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Research Article

Major Resistant Mechanism to Insecticides of Aedes aegypti Mosquito: a Vector of Dengue and Zika Virus in Vietnam

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Abstract

Aedes aegypti, a main vector of dengue fever, is very high density in some areas of Vietnam. These mosquitoes in some cities such as Hanoi (HN), Nhatrang (NT), Dongnai (DN), Kiengiang (KG), Daklak (DL), and Hochiminh (HCM) cities have been found high resistance to Pyrethroid group and DDT. Mosquito populations have been sensitive to malathion insecticide.

Identifying insecticide resistant characteristics by esterase enzyme electrophoresis showed that the first esterase appeared in pyrethroid insecticide resistant mosquito population and not recorded in Bora Bora strain.

Analysis of mutations in by multiplex PCR 1011 and 1016 codons encoding VAL and ISO in 20 and 21 exons showed that mutation in 1016 codons with two types VAL/1016/ISO and VAL/1016/GLY were found in the domain II of the sodium channel gene with 94 *Aedes aegypti* adult female mosquitoes after susceptibility test to alphacypermethrin. The results showed that no mutation in 1011 codon encoding ISO was observed. 14 samples collected from four provinces containing mutations in 1016 codons with two VAL/1016/ISO and VAL/1016/GLY types have been detected.

Introduction

In Vietnam, recent years diseases caused by mosquitoes: Dengue fever caused by Flaviridae virus and transmission main vectors of dengue fever *Aedes aegypti*, more and more increasing number of infected patients death, special in some large cities such as Hanoi, Nhatrang, Dongnai, Kiengiang, Banmethuot, Daklak and Hochiminh(Zika patient) cities [1,7,8]. One of causes was development of insecticide resistance in mosquitoes - a challenge in the world. Genetic resistant characteristics of transmission main vectors of malaria, dengue, filariasis and trypanosomiasis is serious impediment to development in many tropical countries cities [2,8,10,11]. Natural selection occurs quickly. Mutation individuals were selected and conserved to increase resistant clones.

In Vietnam, since 1960, resistant characteristics in Culex quinque fasciatus, *Aedes aegypti* mosquitoes have been detected. In 1975, it was found that *Anopheles epiroticus* (*Anopheles sundaicus*), *An.sinensis, An.vagus* were resistant to DDT, permethrin, and deltamethrin insecticide cities [3,9,8]. Insecticide usage was a selection pressure to population and structure's change. Insecticide resistant observation was performed by susceptibility assay and PCR method was used to detect the resistant level to find a control method of these disease by insect vector; a suitable method selected to waste avoid and pollution environmental were need.

Materials

Time of the study: from 9/2006 to 12/2009

Area of the study: *Aedes aegypti* larvae's were collected at some areas in Hanoi, Nhatrang, Buonmethuot, Bienhoa, Rachgia, Hochiminh, Hoabinh, and Ninhbinh cities.

Susceptibility assay of mosquito adults with six insecticides: Permethrin 0.75%, Alpha-cypermethrin 30 mg/m2, Lambda-cyhalothrin 0.05%, Deltamethrin 0.05%, DDT 4% and Malathion 5% have been performed in Department of Experimental Chemistry cities [4,7,11]. PCR analysis was carried out in Department of Biotechnology in National Institute of Malariology, Parasitology and Entomology (NIMPE), Vietnam.

Nucleotide sequencing was performed in Center of DNA Diagnostics, Institute of Biotechnology, Vietnam Academy of Science and Technology.

Methods

This is the last version of discriminative dose of insecticides for mosquitoes (Table 1)

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Table 1: Diagnostic dose rates of Insecticide impregnated papers available from WHO.

Insecticide	Anophelines	Aedes aegypti	Culex quinquefasciatus
DDT	4%	4%[a]	4%[b]
Fenitrothion	1%[c]		1%[d]
Malathion	5%	0.80%	5%
Bendiocarb	0.10%		
Propoxur	0.1%[c]	0.10%	0.10%
Alpha-cypermethrin	0.05%		
Bifenthrin	0.20%		
Cyfluthrin	0.15%		
Deltamethrin	0.05%		0.03%
Lambda-cyhalothrin	0.05%[e]	0.03%	0.03%
Permethrin	0.75%	0.25%	0.25%
Etofenprox	0.50%		

[a]Half an hour exposure
[b]Four hours exposure
[c]Two hour exposure for Anopheline Larvae
[d]0.1% Anopheline Larvae
[e]Two hour exposure Anopheline Larvae

- *Aedes aegypti* larvaes were collected from natural and personal pool field, classified according to classification table of *Aedes* in Vietnam cities [2,15].
- Larvae process in insect rearing with condition at 27 °C±2 °C and 80% ±10% Relative Humidity (RH), 10 hours/daylight. Larvae food was produced by NIMPE [2,7].
- Susceptibility assay was followed by WHO/CDS/CPS/MAL/98.12 [2,15] with six above insecticides.
- The susceptibility assay was conducted with female mosquito adult supplying with sugar solution.
- The number of mosquitoes for an assay is 100.
- The number of mosquitoes for a control is 20-40.
- Exposure time: the testing tubes were vertically kept under subdued light for 1 hour.
- Dead mosquitoes are counted after 10, 15, 20, 30, 40, 50, 60 minutes. After the exposure time, mosquitoes were transferred to holding-tubes supplying with sugar solution, and maintained in a climatic chamber for 24 hours at 25 °C±2 °C and 80% ± 10% RH.
- If the control tube had the mosquito mortality rate over 20%, the tube should be removed and again test conduct, if the mosquito mortality rate in control tube from 5 to less 20%, change by Abbott's formula.

$$\text{Mortality} = \frac{\%\text{ fact death mortality} - \%\text{ control death mortality}}{100 - \%\text{ control death mortality}} \text{ X } 100$$

The percentage mortality after 24 hours is recorded.

Result assesses:

- Mortality from 98 to 100%: sensitivity.
- Mortality from 80 to 97%: might be resistance and need to confirm again
- Mortality less 80: resistance

Esterase enzyme electrophoresis by Green et al, 1990.

PCR technique

Mutation analysis of Kdr gene of *Aedes aegypti* in 1011 and 1016 codons by a method of Saavedra-Rodriguez K. et al, 2007 [12,13,14] showed in (Table 2).

Table 2: Mutation rates in 1011 and 1016 codons.

	Codon 1011	Codon 1016
Wide	Iso(ATA)	Val(GTA)
C.Bengues et al, 2003	Met(ATG)	Gly(GGA)
K.Saavedra-Rodriguez et al, 2007	Val(GTA)	Iso(ATA)

PCR products were analyzed by 4% agarose electrophoresis in TBE 1X buffer in 45 minutes. DNA extraction kit for nucleotide sequencing was purchased from Fermantas. Nucleotide sequencing was performed by ABI-3100 sequencer.

Result: Tool rates has *Aedes aegypti* larvae in some test provinces (from 2008 to 2009).

In the North: Investigating in Hanoi city with 4 districts, the result showed that vector-bone disease was found in test tools from 20% to 50%. Further, knowledge human about fresh environment to disease control is low, degradation environment.

In special, Tuliem district from March to July in 2007, *Aedes aegypti* was not found in test place, but *Aedes albopictus* was found. However, from March to May in 2009 we found *Aedes aegypti* larvaes in water tool about 50% mortality rate (Table 3).

Table 3: Primer sequences used for PCR

Mutation	Primer	Size (bp)
Val1011		
Val1011 swept	5'-ATTGTATGCTTGTGGGTGACG-3'	
Iso 1011 reverse	5'-[short tail]TACTTACTACTAGATTTCC**AAT**-3'	84
Val 1011 reverse	5'-[long tail]TACTTACTACTAGATTTCC**GAC**-3'	104
Met 1011		
Met 1011 swept	5'-GTCCTGTATTCCGTTCTTTTT-3'	
Iso 1011 reverse	5'-[short tail] TACTTACTACTAGATTT**ACT**-3'	62
Met 1011 reverse	5'-[long tail] TACTTACTACTAGATTT**GCC**-3'	82
Iso 1016		
Val 1016 swept	5'-[long tail] ACAAATTGTTTCCCACCCGCAC**CGG**-3'	102
Iso 1016 swept	5'-[short tail] ACAAATTGTTTCCCACCCGCAC**TGA**-3'	82
Iso 1016 reverse	5'- GGATGAACCGAAATTGGACAAAAGC-3'	
Gly 1016		
Gly 1016 swept	5'-ACCGACAAATTGTTTCCC-3'	
Val 1016 reverse	5'-[short tail] AGCAAGGCTAAGAAAAGGTTAA**TTA**-3'	60
Gly 1016 reverse	5'-[long tail] AGCAAGGCTAAGAAAAGGTTAA**CTC**	80

[short tail]: GCGGGC,

[long tail]: GCGGGCAGGGCGGCGGGGGCGGGGCC

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Table 4: Tool rates had *Aedes aegypti* larvae in some provinces (from 2007 to 2008).

Time	Collecting area	No. water tool to test	No. tool had larvae	Tool rate (%)
4/2008	Thanhxuan District, Hanoi City	50	20	40
6/2007	Dongda District, Hanoi city	50	19	38
8-2007	Hoangmai District, Hanoi city	50	10	20
5/2008	TuliemDistrict, Hanoi city	50	26	50
6/2007	Huunghi commune, Hoabinh city, Hoabinh province	50	0	0
8/2007	Xuanmai commune, Luongson District, Hoabinh province	50	0	0
5/2008	Ninhbinh city, Ninhbinh province	50	0	0
5/2014	Phatdiem commune, Kimson District, Ninhbinh province	50	0	0

Table 5: Susceptibility test result with *Aedes aegypti* population (72nd mosquito generation) in Hanoi from 2008 to 2009.

	Insecticide	Tuliem		Thanhxuan		Dongda		Hoangmai	
		Mortality (%)	assess	Mortality (%)	assess	Mortality (%)	assess	Mortality (%)	assess
1	permethrin 0,75%	80.0	R/S	51	R	65.5	R	79	R
2	alpha-cypermethrin 30mg/m2	83.0	R/S	90	R/S	82	R/S	96	R/S
3	lambda-cyhalothrin 0,05%	63.0	R	74	R	59	R	59	R
4	deltamethrin	97.0	R/S	64.0	R	85.0	R/S	62.0	R
5	DDT 4%	20.0	R	1	R	8.0	R	32	R
6	malathion 5%	99.0	S	98.0	S	98.0	S	98.0	S

Table 6: Tool rate had *Aedes aegypti* larvae in some provinces 2008.

Time	Collecting area	No. water tool to test	No. tool had larvae	Tool rate (%)
9/2008	Nhatrang city, Khanhhoa province	100	62	62,0
8/2008	Buonmethuot city, Daklak province	100	76	76,0
8/2008	Hochiminh city	100	55	55,0
8/2007	Bienhoa city, Dongnai province	100	47	47,0
8/2008	Rachgia city, Kiengiang province	100	62	62,0

Table 7: Susceptibility test result with *Aedes aegypti* populationin Nhatrang city, Khanhhoa province and Banmethuot city, Daklak province.

Insecticide	Nhatrang 9/2008		Daklak 9/2008	
	Mortality after 24 hours (%)	assess	Mortality after 24 hours (%)	assess
permethrin 0,75%	3.03	**R**	14.58	**R**
alpha-cypermethrin 30mg/m2	10.6	R	83.0	R/S
lambda-cyhalothrin 0,05%	10.2	**R**	35.7	**R**
deltamethrin	8.08	**R**	42.85	**R**
DDT 4%	2.0	**R**	0	**R**
malathion 5%	98.0	**S**	98.3	**S**

Table 8: Susceptibility test result with *Aedes aegypti* population in Rachgia- Kiengiang, Bienhoa-Dongnai and Hochiminh cities.

	Insecticide	Kiengiang 9/2008		Dongnai 9/2008		Hochiminh city 9/2008	
		Mortality (%)	assess	Mortality (%)	assess	Mortality (%)	assess
1	permethrin 0,75%	13.5	**R**	6.6	**R**	16.0	**R**
2	alpha-cypermethrin 30mg/m2	47.4	R	55.1	R	16.6	R
3	lambda-cyhalothrin 0,05%	39.3	**R**	30.9	**R**	10.0	**R**
4	deltamethrin	37.7	**R**	24.7	**R**	31.9	**R**
5	DDT 4%	7.0	**R**	6.2	**R**	2.0	**R**
6	malathion 5%	98.3	**S**	98.0	**S**	98.0	**S**

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Table 9: Genotype frequency of *Ae. aegypti* sensitive and resistance mosquitoes with pyrethroid (N= 36) at district of Thanhxuan.

Populations	Allene	Est-1	Est-2	Est-3	Est-4	Est-5	Est-6
Sensitive Bora Bora	a	0	0.81	0.69	0.46	0.92	1
	b	0	0.19	0.31	0.54	0.08	0
Resistance Thanhxuan district	a	0.42	0.73	0.625	0.645	0.625	1
	b	0.58	0.27	0.375	0.355	0.375	0

(Table 4) shows in some test place such as Hoabinh city and Xuanmai commune in Hoabinh province, Phatdiem commune in Ninhbinh province not were found *Aedes aegypti* larvae but had *Aedes albopictus* larvae.

Susceptibility test with *Aedes aegypti* with some insecticides

(Table 5) showed that *Aedes aegypti* at district of Thanh Xuan, Dongda, Hoangmai, Tuliemin Hanoi cities was resistant to permethrin and lambda-cyhalothrin, special highest resistant to DDT. It might be resistant to alpha cypermethrin, and test mosquitoes were sensitive to malathion.

From Center to South

(Table 6) showed that water tool rate in some test cities were high such as Nhatrang city 62.0%, Daklak city 76.0%, Rach gia city of Kiengiang province 62.0%. Fresh environment knowledge, skill larvae to disease control is low, subjective. Special larvae in the roof of house and veranda.

(Table 7) showed that *Aedes aegypti* at Nhatrang city, Khanhhoa province and Buomethuot city, Daklak were resistance with permethrin and lambda-cyhalothrin, special highest resistance with DDT. Might be resistance with alpha cypermethrin and test mosquito were sensitive with malathion.

Need a strategy to mosquito control active to prevent mosquito density, special in summer season.

(Table 8) *Aedes aegypti* population in test places was high resistance with pyrethroid and DDT. Mortality after 24 hours with DDT were highest in Hochiminh city (2.0%) and with alpha cypermethrin were highest in Hochiminh city.

Esterase electrophoresis result

Mutation identified result on kdr gene of *Aedes aegypti:* PCR reaction performed on specific primer at 1011 codon and 1016 codon to reproduction kdr fragment gene of individual from test place, alive and death after susceptibility test.

1011 and 1016 codon mutations.

Mutation analysis: Codon 1011 encoding ISO has mutation A to G to change ISO to VAL (ISO/1011/VAL), or A to G ISO to MET (ISO/1011/MET). Codon 1016 encoding ISO has mutation G to A, to change VAL to ISO (VAL/1016/ISO), or T to G to change VAL to GLY (VAL/1016/GLY).

Codon 1011 has not mutation, has 14 mosquito samples (14, 89%) of 4/6 provinces (66.6%) codon 1016 has mutation with two hetezygote VAL/1016/ISO and VAL/1016/GLY (Table 9). Table 3 showed that has 9 samples-Ae. *Aegypti* from Hanoi city (7.50%) with 5 heterozygote mutation samples VAL/1016/GLY (55.55%), 1 homozygote mutation sample GLY/1016/GLY (11.11%), 1 heterozygote mutation sample VAL/1016/ISO (11.11%) and 2 mix hetezygote mutation samples VAL/1016/ISO and VAL/1016/GLY (Table 10). Khanhhoa province has 2 hetezygote mutation samples AL/1016/GLY (5.88%). Daklak has 1 heterozygote mutation sample VAL/1016/GLY (11.11%). Hochiminh city has 1 mix heterozygote mutation sample VAL/1016/ISO and VAL/1016/GLY (22.22%). Kiengiang and Dongnai provinces have not found mutation in 1011 and 1016 codons (Figures 1-4). Agarose gel electrophoresis of PCR products amplified 1011 and 1016 codons.

Table 10: Type mutation rates of *Ae. aegypti* in individual provinces.

No	Collecting area	Number of samples	Codon 1011						Codon 1016						mix VAL/ 1016/ ISO	Common result	
			ISO to VAL			ISO to MET			VAL to ISO			VAL to GLY					
			ISO/ 1011/ ISO	ISO/ 1011 VAL	VAL/ 1011 VAL	ISO/ 1011/ ISO	ISO/ 1011/ MET	MET/ 1011/ MET	VA/ 1016/ VAL	VAL/ 1016/ ISO	IISO/ 1016 IISO	VA/ 1016/ VAL	VA/ 1016/ GLY	GLY/ 1016/ GLY	VAL/ 1016/ GLY	+	%
1	Hanoi city	24	24	0	0	24	0	0	23	1 11.11%	0	16	5 55.55%	1 11.11%	2 22.22%	9	37.5
2	Hochiminh city	9	9	0	0	9	0	0	9	0	0	7	1 50%	0	1 50%	2	22.22
3	Khanhhoa province	34	34	0	0	34	0	0	34	0	0	32	2 100%	0	0	2	5.88
4	Dongnai province	9	9	0	0	9	0	0	9	0	0	9	0	0	0	0	0
5	Kiengiang province	9	9	0	0	9	0	0	9	0	0	9	0	0	0	0	0
6	Daklak province	9	9	0	0	9	0	0	9	0	0	8	1 100%	0	0	1	11,11
		94	94	0	0	94	9	0	89	1 7.14%	0	80	9 64.28%	1 7.14%	3 21.42	14	14.89

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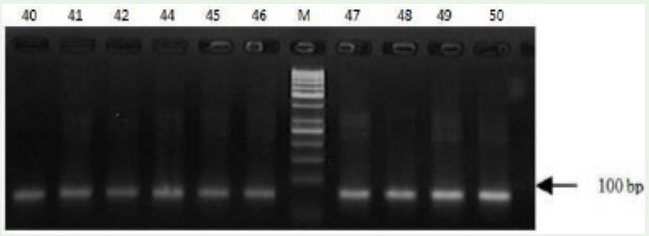

Figure 1: Agarose gel electrophoresis of PCR products in 1011 codon. Wild type Iso/1011/Iso: 40, 41, 42, 45, 46, 47, 48, 49, 50 size product were 80bp. Marker: 100bp.

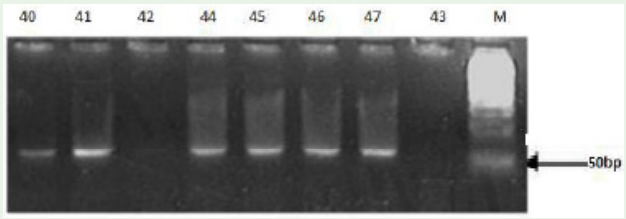

Figure 2: Agarose gel electrophoresis of PCR products in 1011 codon. Wildtype Iso/1011/Iso in lanes 40, 41, 42, 44, 45, 46, 47, 43 size product were 60bp. Marker: 50bp.

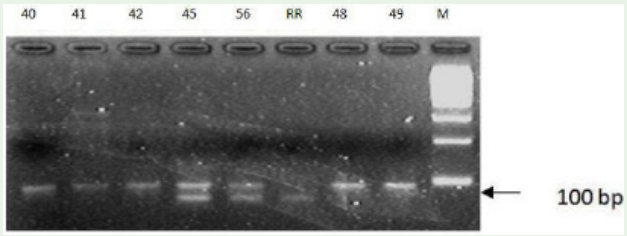

Figure 3: Agarose gel electrophoresis of PCR products amplified 1016 codon. Wild type Val/1016/Val in lanes 40, 41, 42, 48, 49 size product were 102bp. Hetezygote mutation type Val/1016/Iso in lanes 45, 56 size products were 102bp and 80bp. Homozygote mutation type Iso/1016/Iso: RR size productwere 80bp. Marker: 100bp.

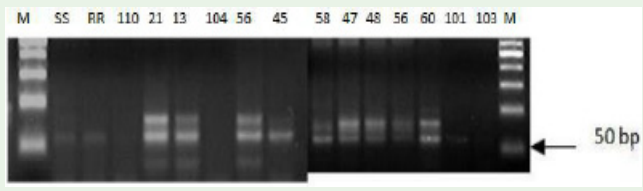

Figure 4: Agarose gel electrophoresis of PCR products amplified PCR 1016 codon. Wild type Val/1016/Val: SS, SR, 110, 45, 101, 103 size product were 60bp. Hetezygote mutation type Val/1016/Gly: 21, 13, 56, 45, 58, 47, 48, 60 size products were 80bp and 60bp. Marker: 50bp.

Compare with previously described of Saavedra-Rodriguez K. et al, 2007 and Bengue C. et al, 2003 we found: 1016 codon mutation on kdr gene of *Aedes aegypti* in Vietnam the same similar site of Aedes aegypti in Latin America of study's Saavedra-Rodriguez K. et al, 2007. We not found 1011 codon mutation that Bengue C. et al, 2003 had mutation in many areas in the world.

According susceptibility test result *Aedes aegypti* population in test places were high resistance with pyrethroid (permethrin, alpha-cypermethrin). Addition kdr mutation gene result showed that: *Aedes aegypti* population were resistance with insecticide, might be has many mutation sites on *Aedes aegypti* mosquito that 1011 and 1016 codons were one of mutation sites involved in insecticide resistance to pyrethroid.

Result

1. *Aedes aegypti* mosquito in test city were resistance against permethrin mortality rates of: 52,25% in Hanoi, 3,03% in Nhatrang city, 16% in Hochiminh city, 13,37% in Kiengiang province, 6,15% in Dongnai province and 14,58% in Daklak province. Resistance against lambda-cypermethrin mortality rates of: 57.7% in Hanoi, 10.2% in Nhatrang city, 10% in Hochiminh city, 39.3% in Kiengiang province, 24.75% in Dongnai province and 42.85% in Daklak province. Might be resistance against deltamethrin in Hanoi city: 82% mortality rate, high resistance with DDT mortality rates of:13.75% in Hanoi city, 2.0% in Nhatrang city, 2% in Hochiminh city, 7% in Kiengiang province, 6.2% in Dongnai province and 0% in Daklak province and tolerant against alpha-cypermethrin mortality rates after 24 hours: 82.25% in Hanoi, 83% in Daklak province, 10.6% in Nhatrang city, 66.6% in Hochiminh city, 47.42% in Kiengiang province, 55.1% in Dongnai province.
2. Tools have larvae: water jar, jar, vase, broken piece of a bowl had *Aedes aegypti* larvae or roof of house, verandra.
3. PCR result showed that not mutation in 1011 codon. Mutation identify in 1016 codon with two VAL/1016/ISO and VAL/1016/GLY types. Total 94 mosquitoes tested and 14 samples had mutation. Almost mutations were heterozygote.

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