BIODIVERSITAS
Volume 19, Number 1, January 2018
Pages: 31-36

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d190105

Detection of *Ace-1* gene with insecticides resistance in *Aedes aegypti* populations from DHF-endemic areas in Padang, Indonesia

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Manuscript received: 7 August 2017. Revision accepted: 17 November 2017.

Abstract. *Hasmiwati, Rusjdi SR, Nofita E. 2018. Detection of* Ace-1 *gene with insecticides resistance in* Aedes aegypti *populations from DHF-endemic areas in Padang, Indonesia. Biodiversitas 19: 31-36. Aedes aegypti* is distributed widely in West Sumatra as a primary vector of Dengue hemorrhagic fever, especially in Padang City. Synthetic insecticide control is one currently used method to prevent mosquito-borne diseases. The extensive, long-term application of Temephos along with inappropriate dosages, have resulted in the development of resistance in *Ae. aegypti* populations. Mutation of the *Ace-1* gene, encoding an acetyl cholinesterase, is one of the mechanisms that confer resistance to organophosphate (OP). The Temephos resistance status of *Ae. aegypti* in Padang city has not yet been studied. This study aimed to investigate the resistance status of *Ae. aegypti* and identify any possible mutation (s) of the *Ace-1* gene in Padang city. *Ae. aegypti* samples were collected in five population in Padang city (Jati (JT), Gunung Pangilun (GP), Lubuk Minturun (LM), Korong Gadang (KG), and Bandar Buat (BB)). The larval susceptibility to Temephos was tested by larval bioassays with Temephos pestanal at 0.02 mg/L dosages. Larval susceptibility was determined by mortality percentage values. The relationship between *Ace-1* genotypes and the resistant phenotype was analyzed by percentage of genotype frequency. Out of five populations, assessed by larval bioassays, JT and GP were resistant to Temephos; LM, KG, and BB were tolerant. A total of 50 individuals from larval bioassays were genotyped for *Ace-1* gene. Our findings showed that *Ace-1* was 495 bp in length. Mutation was not found in the G119S location but in the T506T location. Three alleles in T506T location were detected, including a wild type allele, TT (65.21%), and two mutant alleles, TA (26.08%), AA (8.69%). The use of Temephos showed that some *Ae. aegypti* populations were resistant, others were tolerant, but no population was vulnerable to Temephos. A novel mutation was detected as substitution in T506T location (ACT>ACA).

Keywords: *Aedes aegypti*, insecticide resistance, *Ace-1*gene, susceptibility test

Abbreviations: DHF: Dengue Hemorrhagic Fever, *Ace-1*: Acetyl cholinesterase-1, OP: Organophospate

INTRODUCTION

Prevention and control of Dengue Hemorrhagic Fever (DHF) depends on controlling the mosquito vector, *Aedes aegypti*. Since the Second World War, chemical insecticides have been used widely for controlling vector populations and reducing disease transmission, but their efficacy is now threatened by resistance mechanisms developed by mosquitoes. Resistance to insecticides by *Ae. aegypti* is extensive including most of the currently utilized vector control insecticides (Ranson et al. 2010). The extensive and long-term application of OP (Organophosphate) at inappropriate dosages (1x3 months OP is applied, but furthermore application do not follow the instructions), has resulted in increasing levels of resistance in *Ae. aegypti* populations. Resistance to OP specifically has been linked to mutations in the *Ace* gene, where mutations at specific sites have resulted in decreased sensitivity to insecticides (Poupardin et al. 2014).

Organophosphate is one insecticide commonly used to control *Ae. aegypti*, Temephos or abate are OP used against larvae, and Malathion for adult mosquitoes. Temephos has been utilized worldwide about for 40 years, and resistance to Temephos has been reported in many countries, including Thailand (Saelim et al. 2005), Mexico (Deming et al. 2016), Brazil (Lima et al. 2003), Peru (Rodriguez et al. 2007), Columbia (Ronald MS), El Salvador (Lazcano et al. 2009), Argentina (Llinas et al. 2010), Bolivia (Biber et al. 2010), Venezuela (Rodriguez et al. 2001), Malaysia (Dhang et al. 2008), India (Singh et al. 2014), and Indonesia (Mulyatno et al. 2012). Previous studies have demonstrated resistance to Temephos by *Ae. aegypti*, but the underlying molecular mechanisms of this resistance remain unclear. Hence, monitoring of *Ae. aegypti* resistance is very important to successfully control *Ae. aegypti* populations. The molecular characterization of *Ae. aegypti* underlying resistance mechanism to OP is crucial for tracking down resistance alleles and improving management strategies to OP resistance (Corbel et al. 2013). More than 760 candidate genes were captured and deep sequenced in several populations of the dengue mosquito *Ae. aegypti* displaying distinct genetic backgrounds and various resistance levels to the insecticide (Faucon et al. 2015).

Resistance to OP is determined to two mechanisms, the first is an increase in detoxification enzymes (such as, acetylcholinesterase, glutationtransferase, esterase), and secondly, mutation of *Ace-1* gene encoding acetyl-

cholinesterase. The important indicator of resistance to OP is insensitivity of acetylcholinesterase (*Ache*). *Ache* is widespread in the nervous system; where it functions in the hydrolysis of the neurotransmitter acetylcholine at postsynaptic membranes throughout the central nervous system and peripheral nervous system (Ayad and Georghiou 1975). *Ace-I* is inhibited by OP and carbamate resulting in acetylcholine accumulation which causes signs of intoxication, and eventually death due to respiratory failure (Grisaru et al. 1999). The mutation mechanisms of *Ace-1* gene as target gene of OP have been found in many species of insects such as *Culex tritaeniorhinchus*, *Anopheles gambiae*, *Anopheles coluzzi*, *Anopheles albimanus* (Liebman et al. 2015), *Culex pipiens* (Zhao et al. 2009), *Aedes aegypti* in India (Muthusamy and Shivakumar 2015), and *Drosophila melanogester* (Menozzi et al. 2004).

Organophosphate-resistance studies in insecticide that cause point mutations in Indonesia are very limited and data for West Sumatra are unavailable. The current study identified the status of OP-resistance and the mechanisms of resistance applied over 40 years according to WHO (2016a), and to determine the status of vulnerability and detection of mutations in the *Ace-1* gene, the target gene for OP insecticides in *Ae. aegypti*, vector for DHF in Padang City, West Sumatra, Indonesia.

MATERIALS AND METHODS

Study area

This study was performed from August to December 2016. Five of eleven subdistricts in Padang City, West Sumatra, Indonesia were selected as study locations; each subdistrict was representative of one village. The selection was based on the highest number of DHF cases. Those were Lubuk Minturun village (LM) in Koto Tangah Subdistrict, Jati village (JT) in Padang Timur Subdistrict, Gunung Pangilun village (GP) in Padang Utara Subdistrict, Korong Gadang village (KG) in Kuranji Subdistrict, and Bandar Buat village (BB) in Lubuk Kilangan Subdistrict (Figure 1).

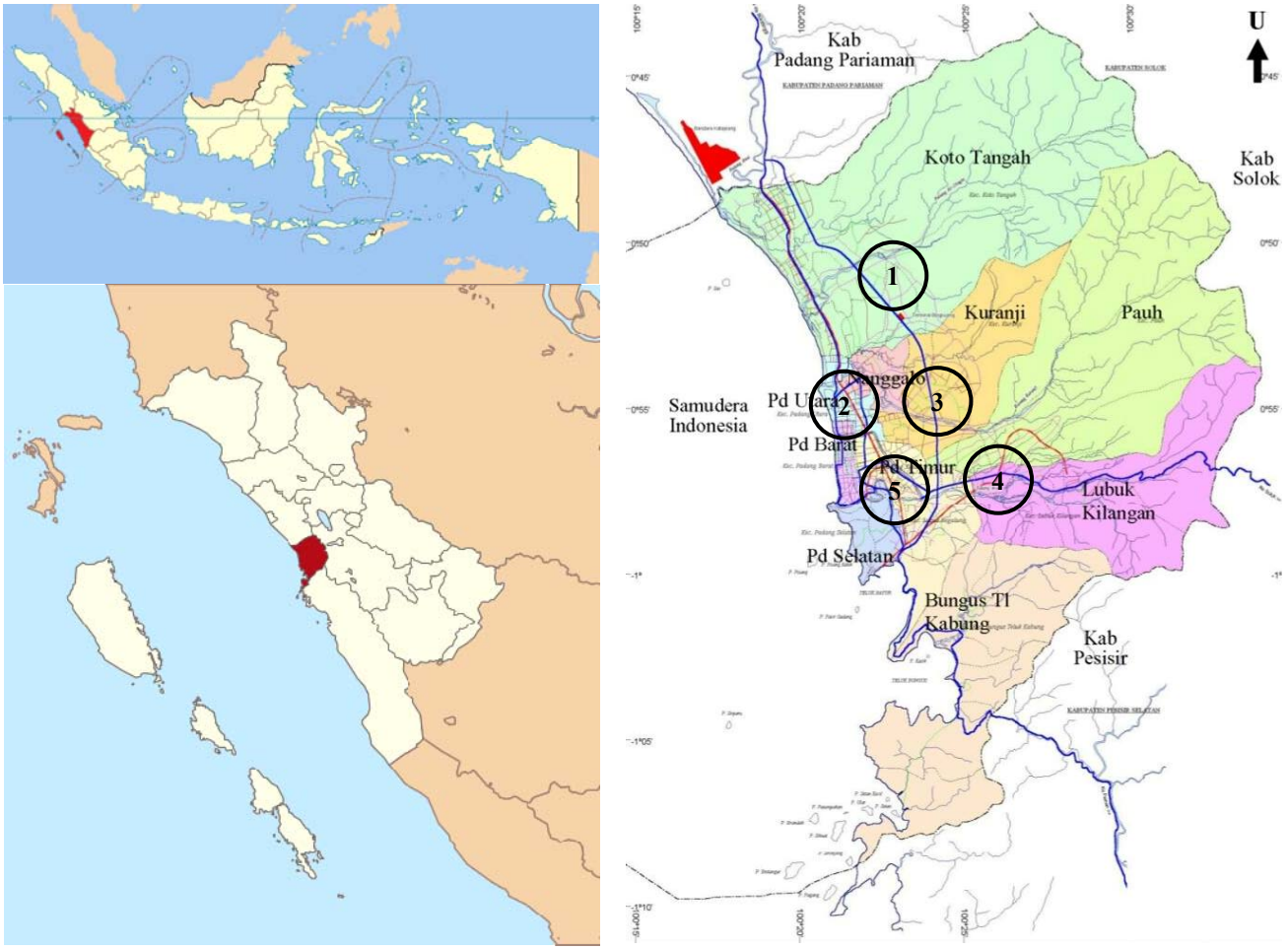

Figure 1. Location of sampling of five populations of *Ae. aegypti* in Padang, West Sumatra, Indonesia. Point 1 (Lubuk Minturun Village in Koto Tangah Subdistrict), point 2 (Gunung Pangilun Village in Padang Utara Subdistrict), point 3 (Korong Gadang Village in Kuranji Subdistrict), point 4 (Bandar Buat Village (BB) in Lubuk Kilangan Subdistrict), and point 5 (Jati Village (JT) in Padang Timur Subdistrict) (Central Bureau of Statistics Padang City 2014)

Procedures

Sample collection

Eggs of *Ae. aegypti* were collected from 100 houses in every village, and kept in the Parasitological Laboratory, Faculty of Medicine, Andalas University. Adult mosquitoes were identified according to Rueda (2004) for susceptibility analysis.

Larval bioassay

The susceptibility test was performed with bioassay method. Temephos pestanal 250 mg 97.5% was used to this study, with 0.02 mg/L dosage of diagnostic standard (WHO recommendation), applied to 20 larvae (3rd or early 4th instars) that were placed in a glass container. Twenty larvae not treated with the 0.02mg/L Temephos pestanal were used as control. Assay was repeated four times to minimize measurement error. Larval mortality was recorded 24 h after treatment. Larval mortality criteria were according to WHO (2016b): (i). 99-100% mortality = Vulnerable. (ii) 80-98% mortality = tolerant/needs verification. (iii) Mortality < 80% = resistant.

Detection of Ace-1 gene and correlation with bioassay

Dead larvae after tested with Temephos from each population were used for DNA extraction with DNA *Purelink genomic DNA Minikit* (Invitrogen, USA). To identify *Ace-1*, genomic DNA was amplified using *Ace-1* primers, forward (5'-CGATAACGAATGGGGAACG-3'), and reverse (5'-TCAGAGGCTCACCGAACACA-3'). Twenty five micro litres DNA cocktail were amplified, and PCR reaction was performed with initial step of denaturation at 94° C for 3 min, followed by 35 cycles of amplification at 94° C for 30 s, 52° C for 30 s, and 72° C for 30 s, with a final elongation at 72° C for 30 s, and cooling at 12° C for 10 min. PCR products were purified and then directly sequenced in both directions with the same primer for PCR amplification, at the position of G119S. The location G119S was examined by sequence analysis, and genotypes were determined.

Data analysis

DNA sequences were edited by using BioEdit v7.0.5 (Ibis Therapeutics, Carlsbad). Mutations and allelic variants were determined by sequence alignment analysis with Geneious Software v7.0 (Biomatters Ltd, Auckland, New Zealand). Correlation between genotype (sum of wild type/mutant heterozygote/homozygote mutant) and phenotype (sum of tolerant (wild type/heterozygote mutant/homozygote mutant/resistant (wild type/ heterozygote mutant/homozygote mutant) of *Ace-1* gene was described based as percentage of frequency from mutation in DNA sequences in five population of *Ae. aegypti* in Padang City.

RESULTS AND DISCUSSIONS

Insecticide susceptibility bioassays

Larval mortality of *Ae. aegypti* to Temephos in five populations in Padang City ranged from 50-96.25% (Table 1). Among the five tested populations, GP (50%) and JT (71.30%) were resistant to Temephos, whereas KG (93.75%), LM (95%), and BB (96.25%) were tolerant to Temephos.

Detection of *Ace-1* gene mutation

DNA was extracted from *Ae. aegypti* larvae from each test location and sequencing of the *Ace-1* gene revealed interesting results. A point mutation was not found at G119S location, where codon GGC encoding glycine alters to AGC encoding serine (glycine to serine, GGC-AGC). But a novel mutation was found at T506T location (codon ACT encoding threonine altered to ACA which also encodes threonine) (Figure 2).

Genotype variation of T506T location in *Ace-I* gene

Of the total of 50 samples, three genotypes were identified, detected as TT, TA, and AA alleles as illustrated in Table 2. TT is the wild type allele, whereas TA are heterozygote mutants, and AA are homozygote mutants. TT was predominantly found in T506T location (f=30, 65.21%), the second was TA (f=12, 26.08%), and the lowest frequency of genotype was AA (f=4, 8.69%).

Correlation between genotype and phenotype (susceptibility status) of T506T location

Analysis of genotype and phenotype correlation of T506T in five populations of *Ae. aegypti* was based on frequency of mutation in DNA sequence alignment.

Table 2. Genotype alleles of T506T location in five populations in Padang, West Sumatra, Indonesia

	Site	Number of sample	Genotypes		
			TT	TA	AA
T506T Location (ACT-ACA)	BB	9	7	2	0
	GP	8	6	1	1
	JT	9	3	4	2
	KG	10	6	3	1
	LM	10	8	2	0
Total		46	30	12	4
			65.21%	26.08%	8.69%

Note: Gunung Pangilun (GP), Jati (JT), Lubuk Minturun (LM), Korong Gadang (KG), and Bandar Buat (BB)

Table 1. Susceptibility status of Ae. *aegypti* larvae to Temephos in five populations in Padang, West Sumatra, Indonesia

Insecticide	Sites	Larval mortality average	Mean mortality rate (%)	Susceptibility status
Temephos	GP	10	50	Resistant
	JT	14.25	71.30	Resistant
	KG	18.75	93.75	Tolerant
	LM	19	95	Tolerant
	BB	19.25	96.25	Tolerant

Note: Larval susceptibility criteria: (i). 99-100% mortality = Vulnerable. (ii) 80-98% mortality = tolerant/needs verification. (iii) Mortality <80% = resistant. Gunung Pangilun (GP), Jati (JT), Lubuk Minturun (LM), Korong Gadang (KG), and Bandar Buat (BB)

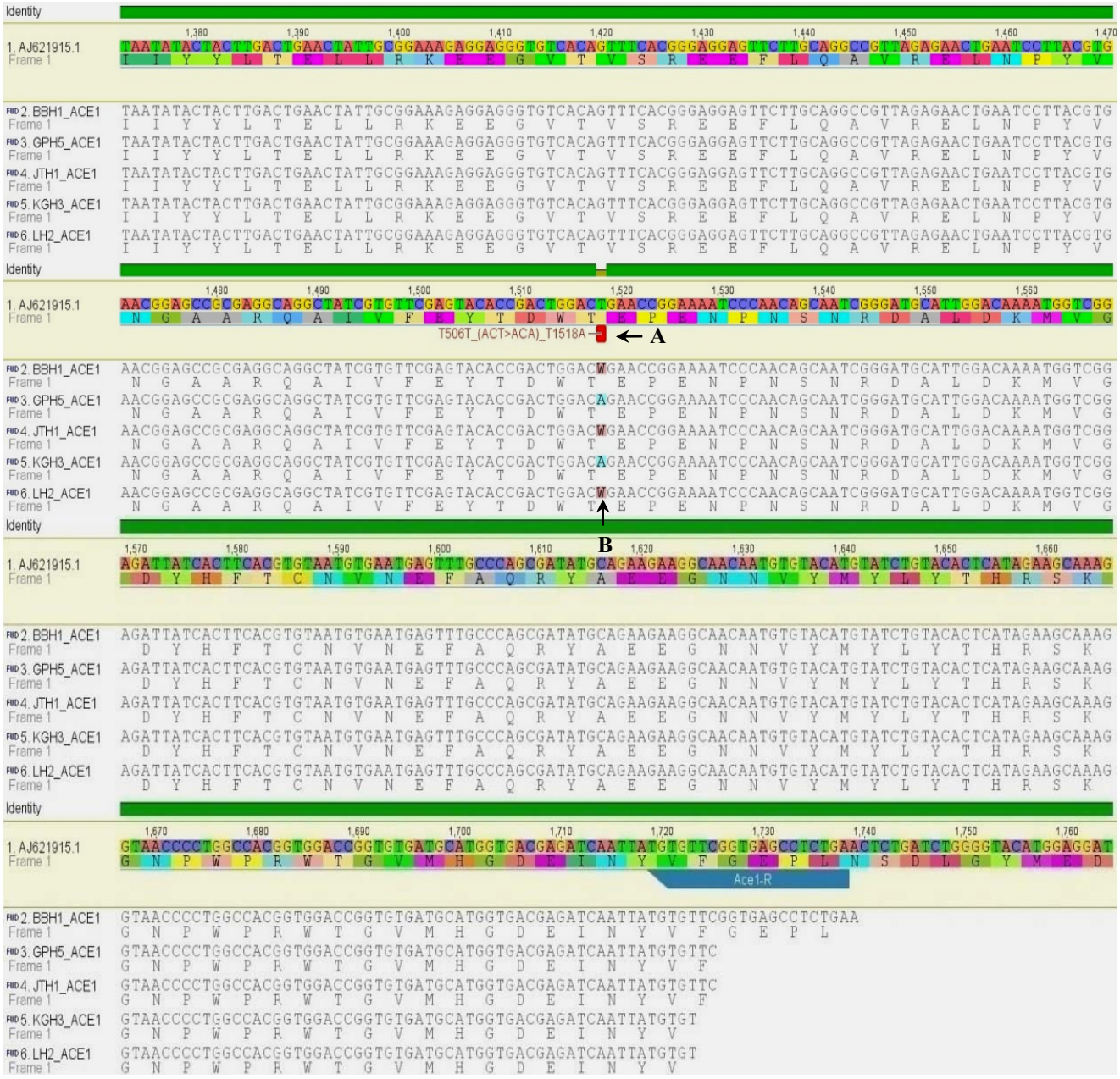

Figure 2. Alignment of *Ace-1* gene sequences (GP, JT, KG, LM and BB population). The sequence of five populations were aligned with the *Ace-1* gene (GenBank accession no. AJ621915.1) (A) Novel mutation occurred in T506T location. (B) Point mutation occurred as substitution in the third nucleotide (ACT-ACA) without altered the amino acid translation

Across the five populations a greater frequency of the TT allele was found in tolerant (34.72%) than in resistant (30.43%) *Ae. aegypti*. While the TA allele dominated in resistant (15.22%) rather than in tolerant populations (10.87%), the AA, allele frequency was the same across both phenotypes (4.35%) (Table 3).

Discussion

Temephos, a widely used organophosphorus insecticide, has been included in the list of WHO as a suitable and safe mosquito larvicide that can be used even in drinking water for controlling most mosquito vectors. The toxicity of this insecticide is low and unlikely to present acute hazard for human (WHO 2006).

Control of *Ae. aegypti* in West Sumatra, especially in Padang city by using Temephos larvicide showed varying results. Grisales (2013) reported that *Ae. aegypti* which has been resistant was also caused by genetic variation, and biological differentiation between vector strains. Previous studies reported only a few larvae were resistant to Temephos in Bangkok Metropolitan District, Thailand (Komalamisra et al. 2011), and the resistance status of *Ae. aegypti* was medium to high in Tanjung Priok and Mampang Prapatan Jakarta (Zulhasril and Lesmana 2010).

Table 3. Phenotype and genotype of T506T location in *Ae. aegypti* population of Padang, West Sumatra, Indonesia

Site	Samples	T506T genotypes					
		TT		TA		AA	
		Phenotypes					
		T	R	T	R	T	R
BB	9	4	3	1	1	0	0
GP	8	4	2	0	1	0	1
JT	9	1	2	1	3	2	0
KG	10	3	3	2	1	0	1
LM	10	4	4	1	1	0	0
Total	46	16	14	5	7	2	2
Frequency (%)		34.72	30.43	10.87	15.22	4.35	4.35

Note: Gunung Pangilun (GP), Jati (JT), Lubuk Minturun (LM), Korong Gadang (KG), and Bandar Buat (BB). Tolerance (T), Resistance (R)

Resistance to Temephos was affected by acetyl cholinesterase (Ache) as synaptic enzyme encoded by *Ace-1* gene in mosquito. Different levels of *A. aegypti* larval resistance to Temephos in all populations are a result of several factors such as existing resistance level, prior exposure to chemicals, the frequency of genes involved in resistance and different resistance mechanism with different method of inheritance (Zulhasri and Lesmana 2010), environmental temperature, host population, virus, and vector bionomics (Polson et al. 2012). Those factors emphasized to the contribution of resistance in vector population. Resistance occurs when naturally occurring genetic mutations allow a small proportion of the population to resist and survive the effects of the insecticide (IRAC 2011).

The susceptibility test based on the WHO recommendation was helpful in getting evidence that mutations have occurred in *Ace-I* which is a target of OP insecticides, because the results phenotypically indicated resistance which was supported by our sequencing revealing the occurrence of mutations in T506T location. The molecular mechanism of *Ae. aegypti* resistance to Temephos and its relationship to mutations in *Ace-I* has not been widely reported, but in *An. gambiae*, *Cx. pipiens,* and *Cx. quinquifasciatus* these mutations have been associated with OP resistance. The mutation was widespread in *Ace-1*, several studies reported mutation in G119S was responsible for organophosphate, but our finding was different from previous studies, and found novel mutation in T506T.

Results of this study suggested that mutation was silent, resulted different genotypes but the alteration in nucleotide did not change codon to translate different amino acid (ACT-ACA: threonine). Nonetheless, the change still affected the *Ae. aegypti* resistance to organophosphate, which confirmed by bioassays results; showed that mosquitoes populations are resistant to Temephos. Similar findings were reported by Soltani (2015) where *An. stephensi* were resistant to Temephos, and found no substitution occurred in G119S. Weill et al. (2004) revealed the possibility of G119S mutation in *Ae. aegypti* is less. It's due to DNA must undergo two independent mutations to result serine.

Assogba et al (2016) found that both heterogeneous (i.e., one susceptible and one resistant *Ace-1* copy) and homogeneous (i.e., identical resistant copies) duplications segregated in field populations. The number of copies in homogeneous duplications was variable and positively correlated with acetylcholinesterase activity and resistance level. Organophosphate resistance is also caused by increasing of *esterase* synthesis, affecting the effectiveness work of insecticide, so reduces the number of active insecticide to achieve the targets.

Studies regarding the maintenance of polymorphisms/ variation of genotypes in the population have been reported for *Cx. pipiens* (Labbe et al. 2007) and *An. gambiae* (Djongbenou et al. 2009). Edi et al. (2014) suggested that *Ace-I* caused of gene duplication in multiple-resistant of *Anopheles gambiae*. Fournier (2005) reported mutation of *acetyl cholinesterase* with the use of insecticides on insect populations.

The current study suggested that regular monitoring is needed to evaluate and understand the dynamics of *Ae. aegypti* at the population level, which is resistant to OP which have applied for over 40 years in Padang city and other DHF endemic areas. Despite the identified mutation being a silent one (T506T) the substitution resulted in different susceptibilities to Temephos where the heterozygote mutant was found at higher frequencies in resistant *Ae. aegypti* populations. Continual monitoring of Temephos resistance will yield useful information to manage insecticide application in control strategy of *Ae. aegypti* as dengue vector and other viral diseases such as Zika, yellow fever, and chikungunya. In conclusion, it is evident that unfortunately some populations of *Ae. aegypti* in Padang City, West Sumatra have developed resistance to Temephos. Novel methods geared towards *Ae. aegypti* control should adjust the usage of insecticides based on the findings, such as different insecticides, or a combination of multiple insecticides. Two novel *Ace-I* mutant heterozygote alleles in T506T location (AA and TA) were detected in Temephos resistant populations of *Ae. aegypti* in Padang City, West Sumatra. Detection and identification are important ways as a basis for developing molecular diagnostics to uncover the resistance mechanism of *Ae. aegypti* have developed to Temephos and other currently used insecticides worldwide.

ACKNOWLEDGEMENTS

The authors would like to thank and appreciation to the Dean of the Faculty of Medicine, University of Andalas, Padang, Indonesia on financing through PNBP funding (Non Tax Revenue) Faculty of Medicine, Andalas University in 2016 with a contract number: 110/BBPT/PNP-FK Unand 2016 dated August 30, 2016. And thanks are also extended to all staff and analyst of the Laboratory of Biomedical, Faculty of Medicine, Andalas University.

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