Molecular Detection of Temephos Insecticide Resistance Mutation in *Ace-1 Gene* of *Aedes aegypti* Populations from Monywa Township, Myanmar

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Aedes aegypti is the main vector of dengue, chikungunya, yellow fever and Zika in human population. Insecticide resistance of mosquito vectors is at a critical tipping point in public health. Temephos which is one of the organophosphate insecticides has been utilized in dengue vector control for more than 40 years all over the world. *Ace-1* gene is linked to temephos resistance and mutations in *Ace-1* gene have been reported in some countries. However, no record for *Ace-1* gene detection of *Ae. aegypti* was found in Myanmar. The present study was conducted to detect the *Ace-1 gene* with temephos insecticide resistance mutations in *Ae. aegypti* populations of dengue endemic areas from Monywa Township. Experimental laboratory based study design was carried out from June, 2019 to May, 2020. *Ae. aegypti* larvae were collected from three study areas; one urban (Myawaddy quarter) and two rural areas (Kamma village and Kyauksitpon village) in Monywa Township. Larval insecticide bioassay test was performed by using the WHO method with temephos 0.02 mg/l (diagnostic dosage). Resistant larvae after tested with temephos were identified *Ace-1* gene by PCR. Total of 45 *Ace-1* gene samples (15 samples in each study area) were sequenced both forward and reverse primers by 3500 series genetic analyzer. *Ae. aegypti* larval mortalities of Kyauksitpon village, Kamma village and Myawaddy quarter were 96.95%, 94.00% and 92.19% after 24 hours exposure with temephos. Mutation was detected in T506T location with 0.13 frequency (13.33%) in *Ae. aegypti* population of Myawaddy quarter. However, mutation in G119S location of *Ace-1* gene was not found in all study populations. This study indicated that the presence of temephos insecticide resistance mutation was appeared in *Ae. aegypti* population. Thus, regular monitoring is essentially needed to evaluate the temephos resistance of *Ae. aegypti* population in Monywa Township and also in other dengue endemic areas in Myanmar where OP insecticide has been widely used.

Keywords: Aedes aegypti, Insecticide resistance, Temephos, *Ace-1* gene, Mutation

INTRODUCTION

Aedes aegypti is the main vector which concerns to the public health as it can transmit various serotypes of viral pathogens causing dengue, chikungunya, yellow fever and Zika. It has become the major indirect cause of morbidity and mortality of human worldwide.[1]

According to the World Health Organization, an estimated 50-100 million dengue infections occur annually, with a 30-fold increase in global incidence observed over the past

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DOI: https://doi.org//10.34299/mhsrj.009116

50 years.[2] In Myanmar, total of 23,273 dengue cases with 112 deaths were reported in 2018. Sagaing Region including Monywa Township was reported 2,513 dengue cases with 16 deaths.[3] During the last five years, Monywa Township was recorded as the highest number of dengue cases within Sagaing Region according to the Public Health Department (Unpublished data).

Immature stages of *Aedes* mosquito are the primary target of the dengue vector control activities. Temephos 1% sand granules (known as Abate) is the organophosphate (OP) insecticide which targets the nervous system of the larva and applied as the main mosquito larvicide world widely for more than 40 years.[4] In addition, temephos has been extensively used for dengue control in Myanmar.[5] Meanwhile, resistance to temephos has been reported in many countries including Brazil, Cuba, Argentina, Venezuela, Thailand, Malaysia, India, and Indonesia where temephos has been used as larvicide for many years.[6-13] However, the two studies in Myanmar were reported no resistance to temephos in *Ae. aegypti* populations from Mandalay and Yangon in 2013 and 2014.[14, 15]

Resistance to organophosphate insecticide (OP) is determined by two mechanisms, the first is an increase in detoxification enzymes (such as, acetylcholinesterase, glutation-transferase, esterase), and secondly, mutation of *Ace-1* gene encoding AChE. Acetylcho-linesterase (AChE) is an enzyme which is widespread in the nervous system; where its action serves to stop the excitation of a nerve after transmission of an impulse.[16] AChE is a common resistance mechanism to OP and carbamate insecticide in insects. The important indicator of resistance to OP is insensitivity of AChE.[17]

The mutation mechanisms of *Ace-1* gene as target gene of OP have been found in many species of insects such as *Anopheles gambiae*, *Anopheles coluzzi*, *Anopheles albimanus, Culex pipiens, Ae. aegypti,* and *Drosophila melanogaster*.[18-22] Most insects, including mosquitoes, carry two genes encoding AChE; *Ace-1*and *Ace-2*, but *Ace-1* is linked to insecticide resistance and the function of *Ace-2* is still unknown.[17]

Insecticide resistance of mosquito vectors is at a critical tipping point in public health. Nowadays, resistances of some mosquito populations are showing to all insecticide classes, and hence the impact of this resistance is escalating every year.[23] In *Ae. aegypti* population, G119S and T506T mutations of *Ace-1* gene have been reported in India and Indonesia.[16, 21] Nevertheless, as the first time in Myanmar, the current study identified the organophosphate insecticide resistance *Ace-1* gene in *Ae. aegypti* population of Monywa Township.

MATERIALS AND METHODS

The present study was an experimental laboratory-based study and conducted from June, 2019 to May, 2020. The field activity was conducted in Monywa Township and the laboratory activities were conducted in Medical Entomology Research Division, Department of Medical Research (Pyin Oo Lwin Branch) and Bioinformatic Division, Advanced Molecular Research Center in Department of Medical Research (Head Quarter, Yangon). Monywa is located in the central dry zone of Myanmar (22.1216°N, 95.1536° E) with the population of 372,095 inhabited in 26 quarters and 57 villages.[24] According to the recommendation of Vector Born Disease Control (VBDC) unit of Sagaing Region and based on the number of reported dengue cases in 2018, three areas (Myawaddy quarter, Kamma village and Kyauksitpon village) in Monywa Township were selected in the present study.

Sample collection

Dengue vector *Aedes* larvae were collected from indoor and outdoor water containers of 50 houses in each selected area and stored in plastic boxes. These field collected larvae were transferred to insectarium of Medical Entomology Research Division, Department of Medical Research (Pyin Oo Lwin Branch) for rearing to the adult stage.

Mosquito colonization

Collected *Aedes* larvae from three study areas were kept in each plastic tray (35×25 cm) containing two liters tap water. Larvae were fed fish food pellets (Tetra, Germany). Pupae were picked from the larval trays and transferred to a cup containing tap water that were maintained in mosquito cages (45×45×40cm) to allow emergence of adult mosquitoes. Adult mosquitoes of *Ae. aegypti* were morphologically identified by using the pictorial keys[25] such as scutum, vertex and hind leg, and move to separate cages. Adults were provided with 10% sucrose solution soaked in cotton wool. Female mosquitoes were regularly provided with blood meal from mice, after maturity to lay eggs. And then, plastic cups (7.5 cm diameter) that contained 50 ml tap water, lined with filter paper were placed inside the cages for oviposition. When filter paper found eggs, it was transferred to a plastic tray filled with the tab water for larval hatching to produce F1 generation. Rearing conditions for all stages of mosquitoes were 27±2°C and 75-85% relative humidity.

Insecticide

For larval bioassay testing, diagnostic dosage, 0.02 mg/l of temephos WHO (1981) was prepared from technical grade of temephos with 96.9% (PESTANAL™, Sigma-Aldrich).[26]

Larval susceptibility testing

Larval bioassay was performed by using the WHO (2016) method. *Ae. aegypti* larvae of F1 generation except small or damage larvae were used in susceptibility test. Twenty-five late third or early fourth instar larvae were introduced into a 500 ml paper cup containing 250 ml of tap water and the concentration 0.02 mg/l of temephos. Each bioassay included the control group which received 1 ml of ethanol because temephos was diluted by ethanol. Three replicates were kept for the concentration along with the control. All the testing cups were held in a laboratory at room temperature (27°C±2°C). Larval mortality was recorded every 10 minutes until 120 minutes (2 hours) and after 24 hours of exposure.[27]

DNA extraction from mosquito specimen

As a next step, AccuPrep Genomic DNA Extraction Kit (BIONEER) was used to extract DNA from each alive larva collected from the larval susceptibility test. The procedure was carried out as follow: the individual sample was homogenized with 200 µl of Tissue Lysis Buffer and 20 µl of Proteinase K and 10 µl of RNasa A were added. The sample was mixed by vortex mixer and was incubated at 60°C for one hour. After that 200 µl of Binding Buffer were added and the sample was again incubated at 60°C for 10 minutes. And then 400 µl of absolute ethanol were added to the sample and mixing performed by pipetting. The lysate was transferred to a filter column in a micro centrifuge tube. After centrifugation at 8,000 rpm for 1 minute, the filter was washed with Wash buffer 1 and then Wash buffer 2. Finally, the genomic DNA was eluted by use of 200 µl elution buffer.[28]

DNA amplification and sequencing

Genomic DNA was amplified using *Ace-1* primers, forward (5'CGATAACGAATGG GGAACG-3'), and reverse (5'-TCAGAGGC TCACCGAACACA-3') to identify *Ace-1*. Those primers amplify a region of 500 bp of the gene (GenBank accession Number: EF209048).[21] In amplification, AccuPower® PCR premix (BIONEER) containing DNA polymerase, dNTPs and reaction buffer was used. For PCR reaction, total volume of 30 µl which were 2 µl of template DNA, 2 µl of forward and reverse primers, and 26 µl of distilled water (DW) were added in each premix tube. The PCR conditions were single cycle of 3 min denaturation at 95°C followed by 40 cycles of the amplification consisted of 30 sec at 94°C, 30 sec at 56°C, and 30 sec at 72°C and final extension step of 30 sec at 72°C. The DNA fragments were visualized in gel documentation system under UV trans-illumination.[16] To detect the mutations at G119S and T506T locations, total of 45 *Ace-1* gene samples (15 samples in each study area) were purified using a PureLink® PCR Purification Kit from Invitrogen (Carlsbad, CA 92008, United States) and sequenced both

directions with respective forward and reverse primers by 3500 series genetic analyzer.

Sequencing analysis and statistic

Entomological quantitative data were managed in Microsoft Excel and mortality rate for larval susceptibility was calculated by SPSS software (Version 25). Nucleotide sequences of *Ace-1* gene were analyzed using MegAlign software (DNAStar Lasergene, version 7.1).

RESULTS

Larval susceptibility

For larval insecticide susceptibility test, F1 generation of 1050 *Ae. aegypti* larvae from each study place were tested applying WHO larval bioassay test procedure. The same number of larvae were also used for control group. Mortality percentages of *Ae. aegypti* larvae against temephos (diagnostic dosage 0.02 mg/l) from 3 study areas (Kyauksitpon village, Kamma village and Myawaddy quarter) were 96.95%, 94.00% and 92.19% respectively. No mortality was detected in control group of all strains (Table 1).

Table 1. Temephos insecticide susceptibility status of *Aedes aegypti* larvae (after 24 hours exposure to diagnostic dosage 0.02 mg/l) from three study areas of Monywa Township

Study area	Test			Control		
	Total no. of larvae					
	Tested	Dead (%)	Alive (%)	Tested	Dead (%)	Alive (%)
Kyauksitpon village	1050	1018 (96.95)	32 (3.14)	525	0 (0)	0 (0)
Kamma . village	1050	987 (94.00)	63 (6.38)	525	0 (0)	0 (0)
Myawaddy quarter	1050	968 (92.19)	82 (8.47)	525	0 (0)	0 (0)

Ace-1 gene mutation

The total of 45 *Ace-1* gene sample from three different study areas were sequenced to find the mutation of gene after DNA amplification. The sample sequences were detected on 500 bp fragment of the *Ace-1* gene (nucleotide position from 1258 to 1739) after measuring the quality of sequences. All samples were aligned with the reference sequence of partial *Ace-1* gene of Rockefeller strain (Sequence ID: emb|AJ621915.1|). Mutation in G119S location (glycine to serine, GGC-AGC) of *Ace-1* gene was not found when compared to the reference sequence. However, mutation was detected in T506T position, where codon ACT encoding threonine altered to ACA which also encodes threonine. The T506T mutation was identified two homozygous individuals in an only Myawaddy quarter, with 0.13 frequency (13.33%) (Fig. 1).

DISCUSSION

Temephos is a non-systemic organophosphorus insecticide, mainly used as a larvicide to control mosquitoes, including in domestic water containers and it could be used for storing drinking-water. The toxicity of this insecticide was low and unlikely to present acute hazard for human.[29] An important indicator of insecticide resistance to organophosphate is the loss of acetyl cholinesterase (AChE) sensitivity. AChE is a widely distributed enzyme within the nervous system, which mediates hydrolysis of the neurotransmitter acetylcholine throughout the central and peripheral nervous systems at the postsynaptic membrane through terminating nerve impulses.[30]

In this study, the results from the bioassay of *Ae. aegypti* larval susceptibility showed that Kyauksitpon village was 96.95%, and followed by Kamma village was 94.00% and Myawaddy quarter was 92.19% after 24 hours exposure with temephos. One study described that the mortality rates of *Ae. aegypti* larvae from three study areas in Mandalay District were 100% to temephos (0.02 mg/l), after 24 hours exposure.[14] Therefore, the mortality rates of all populations in this study were lower than previous study.

A study from Indonesia reported that the larval mortalities of *Ae. aegypti* from five populations in Padang City ranged from

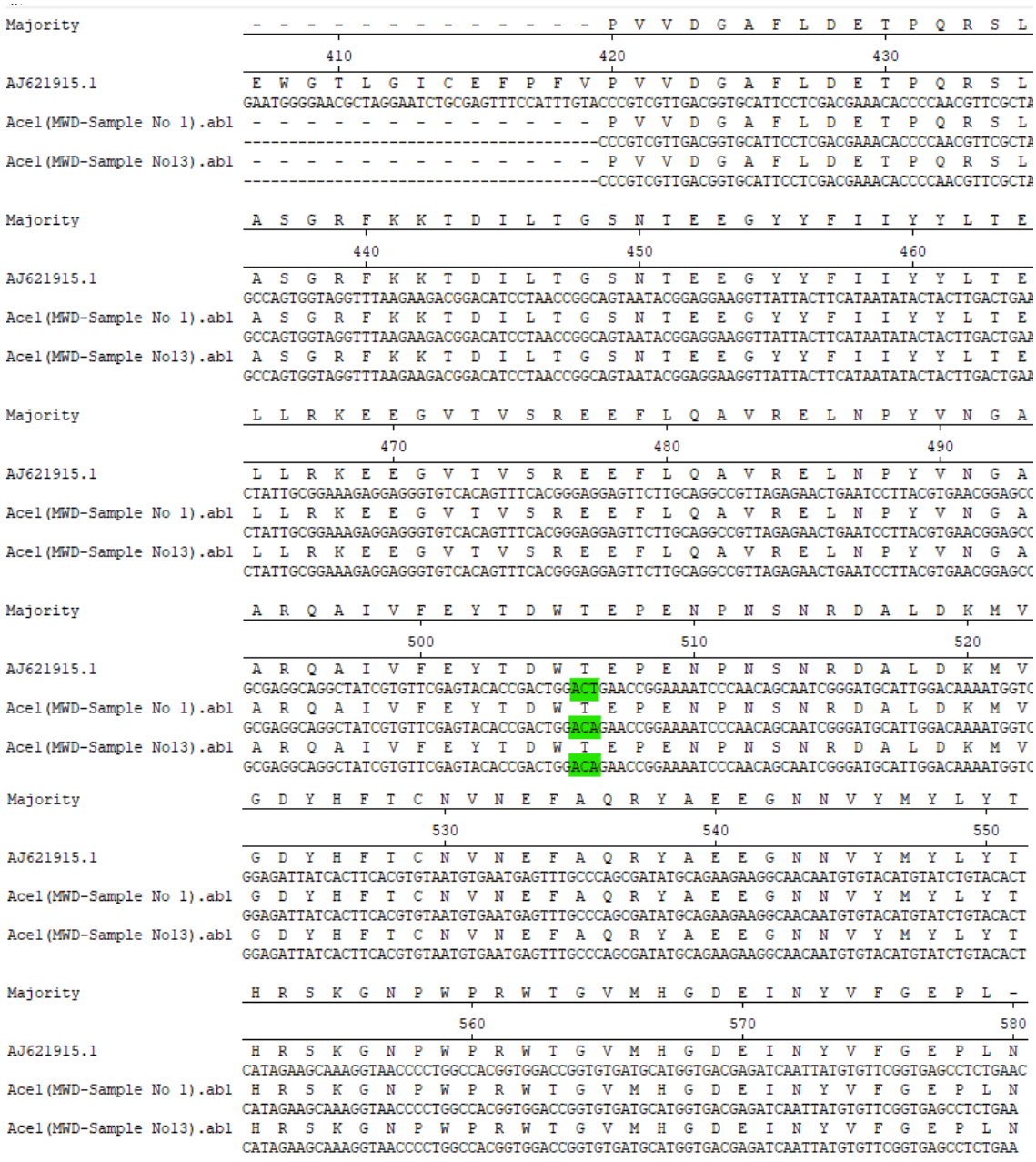

Fig. 1. Alignment of *Ace-1* gene of *Aedes aegypti* larvae (Myawaddy quarter) compared with Rockefeller strain sequence and occurring of the mutation at T506T position

50 to 96.25% that indicated resistance and possible resistance to temephos (0.02 mg/l).[16] Only one strain of *Ae. aegypti* larvae from Cambodia was susceptible to 0.02 mg/l temephos with 99.5% mortality, and one strain of *Ae. aegypti* showed resistance to temephos with 93.33% mortality.[31] Temephos resistance levels in all *Ae. aegypti* larval populations are varied depending on the 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, environmental temperature, host population, virus, and vector bionomics.[16] The results of the current study described the occurrence of mutations in T506T location from Myawaddy quarter *Ae. aegypti* population. Although nucleotide was altered, codon did

not change to different amino acid. Therefore, the type of mutation was silent (ACT-ACA: threonine). However, G119S mutation in *Ace-1* gene was not found in the present study. The larval susceptible results phenotypically showed that Myawaddy quarter population was the lowest percentage of larval mortality rate among three populations in the present study. The results of mutation in *Ace-1* gene were relevant with the finding of larval bioassay. Myawaddy quarter, which is a part of Monywa City, is an urban area and other two villages, Kamma and Kyauksitpon villages, are rural areas. The results indicated that the mosquito population of Myawaddy quarter was highly treated with OP insecticide for vector control intervention than other two study areas (Unpublished data).

Similar findings were reported by Hasmiwati, *et al* from Indonesia where *Ae. aegypti* was resistant to temephos and a novel mutation was found at T506T location in *Ace-1* gene, but substitution in G119S was not occurred. It also described that, the possibility of G119S mutation was less likely to occur in *Ae. aegypti* population than in other mosquito species.[16] Another study of Iran showed lack of the G119S mutation in ACE-1 gene for organophosphate insecticide resistance in the temephos-selected strain of *An. stephensi*.[32] Globally, studies of *Ace -1* gene mutations that related to the resistance of *Ae. aegypti* against organophosphate and carbamate insecticides are relatively limited.[33] The molecular mechanisms were not well-characterized despite increasing reports of temephos resistance in *Ae. aegypti*.[34] India study reported a mutation in the G119S codon in the *Ae. aegypti* mosquito from Namakkal area, in 2015.[21] Although organophosphate and carbamate has been used for more than two decades, the mutation of G119S codon has never been reported in Southeast Asia.[33]

In conclusion, *Ace-1* gene mutations of *Ae. aegypti* larval population in Myawaddy quarter were recorded at T506T location as silent mutation. Therefore, regular monitoring is needed to evaluate the temephos resistance of *Ae. aegypti* population in Monywa Township and also in other dengue endemic areas in Myanmar where OP insecticide has been widely used. To success the implementation of insecticide resistance management strategies, the early detection of insecticide resistance gene is essential. The findings of the present study provided to Vector Borne Disease Control Program (VBDC) as the information of temephos resistant mechanisms.

ACKNOWLEDGEMENT

We would like to express our sincere gratitude to the Department of Medical Research, Ministry of Health in Myanmar for providing funding support to conduct this research. We are also grateful to the staff of Medical Entomology Research Division (Pyin Oo Lwin Branch), and Bioinformatic Division, Advanced Molecular Research Center in Department of Medical Research (Head Quarter, Yangon), and Vector Borne Disease Control Unit (Sagaing Region) for their collaboration in the study.

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