# Evaluation of pyrethroid efficacy for control of dengue vector mosquitoes in Taiwan

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## Summary

Dengue fever is a serious mosquito-borne disease in Taiwan which the number of indigenous cases in last year was up to 15,708. Vector control was used to reduce local mosquito density, and thus to reduce the number of dengue cases. One of the important measures is the application of insecticides to kill flying adults. The pyrethroids were the most commonly used insecticides because of their low mammal and rapid mosquito toxicities. The objective of this study is to use PCR methods to examine the resistance frequency of 5 loci of voltage-gated sodium channel in local *Aedes aegypti* populations in dengue endemic area in Taiwan. We genotyped five positions of Vssc and found 4 amino acid substitutions in local mosquito populations in southern Taiwan with varied frequencies (0-0.90) within districts compared with the reference sequence. The 3 major substitutions were a serine to phenylalanine at codon 989, a valine to glycine at codon 1016 in the domain II, and a phenylalanine to cysteine at codon 1534 in the domain III. The overall frequencies of 989, 1016, 1534, and 1763 alleles were 0.28, 0.38, 0.24, and 0.09, respectively. In Tainan City, the overall frequencies of 989, 1016, 1534, and 1763 alleles were 0.34, 0.50, 0.19, and 0.14, respectively. Among districts, the West-Central district had higher frequencies (0.65, 0.90, and 0.20) than other districts in 989, 1016, and 1763 alleles in the same city. Moreover, the high frequency (0.9) in 1016 was all homologous mutations. In Kaohsiung City, the overall frequencies of 989, 1016, 1534, and 1763 alleles were 0.23, 0.28, 0.29, and 0.06, respectively. Among districts, the Cianjhen District had higher frequencies (0.45, 0.53, 0.45, and 0.10) than other districts in all 4 alleles in the same city. However, most of them were heterologous mutations. This results only preliminary, more samples are needed to clear the resistance status in Taiwan mosquito population.

## I. Purpose

Dengue has emerged as a worldwide problem only since the 1950s and as a Taiwan problem since 1987. Since 2004, a dengue outbreak of 202-2,000 cases occurred every year. In 2014, the number of dengue cases is over 15,708. No dengue vaccine or specific medications to treat a dengue infection is available for the time-being. The main strategy to interrupt or prevent transmission is to combat with

mosquito vectors, *Aedes aegypti* L. and *Ae. albopcitus* Skuse. One of the important control measures is the application of insecticides to kill flying adults. Among these insecticides, the pyrethroid has a high and rapid toxicity against mosquitoes but low toxicity to mammals. Therefore, pyrethroids are commonly used in Taiwan households and dengue control programs. The heavy and intensive uses of the permethrin led to the serious problem of the premethrin resistance in southern Taiwan since 2001 (Lin et al. 2002). The conventional bioassay to detect the resistance requires many field-collected mosquitoes and times to complete one trial. Through the technology of the molecular methods, the resistance can be detected in voltage-gated sodium channel by PCR using few samples within 1-2 days.

Pyrethroid insecticides target the voltage-sensitive sodium channel(Vssc). Amino acid substitution in the Vssc gene would affect the affinity of Vssc for pyrethroids and result in resistance to this insecticide in pest (Davies et al., 2007; Rinkevich et al., 2013). The mutation of specific amino acid and caused pyrethroid resistance was the mechanism called ‘‘knockdown resistance (kdr)’’. Previously report showed several amino acid substitutions involved in kdr have been found in *Ae. aegypti* Vssc gene: the S989P, I1011M/V, V1016G/I, F1534C, and D1763Y(Chang et al., 2009; Jintana Yanola et al., 2010; Kawada et al., 2009; Rajatileka et al., 2008; Saavedra-Rodriguez et al., 2007; Srisawat et al., 2010). Genotyping of the Vssc gene would halp us to know the allele frequencies in *Ae. aegypti* from the dengue outbreak region in Taiwan.

In this study, we investigated kdr mutation in the Vssc gene of *Ae. aegypti* collected from southern Taiwan, which was the first report for studying 5 specific amino acid positions to identify the candidates for knockdown resistance to pyrethroid.

## II. Methods

*Aedes aegypti* were collected between July to October in 2014 from 4 districts (West Central District, North District, South District and Yong Kong District) of Tainan City and 4 districts (Siaogang District, Fongshan District, Sanmin District and Cianjhen District) of Kaohsiung City, southern Taiwan. In each district, 10-20 mosquitoes in the first to six generations were used and the detail information were showed in table 1. Mosquito colonies were maintained in an insectary at 25$^{o}$C with a photoperiod of 10:14 (Light:Dark) h. Larvae were reared in a plastic pan (21 by 14 by 7 cm) containing 450 mL of deionized water. A sufficient amount of food (yeast powder and pig liver; 1:1 by weight) was provided daily. Adult mosquitoes were kept in an acrylic cage (29 by 20 by 20 cm) and were provided with a 10% sucrose solution.

Table 1. The collection sites and generations of field-collected *Aedes aegypti* used in this study.

| Location | Generation(s) | Number |
|---|---|---|
| Tainan-West Central District | 1 | 10 |
| Tainan-North District | 4 | 10 |
| Tainan-South District | 2 | 10 |
| Tainan-Yong Kong District | 1 | 10 |
| Kaohsiung-Siaogang District | 1 | 10 |
| Kaohsiung-Fongshan District | 1 | 10 |
| Kaohsiung-Sanmin District | 6 | 10 |
| Kaohsiung-Cianjhen District | 1 | 20 |

The study method was learned from Dr. Takashi Tomita (Department of Medical Entomology, National Institute of Infectious Diseases) last December by Dr. Tien-Huang Chen. We briefly described below. Single male mosquito with a 3 mm glass ball in a tube was homogenized in a TissueLyzer (Qiagen GmbH, Hilden, Germany) with two cycles for 90 sec at a frequency of 30 Hz. Male mosquitoes was used to avoid gene contamination derived from sperm DNA in females and no gender bias in this species (Kasai et al. 2014). Genomic DNA was extracted from the individual mosquito extraction using the QIAmp genomic DNA Mini Kit (Qiagen Gmbh, Hilden, Germany) according to the manufacturer's instructions. We perform PCR to amplify partial DNA fragments of VGSC gene domains II, III, and IV by using 2X PCR master mix solution (i-pfu)(iNtRON Biotechnology, Korea) and three primer sets (table 2). The PCR conditions used were as following: initial denaturation at 95$^{o}$C for 5 min, followed by 35 cycles of 94 $^{o}$C for 30 s, 55 $^{o}$C for 30 s, 72 $^{o}$C for 1 min, and a final extension step at 72 $^{o}$C for 10 min. 5 amino acid loci were targeted to identify the candidates of kdr, in *Ae. aegypti*, according to the reference sequence (GenBank: AAB47604 and AAB47605), the amino acid loci in VGSC gene associated with pyrethroid resistance namely S989P, I1011M or V, V1016G or I, F1534C, and D1763Y. The PCR products were directly sequenced with the primers: AaSCF3 (forward primer for domain II), AaSCR22 (reverse primer for domain II), AaSCR8 (reverse primer for domain III), and AlSCF7 (forward primer for domain IV).

Table 2. The PCR primer sequences used in this study. Primer

| Name | Sequence | Target site |
|---|---|---|
| AaSCF20 | GACAATGTGGATCGCTTCCC | PCR for domain II (Forward) |

| | | |
|---|---|---|
| AaSCR21 | GCAATCTGGCTTGTTAACTTG | PCR for domain II (Reverse) |
| AaSCF7 | GAGAACTCGCCGATGAACTT | PCR for domain III (Forward) |
| AaSCR7 | GACGACGAAATCGAACAGGT | PCR for domain III (Reverse) |
| AlSCF6 | TCGAGAAGTACTTCGTGTCG | PCR for domain IV (Forward) |
| AlSCR8 | AACAGCAGGATCATGCTCTG | PCR for domain IV (Reverse) |
| AaSCF3 | GTGGAACTTCACCGACTTCA | Sequencing for domain II (Forward) |
| AaSCR22 | TTCACGAACTTGAGCGCGTTG | Sequencing for domain II (Reverse) |
| AaSCR8 | TAGCTTTCAGCGGCTTCTTC | Sequencing for domain III (Forward) |
| AlSCF7 | AGGTATCCGAACGTTGCTGT | Sequencing for domain IV (Forward) |

## III. Results

We genotyped five positions of Vssc and found 4 amino acid substitutions in local mosquito populations in southern Taiwan with varied frequencies (0-0.90) within districts compared with the reference sequence (Table 3). At codon 1011, no amino acid substitution occurred, all the sample was wild type. The 3 major substitutions were a serine to phenylalanine at codon 989, a valine to glycine at codon 1016 in the domain II, and a phenylalanine to cysteine at codon 1534 in the domain III. The overall frequencies of 989, 1016, 1534, and 1763 alleles were 0.28, 0.38, 0.24, and 0.09, respectively. In Tainan City, the overall frequencies of 989, 1016, 1534, and 1763 alleles were 0.34, 0.50, 0.19, and 0.14, respectively. Among districts, the West-Central district had higher frequencies (0.65, 0.90, and 0.20) than other districts in 989, 1016, and 1763 alleles in the same city. Moreover, the high frequency (0.9) in 1016 was all homologous mutations. In Kaohsiung City, the overall frequencies of 989, 1016, 1534, and 1763 alleles were 0.23, 0.28, 0.29, and 0.06, respectively. Among districts, the Cianjhen District had higher frequencies (0.45, 0.53, 0.45, and 0.10) than other districts in all 4 alleles in the same city. However, most of them were heterologous mutations. We also combined the frequencies of P989+G1016 to see the frequencies of simultaneous mutations. The overall P989+G1016 frequencies in Tainan and Kaohsiung mosquito populations were 50.0% and 40.0% respectively, and the mosquitoes collected from the West Central District and Cianjhen District had higher frequencies (90.0% and 80.0%) than overall frequencies of the same city (table 4). The sample frequencies to occur mutation only in P989 or G1016 were 15.0, 0.0, 6.0, and 5.0% collected in Tainan City, West Central District, Kaohsiung City, and Cianjhen District, respectively.

Table 3. Vssc genotypes of S989P, V1016G/I, F1534C, D1763Y, and I1011M/V sites in field-collected *Ae. aegypti* from Tainan City and Koahsiung City.

| Site | 989 | | | 1016 | | | | 1534 | | | | 1763 | | | | 1011 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | S/S | S/P | P/P | P Frequency | V/V | V/G | G/G | F Frequency | F/F | F/C | C/C | C Frequency | D/D | D/Y | Y/Y | Y Frequency | I/I |
| West Central District, Tainan | 1 | 5 | 4 | 0.65 | 1 | 0 | 9 | 0.90 | 8 | 2 | 0 | 0.10 | 6 | 4 | 0 | 0.20 | 10 |
| North District, Tainan | 3 | 5 | 2 | 0.45 | 4 | 3 | 3 | 0.45 | 4 | 5 | 1 | 0.35 | 9 | 1 | 0 | 0.05 | 10 |
| South District, Tainan | 5 | 5 | 0 | 0.25 | 3 | 4 | 3 | 0.50 | 5 | 4 | 1 | 0.30 | 7 | 3 | 0 | 0.15 | 10 |
| Yong Kong District, Tainan | 10 | 0 | 0 | 0.00 | 7 | 3 | 0 | 0.15 | 10 | 0 | 0 | 0.00 | 7 | 3 | 0 | 0.15 | 10 |
| Tainan City total | 19 | 15 | 6 | 0.34 | 15 | 10 | 15 | 0.50 | 27 | 11 | 2 | 0.19 | 29 | 11 | 0 | 0.14 | 40 |
| Siaogang District, Kaohsiung | 10 | 0 | 0 | 0.00 | 9 | 1 | 0 | 0.05 | 10 | 0 | 0 | 0.00 | 9 | 1 | 0 | 0.05 | 10 |
| Fongshan District, Kaohsiung | 7 | 2 | 1 | 0.20 | 7 | 2 | 1 | 0.20 | 5 | 4 | 1 | 0.30 | 10 | 0 | 0 | 0.00 | 10 |
| Sanmin District, Kaohsiung | 9 | 1 | 0 | 0.05 | 8 | 2 | 0 | 0.10 | 7 | 1 | 2 | 0.25 | 9 | 1 | 0 | 0.05 | 10 |
| Cianjhen District, Kaohsiung | 4 | 14 | 2 | 0.45 | 3 | 13 | 4 | 0.53 | 4 | 14 | 2 | 0.45 | 17 | 2 | 1 | 0.10 | 20 |
| Kaohsiung City total | 30 | 17 | 3 | 0.23 | 27 | 18 | 5 | 0.28 | 26 | 19 | 5 | 0.29 | 45 | 4 | 1 | 0.06 | 50 |
| Total | 49 | 32 | 9 | 0.28 | 42 | 28 | 20 | 0.38 | 53 | 30 | 7 | 0.24 | 74 | 15 | 1 | 0.09 | 90 |

Table 4. Frequencies of P989+G1016 of the voltage-sensitive sodium channel gene in *Aedes aegypti*.

| Amino acid location | Tainan City (%) | West Central District, Tainan(%) | Kaohsiung City(%) | Cianjhen District, Kaohsiung (%) |
|---|---|---|---|---|
| 989 S/P+1016 V/G | 7 (17.5) | 0 | 15 (30.0) | 12 (60.0) |
| 989 S/P+1016 G/G | 7 (17.5) | 5 (50.0) | 2 (4.0) | 2 (10.0) |
| 989 P/P+1016 V/G | 0 | 0 | 0 | 0 |
| 989 P/P+1016 G/G | 6 (15.0) | 4 (40.0) | 3 (6.0) | 2 (10.0) |
| 989 S/P+1016 V/V | 1 (2.5) | 0 | 0 | 0 |
| 989 P/P+1016 V/V | 0 | 0 | 0 | 0 |
| 989 S/S+1016 G/G | 2 (5.0) | 1 (10.0) | 0 | 0 |
| 989 S/S+1016 V/G | 3 (7.5) | 0 | 3 (6.0) | 1 (5.0) |
| 989 S/S+1016 V/V | 14 (35.0) | 0 | 27 (54.0) | 3 (15.0) |
| P989+G1016 | 20 (50.0) | 9 (90.0) | 20 (40.0) | 16 (80.0) |
| P989 or G1016 only | 6 (15.0) | 0 | 3 (6.0) | 1(5.0) |
| Total | 40 | 10 | 50 | 20 |

## IV. Discussion

We collected 40 and 50 male adult mosquitoes from Tainan and Kaohsiung city, and genotyped 5 specific loci of the Vssc gene, which were potentially conferring the mosquito resistance against pyrethroid. In previously study, the V1016G and F1534C mutated site of Vssc gene caused strongly correlated with resistance to pyrethroid in *Ae. aegypti* (Harris et al., 2010; Hu et al., 2011; Saavedra-Rodriguez et al., 2008; Saavedra-Rodriguez et al., 2007). The V1016G and F1534C mutant in *Ae. aegypti* also confirmed in many Southeast Asia countries, like Vietnam (Kawada et al., 2009), Thailand (Stenhouse et al., 2013), and Singapore (Kasai et al., 2014). In Taiwan, a report has been showed that they found the V1016G mutant exists in *Ae. aegypti*, and Hirata et al. mentioned the F1534C mutation of Vssc in *Ae. aegypti* distributed in some Southeast countries except for Taiwan (Hirata et al., 2014). In our study, the tested 5 specific loci of Vssc gene in *Ae. aegypti* from southern Taiwan had 4 mutated sites except for I1011M/V, so we found that there also existed the heterozygous and homozygous F1534C mutated Vssc gene of *Ae. aegypti* in Taiwan.

S989P was usually accompanied by V1016G (Srisawat et al., 2010), which we obtained the similar results in our study. We also detected the higher mutated Vssc gene in mosquitoes collected in the epidemic hot spot regions, which indicated the selection pressures of insecticides in those areas by dengue control. However, it is surprisingly to found that lower gene mutation in mosquito populations in Kaohsiung

City than in Tainan City. Although Kaohsiung City (14,969 indigenous cases) had more dengue cases last year than Tainan City (155 indigenous cases). More samples or information are needed to clear this point. Few mosquito samples showed F1534C mutation, which an investigation in Cayman Islands demonstrated that F1534C mutation was also correlated with permethrin resistance (Harris et al., 2010). In addition, an electrophysiological study also demonstrated that the F1534C mutation of Vssc gene would lower the sensitivity to type I pyrethroids, but not type II (Hu et al., 2011). Therefore, the presence of F1534C mutant strain of *Ae. aegypti* in nature field may also affect the efficiency of using type I pyrethroids in vector control.

Up to date, the mutation sites of V1016G, V1016I, and F1534C have been shown to correlate with pyrethroid resistance in many countries, and the current study showed that the other dengue vector, *Ae. albopictus,* was not an exception (Kasai et al., 2011). Therefore, it was important to conduct surveillance on Vssc kdr mutations in the field population of *Aedes* vectors in dengue epidemic areas in Taiwan. Early detection of the mutation sites would assist us in developing the appropriate vector control strategies, and using the suitable insecticides to effectively control mosquitoes in the field.

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