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Susceptibility of *Aedes albopictus* from dengue outbreak areas to temephos and *Bacillus thuringiensis* subsp. *israelensis*

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ABSTRACT

Objective: To monitor the current duration of the application rates in vector programme and the level of *Aedes albopictus* larvae susceptibility from three selected areas in northeast district of Penang on two selected larvicides, temephos and *Bacillus thuringiensis* subsp. *israelensis* (Bti) which are commonly used by Penang Health Department for vector control.

Methods: The mosquito larvae were tested against two types of larvicides: (1) temephos (Abate®) with diagnostic dosage (0.012 mg/L) and operational dosage (1 mg/L) and (2) *Bacillus thuringiensis* subsp. *israelensis* (VectoBac® WG) with operational dosage ranging from 6000 international toxic units per L to 24000 international toxic unit per L. A total of 20 late third and early forth instar larvae were selected and transferred into paper cup sized 300 mL using wide-mouthed pipette. The larvae were distributed into each 300 mL paper cup containing 50 mL of aged tap water. The experiment was replicated five times for each concentration tested. Each test was repeated three times. The mortality was recorded after 24 h of exposure and recorded lethal time was based on 2 h for temephos and 6 h for Bti. The control consisted of ethanol for temephos and only seasoned water for Bti.

Results: The result showed that *Aedes albopictus* from Flat Hamna, Kampung Sungai Gelugor and Kampung Tanjung Tokong were still susceptible to Bti and temephos. However, higher lethal time and resistance ratio were detected in strain from Flat Hamna which was a known dengue hot spot area in northeast of Penang.

Conclusions: The application of temephos and Bti in vector control activity in these selected localities is still relevant in the control of *Aedes* larvae populations.

1. Introduction

Insecticide is a toxic product and used to kill pest insects or eliminate diseases carrying pests. Natural insecticide can also be derived from natural plants [1]. An insecticide has to be applied in the living place or habitat of the target insect to ensure that the insecticide is touched or digested by the pest insects. Most of the insecticides are nerve poison and kill the insect by attacking the specific part in the metabolism mechanism inside the insect's body [2]. However, the excessive use of the insecticide brings several problems to the environment. The most problematic case is the environmental contamination through basic food chain, which endangers the insect, wildlife, and human being. Many insects or animals are at risk as they rely upon the main source of food contaminated with insecticides.

Globally, temephos is the most widely used as it is easy to handle, cheap in price, has good residual effect, has low toxicity to mammalian and safe to apply to drinking water [3–5].

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Temephos is one of a few organophosphates registered and produced commercially to control *Aedes* mosquito larvae as a larvicide used [6]. Larvicidal activity is very important in vector control management because the effectiveness in polluted water, has a long residual activity and can be used on any stage of larva. Since 1970, it has been used against mosquito larvae in stagnant water and in the vector management of dengue fever, malaria and filariasis especially in Thailand [7] and Malaysia since 1973 [8]. The larval control still primarily utilised the temephos, despite the known existence of temephos resistant populations in many parts of the world [9].

Bacillus thuringiensis subsp. *israelensis* (Bti) is a biological larvicide and widely used to control mosquitoes and black flies [10,11]. Bti showed a fast killing effect with a good toxicology profile with the absence of cross-resistance with conventionally used pesticides [12]. Product with Bti causes no harm and is safe to the bird, man, fish and mammals [13,14]. The mode of action of endotoxin to kill larvae is still unclear but the sequence of toxin activation is well studied [15]. Once the bacteria are ingested, the crystal will be dissolved in the naturally alkaline pH in the larval midgut and the endotoxin attached were activated. The larvae usually die within 2 or 3 days [16].

One of the major problems in vector control is development of resistance to existing insecticides in the vectors. Recently, resistance to temephos has been previously reported in Malaysia and other country [17–19]. The widespread use of insecticide has led to insecticide resistance in mosquitoes and become another problem for the ability to control disease. Thus, Bti is an alternative candidate to manage the resistance to temephos [4]. It contains four different larvicidal proteins, each acting in different ways to make it difficult to develop resistance [20]. Furthermore, there is no consistent resistance which has been detected after a long-term treatment with Bti [21], but only a moderate Bti resistance was reported locally [22].

Laboratory bioassay can only detect resistance when it presents in high frequencies in vector population. The early detection of the resistance can improve the vector efficacy by improving the implementation of alternative control strategies. Diagnostic dosage was used to determine insecticide resistance against *Anopheles*, *Culex quinquefasciatus* and *Aedes aegypti* (*Ae. aegypti*) [23]. It is a standard test for detecting and measuring resistance, which should be available to the researcher committees to make a comparison between other countries in order to verify the standard dosage for all insecticides. The baseline of diagnostic dose is possible to be changed due to the current resistance status data. The mortality assay based on time is one of the convenient procedures which is easy, time-saving and feasible to determine insect susceptibility in the field population [24].

Therefore, the purpose of this study is to monitor the current duration of the application rates in vector programme and the level of *Aedes albopictus* (*Ae. albopictus*) larvae susceptibility from three selected areas in northeast district of Penang on two selected larvicides, temephos and Bti which are commonly used by Penang Health Department for vector control.

2. Materials and methods

2.1. Tested mosquitoes

The wild strain of *Ae. albopictus* mosquitoes from the three selected areas with the highest reported dengue fever cases in northeast district of Penang were collected from the majority (> 90%) of natural breeding sites such as tyres, discarded tins, cans and flower pots. Collections were carried out from three selected areas on Penang Island: Flat Hamna (FH) Sungai Dua, Kampung Sungai Gelugor (KSG) and Kampung Tanjung Tokong (KTT) based on the methodologies described previously [25]. Collected samples of *Aedes* larvae were brought back to the laboratory, identified and were used in the bioassay test. *Ae. albopictus* VCRU strain (susceptible strain), which served as control reference baseline was obtained from insectarium of Vector Control Research Unit, Universiti Sains Malaysia (5°21′ N, 100°18′ E). This susceptible strain was colonised since 1980s for more than 800 generations.

2.2. Temephos bioassay

Larvicide testing was prepared according to the World Health Organization (WHO) procedure with a modification on the applied dose of commercial Abate® 1.1G [1.1% w/w, registered by BASF (Malaysia) Sdn. Bhd] [8]. The mosquito larvae were tested against two different dosages of temephos: (1) recommended dose at 0.012 mg/L and (2) operational dose that has been used by Department Health of Penang at 1 mg/L and prepared with ethanol as solvent. A total of 20 late third and early fourth instar larvae were selected and transferred into paper cup sized 300 mL using wide-mouthed pipette. Larvae were left for 1 h prior to the experiment to permit acclimatization and no additional food was offered. After that period, any abnormal larvae were replaced with healthy ones. The larvae were distributed into each 300 mL paper cup containing 50 mL of aged tap water. The experiment was replicated five times for each concentration tested. Each test was repeated three times.

The test solution was prepared by adding appropriate amount of temephos in solvent and stirred for 30 s with a glass rod. Fifteen minutes after solution has been prepared (to allow the agent to mix well in the solvent), the mosquito larvae were introduced into each cup and water was further added to make up the final volume of 200 mL. The control (untreated) consisted of 1 mL of ethanol for temephos. Larval bioassay tests were run under laboratory condition at temperature of (26 ± 2) °C and (60 ± 20)% relative humidity. Cumulative larval mortality was recorded for 2 h with interval of 5 min for operational dosage of temephos (1 mg/L). Larval mortality after 24 h was also recorded after the exposure to temephos. The mortality of *Ae. albopictus* was recorded after 24 h of exposure and was presented as percentage, whereas, lethal time (LT) was based on 2 h. Lethal data were log-transformed prior to statistical analysis to fulfil the assumption of probit analysis [26]. The resistance ratio (RR) was calculated by dividing the LT of the field strain by the LT of the susceptible strain.

$$\text{RR of } LT_{50} = \frac{LT_{50} \text{ of field strain}}{LT_{50} \text{ of laboratory strain}}$$

2.3. Bti bioassays

VectoBac® WG was a commercial biolarvicidal formulation of Bti with a potency of formulation 3000 international toxic unit (ITU)/mg against *Ae. aegypti*. The larval bioassay tests were conducted with slight modifications as previously described [27–

30]. The recommended dose by the manufacturer was 2–8 g/1000 L (equivalent to 6000 ITU/L–24000 ITU/L). The operational dose used for Bti application for control program in Penang was based on the recommended dose by the manufacturer as mentioned on the label. Three concentrations were used in this study, which were 6000, 15000 and 24000 ITU/L with water as the solvent. The test of Bti was assessed for 10 min–6 h after Bti applications and 24 h after exposure to all of the concentrations tested. For each bioassay, 20 larvae per cup were exposed to different concentrations of Bti. The appropriate dilution from stock solution was added to the water in the cups to obtain the desired target doses. Five cups per concentration (100 larvae) were performed for each concentration tested. Each bioassay was repeated three times and a control group was tested using water. The mean LT and the RR were obtained for each sample, as described above.

3. Results

3.1. Toxicity of insecticide in 24 h

The study was performed based on three strains of *Ae. albopictus* colonies collected from different localities and VCRU susceptible strain was used as a reference strain. The susceptibility tests of temephos were based on the number of *Ae. albopictus* mortality for FH, KSG and KTT strain. All of the strains showed 100% mortality on 0.012 mg/L (WHO diagnostic dose) and 1 mg/L (operational dose for Health Department of Penang) dosages (Table 1). Recommended dose for temephos proposed by WHO (0.012 mg/L) and Health Department of Penang (1 mg/L) which was 83.3% higher than WHO dose, gave the same result of 100% mortality after 24 h post-treatment. Test on Bti also showed 100% mortality against 6000, 15000 and 24000 ITU/L after 24 h post-exposure for all strains tested (Table 2). This indicated that the Bti dosage recommended by manufacturer was still effective against *Ae. albopictus* larvae for all strains.

3.2. Temephos

The result showed the VCRU susceptible strain had the lowest LT values compared to other field strains (FH, KSG and KTT). The LT_{50} values against *Ae. albopictus* from VCRU, FH, KSG and KTT ranged from 36.44 min to 68.31 min. Wild strains required longer time to be killed compared to the laboratory strain (36.44 min). The LT_{50} of the operational dose against *Ae. albopictus* from VCRU, FH, KSG and KTT were 36.44, 68.31, 64.86 and 50.61 min respectively. All of the LT_{50} values were higher for the field strains (Table 1).

The results obtained from bioassay test revealed that FH strains had significantly longer LT when tested with operational dosage (1 mg/L) compared to VCRU and KTT strain (non-overlapping of 50% and 95% CLs, $P < 0.01$; Table 1). However, the values of LT_{50} for FH strain showed no statistical difference compared to KSG strain (overlapping of 50% and 95% CLs, $P < 0.01$; Table 1). Our result indicated that low mortality was recorded within the first 2 h after treatment with 0.012 mg/L temephos dose in all of field strains due to the lower concentration compared to operational dose for Health Department of Penang (1 mg/L).

Table 2
Larval mortality of *Ae. albopictus* strains to different doses of VectoBac® WG (Bti) after 24 h of continuous exposure.

Strains	24 h Post-exposure mortality (%)		
	6000 ITU/L	15000 ITU/L	24000 ITU/L
VCRU laboratory strain	100	100	100
FH	100	100	100
KSG	100	100	100
KTT	100	100	100

3.3. VectoBac® WG susceptibility

The results obtained from Bti bioassay revealed that FH strains had significantly longer LT in all three dosages tested compared to other strains (non-overlapping of 50% and 95% CLs, $P < 0.01$; Table 3), which indicated that FH strain was less tolerant to Bti. The result showed the VCRU susceptible strain had the lowest LT values compared to other three field strains (FH, KSG and KTT). The LT_{50} of the 6000 ITU/L against *Ae. albopictus* for VCRU, FH, KSG and KTT were 52.70, 107.06, 88.56 and 59.81 min respectively followed by 15000 ITU/L (26.09, 63.92, 46.10, 32.94 min) and 24000 ITU/L (20.65, 40.90, 34.87, 27.96 min). All the LT_{50} values were higher for the field strains and the LT reduced with the increasing dose of Bti. However, the LT values between each strain showed significant differences between tested doses ($F = 1692.59$, $df = 2$, $P = 0.00$). Generally, the susceptibility decreased in order of VCRU strain > KTT > KSG > FH.

3.4. Resistance of mosquito to temephos and Bti

All mosquitoes found to be still susceptible against temephos at operational dosage (1 mg/L) (Table 1). *Ae. albopictus* from FH showed the highest value of RR_{50} with 1.87 folds followed by KSG and KTT with 1.77 and 1.39 respectively. The level of susceptibility of *Ae. albopictus* larvae strains from FH, KSG and KTT was generally considered susceptible to Bti (Table 3). At 6000 ITU/L Bti (VectoBac® WG), FH strain had the highest value of RR at 2.03 followed with KSG and KTT at RR 1.68 and

Table 1
Larval susceptibility of *Ae. albopictus* strains after 24 h of continuous exposure to temephos.

Strains	% Mortality after 24 h		LT_{50} (95% CL)[a]	LT_{95} (95% CL)[b]	RR_{50}	RR_{95}
	0.012 mg/L	1 mg/L				
VCRU laboratory strain	100	100	36.44 (31.69–40.03)	56.50 (48.46–89.89)	–	–
FH	100	100	68.31 (65.78–70.99)	89.72 (83.99–99.70)	1.87	1.59
KSG	100	100	64.86 (62.36–67.47)	91.66 (85.29–108.68)	1.77	1.62
KTT	100	100	50.61 (42.04–56.39)	65.43 (57.97–180.57)	1.39	1.15

[a,b]: The LT required to kill 50%/95% of larvae using 1 mg/L of temephos. CL: Confidence limit.

Table 3
LT and RR of *Ae. albopictus* from different localities to VectoBac® WG (Bti).

Dose (ITU/L)	Locality	LT_{50} (95% CL)	RR	LT_{95} (95% CL)	RR
6000	FH	107.06 (101.31–112.18)	2.03	168.20 (155.78–188.05)	1.78
	KSG	88.56 (86.24–90.78)	1.68	199.10 (189.62–210.50)	2.10
	KTT	59.81 (52.20–67.15)	1.13	101.96 (85.23–156.64)	1.08
	VCRU	52.70 (48.17–56.90)	1.00	94.68 (83.02–117.89)	1.00
15000	FH	63.92 (57.96–71.05)	2.45	115.51 (96.86–160.60)	2.02
	KSG	46.10 (45.15–46.97)	1.77	71.53 (69.28–74.29)	1.25
	KTT	32.94 (29.91–35.44)	1.26	59.20 (52.98–70.70)	1.04
	VCRU	26.09 (24.25–27.82)	1.00	57.12 (49.87–70.00)	1.00
24000	FH	40.90 (37.83–43.41)	1.98	83.75 (76.70–94.77)	2.63
	KSG	34.87 (31.18–37.69)	1.69	63.49 (57.50–74.15)	1.99
	KTT	27.96 (22.73–30.86)	1.35	45.75 (38.84–80.74)	1.44
	VCRU	20.65 (19.26–21.67)	1.00	31.82 (29.52–36.01)	1.00

1.13 respectively. For 15000 ITU/L, FH, KSG and KTT showed RR of 2.45, 1.77 and 1.26 respectively, while RR against 24000 ITU/L at LT_{50} for all three localities was 1.98, 1.69 and 1.35 respectively (Table 3).

4. Discussion

Penang Island has experienced increased breeding of *Ae. albopictus* which supposedly leads to an increase in the number of dengue fever cases. There were 878 cases during first half of 2014, an increase of 175% compared to 2013 [31]. The present study indicated that both the larvicide are still effective against *Ae. albopictus* larvae as reported previously [17,32]. In comparison, the standard larvicides used in mosquito control are still effective against the United States, Thailand and India population but the monitoring and the need of development of new tools is still in the first priority [33]. Detection of resistance in larvae often forestall resistance in adult mosquito. It can also show that the resistance in the larvae can be expressed and determined in the adult mosquito population.

In Malaysia, temephos has been introduced since the first nationwide dengue fever outbreak in 1973 [34]. The first study on temephos resistance in *Ae. aegypti* was conducted in Kuala Lumpur in 1984 and resistance was not detected [35]. A re-evaluation was carried out in Jinjang, Kuala Lumpur in 1989 and it was found that *Ae. aegypti* started to develop low resistance towards temephos [36]. However, other study has found that the larvae of *Ae. albopictus* were less susceptible to temephos than in *Ae. aegypti* [37]. *Aedes* mosquitoes have a potential to develop resistance towards temephos under selection pressure, which is found to be correlated with the time and fitness costs [38–40].

Temephos is used extensively and intensively in dengue outbreak areas. This seems to enhance the development of resistance in the field strains. The FH and KSG areas have been treated repeatedly with operational dose of temephos. Despite this, *Ae. albopictus* larvae are still considered susceptible to temephos with complete mortality in 24 h as shown in our study. In this study, FH strain, has shown the possibility to develop faster resistance towards temephos in the future as the RR_{50} was the highest among the strains. Similar findings were reported by Chen *et al.* [17]. There is no failure in control activity (100% mortality in 24 h) in Argentina, but the lethal concentration and RR values show incipient resistance. It is therefore crucial to continuously monitor the nationwide temephos resistance status of *Ae. albopictus* prior to emergence of high level of resistance [41].

In the current study, *Ae. albopictus* was susceptible to VectoBac® WG in all concentrations tested. In Penang, Bti has been used to control dengue vectors since 2003 and such use was intensified in 2010. It was used as an alternative larvicide other than temephos. In Malaysia, as in other parts, there is no report of resistance among *Aedes* mosquitoes against Bti. Bti is applied in the field to supplement temephos in order to optimize the efficiency of the larviciding program. This is similar with the finding in Lahore, Pakistan, which reported the exposure against Bti causing RR of 1.97 and 2.22 [25]. No cross-resistance between Bti and temephos has been reported so far [4]. However, low resistance to Bti has been reported from other countries [42].

Resistance against Bti was reported due to reduction of the toxin binding to epithelial lining in the insect gut or the enhancement of the digestion process of Bti by the gut protease [25,43]. The frequency of gene resistance may be changed if they are occasionally exposed or routinely exposed to the insecticide which brings an advantage to the resistance gene. Other study also suggested that resistance also occurred from the standing genetic variation in the affected areas [44]. The flow of the low genetic variation from treated areas can cause Bti resistance in other population [45]. The resistance against Bti has also been found in the laboratory [43,44]. Development of resistance to Bti is related to the mosquito habitat types which is an important factor in determining the effectiveness of Bti.

An attempt has been made to evaluate the effectiveness of temephos and Bti against mosquito larvae. The monitoring of resistance status must be initiated and fully documented. The vector control programme will not succeed without any extra resistance status information, which becomes one of the limitation in vector control activities. This study provided a baseline reference for the future monitoring on the resistance status of *Ae. albopictus* in FH, KSG and KTT. It is very important to monitor the development of resistance in dengue outbreak areas. The occurrence of resistance should be mapped out carefully. However, we preferred source reduction as one of the most effective strategies. Besides, this method is more direct and simple to reduce the mosquito population for a long-term activity. The application of temephos and VectoBac® WG in vector control management in FH, KSG and KTT is still relevant and serves as an effective tool to control *Aedes* populations.

Conflict of interest statement

We declare that we have no conflict of interest.

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