent to sequencing, with five being homozygous and one heterozygous (Chilpancingo sample). This mutation is very prevalent in the Cayman Islands, and is also found in Southeast Asia and recently in populations of *Ae. albopictus* from Singapore [10,25,26]. Recently this mutation has been added a new report of a novel mutation in *Ae. aegypti* in a DDT／permethrin-resistant strain (PMD-R) from Thailand [27] and shows it confers resistance to type II but not type I PY [26]. This mutation has also been found associated with pyrethroid resistance in Vietnam, Cayman Island [10, 28] and in this study. The phenotypic effect of having both F1534C and V1016I alleles is not yet known but the high frequencies of both these alleles in the Caribbean suggests a selective advantage [10]. Additional polymorphisms have been identified in sodium channel of *Ae. aegypti*, but, with the exception of I1011M in Brazil [9], none of these have been conclusively linked to resistance.

The presence of the kdr mutations has important implications for the control to vector borne diseases, since the continuous use of pyrethroid insecticides may promote a dramatic increase of this mutation in Mexico [4]. Our results demonstrated the existence of DDT and PYR cross-resistance and multi-resistance in which both mechanisms act (metabolic and target site point mutation). As a consequence of the high levels of PY resistance in the Guerrero state, this study results has already help to implement more effective resistance management strategies in this major disease vector. As part of the integral management by the Vector Control Program, a switch to

an organophosphate (chlorpirifos) and a carbamate (bendiocarb) insecticide as adulticide for dengue control was recently implemented for ULV applications and the latter for indoor spraying. However, the status of susceptibility to pyrethroid insecticides in populations of *Ae. aegypti* from the Guerrero state, generates the need of continuously monitor the effectiveness of actions by the Vector Control Program.

ACKNOWLEDGMENTS

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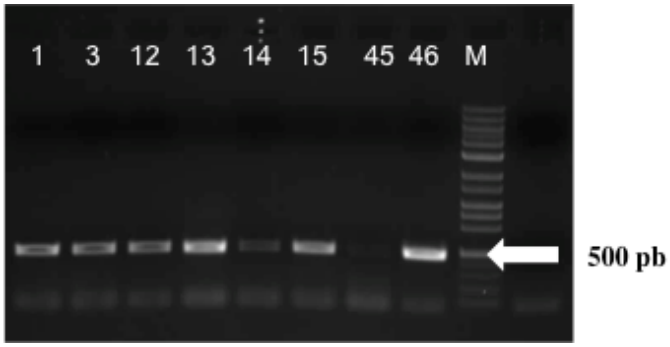
ANEXOS

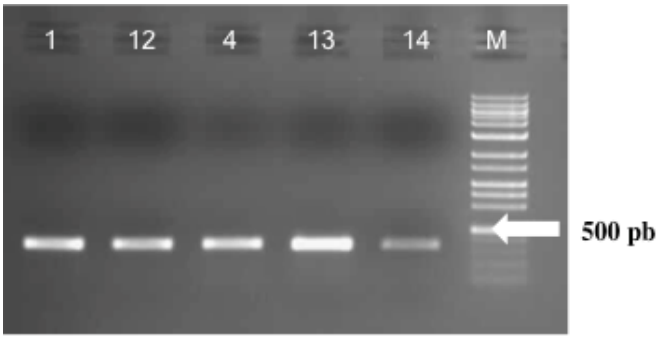
1. Pruebas de susceptibilidad de la OMS para las localidades y colonias seleccionadas en el estudio.

Localidad	Colonia	Piretroides						Organofosforado						Carbamato					
		Permetrina 0.816%			Deltametrina 0.034%			Malation 0.80%			2.17%			Propoxur 0.10%			0.47%		
		n	Muertos	%	n	Muertos	%	n	Muertos	%	n	Muertos	%	n	Muertos	%	n	Muertos	%
Iguala	Tierra y Libertad	193	89	**46.1**	201	143	**71.1**	305	303	**99.3**	307	307	**100**	316	283	**89.6**	315	315	**100**
	Santa Cruz	314	35	**11.1**	311	20	**6.4**	314	139	**55.7**	307	307	**100**	311	221	**71.1**	308	302	**98.1**
	San José	321	31	**9.7**	326	42	**12.9**	310	251	**81**				216	167	**77.3**			
	Luis Quintero	204	13	**6.4**	210	35	**16.7**												
	Acatempan	302	19	**6.3**	302	66	**21.9**	258	32	**12.4**	305	291	**95.4**	311	85	**27.3**	213	213	**100**
	Chapultepec	212	12	**5.7**	305	39	**12.8**	315	307	**97.5**				320	295	**92.2**			
	Mirador	300	14	**4.7**	200	30	**15**	312	147	**47.1**				203	55	**27.1**	309	309	**100**
Acapulco	H. Moderno	315	58	**18.4**	311	20	**6.4**												
	Progreso	308	20	**6.5**	207	27	**13**	103	66	**64.1**	309	297	**96.1**	306	99	**32.4**	308	297	**96.4**
	Benito Juarez	200	14	**7**	200	7	**3.5**	314	302	**96.2**	300	300	**100**	308	160	**51.9**	311	311	**100**
	Renacimiento	312	18	**5.8**	105	11	**10.5**												
Chilpancingo	Tatagildo	314	51	**16.2**	204	28	**13.7**	103	33	**32**	103	103	**100**	104	47	**45.2**	104	111	**97.1**
	San Mateo	203	32	**15.8**	105	12	**11.4**	239	74	**31**				100	44	**44**			
Tecpan de Galeana	Colonia Pri	318	47	**14.8**	312	89	**28.5**	323	136	**42.1**	305	302	**99**	308	94	**30.5**	313	220	**70.3**
Tlapa	Jardín Niños	210	33	**15.7**	201	103	**51.2**	210	104	**49.5**	313	257	**82.1**	210	178	**84.8**	207	207	**100**
	Caltitlán	202	31	**15.3**	201	79	**39.3**	309	216	**69.9**	311	311	**100**	209	77	**36.8**	300	295	**98.3**
Zihuatanejo	Morelos	309	81	**26.2**	320	107	**33.4**	311	205	**65.9**	315	315	**100**	306	204	**66.7**	337	310	**92**
	Primer paso	315	24	**7.6**	308	54	**17.5**	318	254	**79.9**	308	307	**99.7**	310	181	**58.4**	322	319	**99.1**
	Hujal	300	19	**6.3**	200	7	**3.5**	310	170	**54.8**	306	306	**100**	308	198	**64.3**	304	294	**96.7**
	Zapata	300	16	**5.3**	198	62	**31.3**	311	128	**41.2**	308	308	**100**	314	122	**38.9**	311	275	**88.4**
New Orleans (Control -)		311	309	**99.4**	307	301	**98**	300	300	**100**	300	300	**100**	300	300	**100**	300	300	**100**
IMUS F-10 (Control +)		307	89	**29**	321	81	**25.2**	316	315	**99.7**	320	320	**100**	317	203	**64**	316	316	**100**

2. GELES DE AGAROSA DE LA AMPLIFICACIÓN DE LOS EXONES 20-21 y 31 DEL CANAL DE SODIO



Geles de agarosa (2%) del dominio IIS6 (exon 20-21), 1-Progreso PER, 23-Renacimiento PER, 12-Luis Quintero PER, 13- Tatagildo PER, 14-Morelos PER, 15-Col. Pri PER, 45-Caltitlan DeL dead, 46- Santa Cruz PER, M- Marcador Molecular marker, , PER (sobreviviente a permetrina), Del (sobreviviente a deltametrina)



Geles de agarosa (2%) del dominio IIIS6 (exon 31), 1-Progreso PER, 12-Luis Quintero PER, 4-Renacimiento DEL, 13- Tatagildo PER, 14-Morelos PER, M-Molecular marker.

3. GENOTIPOS KDR ENCONTRADOS EN MOSQUITOS *Ae. aegypti* DEL ESTADO DE GUERRERO.

Genotipos Kdr encontrados en *Ae. Aegypti* sobrevivientes a la exposición a piretroides.

Muestra	Localidad	Dominio IIS6 Exón 20-21 Posición 1016			Dominio IIIS6 Exón 31 Posición 1534		
		V/V	V/I	I/I	F/F	F/C	C/C
Progreso PER	Acapulco			ATA/ATA			TGC/TGC
Renacimiento DEL	Acapulco	NA					TGC/TGC
Luis Quintero PER	Iguala			ATA/ATA			TGC/TGC
Tatagildo PER	Chilpancingo	GTA/GTA				TCC/TGC	
Morelos PER	Zihuatanejo	NA					TGC/TGC
Colonia PRI PER	Tecpan	NA	NA	NA			TGC/TGC

NR (No PCR)

4. Ensayo de HOLA (Hot Oligonucleotide Assay)

Detección de los SNP

- Remover el TMB para que alcance temperatura ambiente
- Descongelar una alícuota de la solución BSA y el conjugado HSP anti fluoresceína AB solución stock.
- Hacer el Buffer de lavado 1.

1. Adicionar 20µl de TNE de 20 µl de la reacción de PCR (Utilizar material esterilizado para evitar contaminación).

2. Colocar los 40µl en un pozo de la placa de estreptavidina y permitir incubar a temperatura ambiente por 30 minutos en la oscuridad.

- Diluir 5µl del conjugado HSP anti fluoresceína Ab solución stock en 10 ml de la solución BSA.

3. Remover cuidadosamente la mezcla de cada pozo usando una pipeta multicanal para evitar contaminación.

4. Lavar 2 veces con 200µl del buffer de lavado 1 recién preparado.

5. Lavar 2 veces con 200µl del buffer de lavado 2.

6. Adicionar 40µl de 75mU/ml del conjugado HSP anti fluoresceína Ab solución e incubar a temperatura ambiente por 30 minutos.

7. Lavar 3 veces con 250µl del buffer de lavado 2.

8. Remover trazas finales colocando la placa boca abajo en papel toalla.

9. Adcionar 100µl de solución TMB.

10. Registrar visualmente los cambios de color después de 5 minutos o leer el plato en un lector de Elisa a 650nm.

Detalles de los reagentes y soluciones para el HOLA

- Conjugado HSP anti fluoresceína AB solución- Roche 1 426 346
- Solución TMB (BM Blue pod Susbtrate)- Roche 1 484 281

Soluciones y Buffers

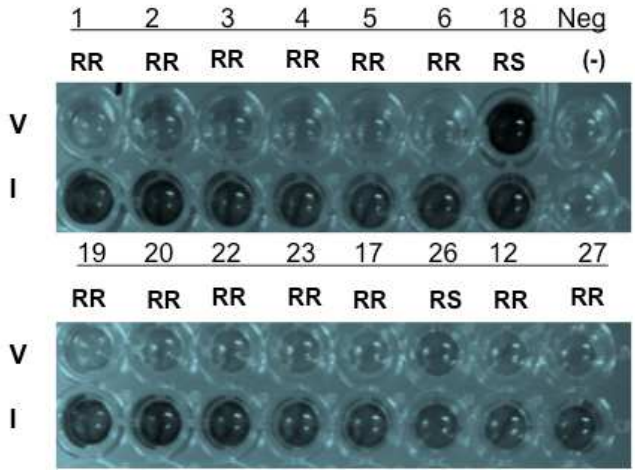
- **TE Buffer**
  - 100mM Tris-HCI pH8.0 (4ml 0.5M Tris HCl pH8.0)
  - 10mM EDTA (400ml de 0.5M EDTA pH8.0)
  - 15.6ml ddH_2O
- **Solución stock de Estreptavidina** 1mg/mi- disolver en 1 mg en 1ml de ddH_2O, almacenar y refrigerar.
  - La solución de trabajo de Estreptavidina 5µl/ml- adicionar del stock 10 ml de ddH_2O.
- **PBS** (Buffer fosfato salino- 1x PBS, 0.1% v/v Tween 20)
  - 300 ml 5M NaCl
  - 680 ml de dH_2O
  - 9.5ml 1M de fosfato sódico dibásico
  - Llevar a pH7.2 con fosfato sódico monobásico
  - Revolver dentro de 2 botellas de 500ml
  - Llevar al autoclave
  - Adicionar 500µl de Tween 20 a cada botella de 500ml
- **Solución de Bloqueo** (1x PBS, 01%v/v Tween 20, 2%v/v BSA)
  - 10g polvo de BSA en 500 ml de PBS, almacenar a 4ªC.
- **TNE** (10mM Tris-HCl pH7.5, 1mM EDTA pH8.0, 0.2 NaCl)

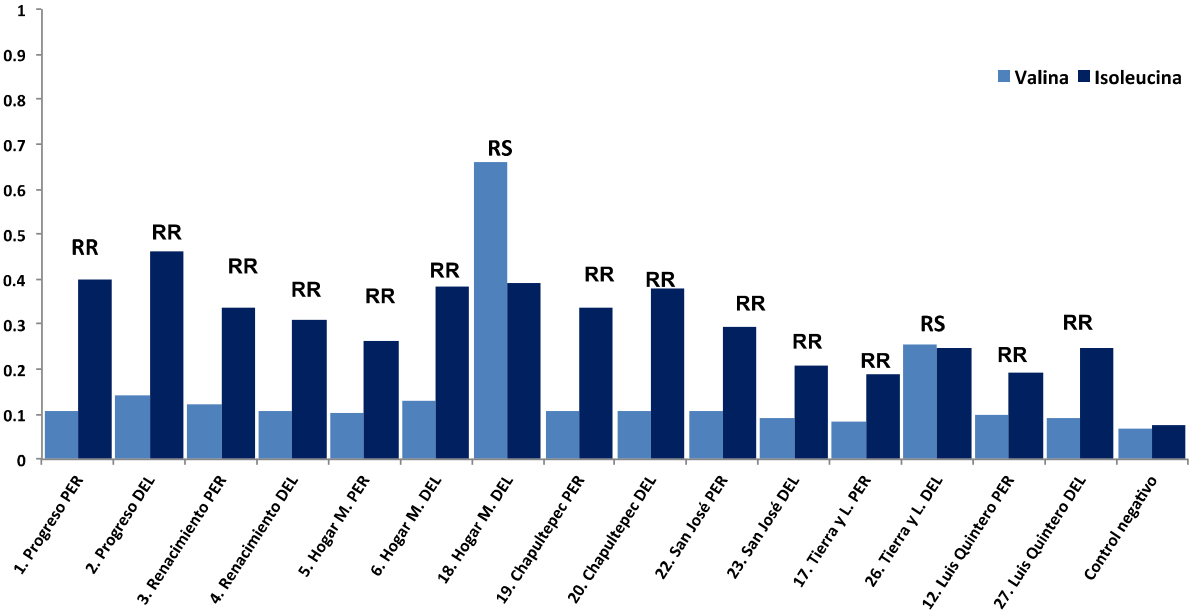
- 4ml de Tris-HCl 0.25M pH7.5
- 200µl de EDTA 0.5M pH8.0
- 4ml de NaCl 5M
- 91.8ml dH_2O
- Llevar a autoclave.

- **Buffer de lavado 1** (10mM NaOH, 0.05% v/v Tween 20)
  - 500µl de NaOH 2M
  - 50µl de Tween 20
  - 99.5ml de dH_2O
- **Buffer de lavado 2** (0.1M Tris-HCl pH7.5, NaCl 0.15M, 0.05% v/v de Tween 20)
  - 100ml Tris-HCl pH7.5 1M
  - 30ml 5M NaCl
  - 870ml dH_2O
  - Llevar a autoclave
  - Adicionar 250µl de Tween 20 a cada botella de 500ml
- **1% Solución de BSA**
  - 1g de polvo de BSA
  - 100ml de Buffer de lavado 2
  - Hacer alícuotas de 10ml y congelar.
- **Solución stock del anticuerpo Anti-Fluoresceína** – Re suspendida a 150U/ml en 1% BSA

  - Solución de trabajo del anticuerpo Anti-Fluoresceína (75mU/ml)- Adicionar 5µl de la solución stock a una alícuota de 10ml de la solución BSA al 1%.

5. EJEMPLO DE UN RESULTADO DE HOLA





RESULTADO DE HOLA: V=Valina, I=Isoeucina, RR=Homocigoto Resistaente RS= Heterocigoto Susceptible, PER (sobreviviente permetrina), Del (sobreviviente deltametrina) 1. Progreso PER, 2. Progreso DEL, 3. Renacimiento PER, 4. Renacimiento DEL, 5. Hogar Moderno PER, 6. Hogar M DEL, 18. Hogar M. DEL muerto, 19. Chapultepec PER, 20. Chapultepec DEL, 22. San jose PER, 23. San jose DEL, 17. Tierra y Libertad PER, 26. Tierra y Libertad DEL dead, 12. Luis Quintero PER, 27 Luis Quindero DEL.

6. Presentación en eventos Cientícos

- Octubre 3 al 6, 7th European Congress on Tropical Medicine and International Health. Expositor con el póster: Insecticide resistance mechanisms in *Aedes aegypti* populations from localities with high dengue transmission risk of Guerrero, Mexico. Barcelona España.
- Septiembre 27, XX Congreso Latinoamericano de pasitología y XV Congreso Colombiano de parasitología y Medicina Tropical. Expositor con el poster "Mecanismos de resistencia a insecticidas organofosforados, Carbamatos y piretroides en *Aedes aegypti* de localidades con alto riesgo para la transmisión de dengue en el Estado de Guerrero, México. Bogotá, Colombia.
- Junio 2011, XLVI Congreso Nacional de Entomología. Sociedad Mexicana de Entomología. Expositor con el poster "Resistencia a insecticidas piretroides mediada por mutaciones kdr en *aedes aegypti* del estado de Guerrero, México". Cancún-Riviera Maya, Quintana Roo, México.
- Marzo 2011, XIV Congreso en investigación en Salud Pública. Instituto Nacional de Salud Pública de México. Expositor con el poster "Situación actual de la resistencia a insecticidas de *Aedes aegypti* del estado de Guerrero". Cuernavaca, Morelos, México.

8. Artículos Derivados del Trabajo.

Resistencia a insecticidas piretroides mediada por mutaciones kdr en *aedes aegypti* del estado de Guerrero, México. Publicado en memorias del XLVI Congreso Nacional de Entomología. Sociedad Mexicana de Entomología. Cancún-Riviera Maya, Quintana Roo; México. 26-29 de junio de 2011. Pp