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RESEARCH ARTICLE

Evaluation of insecticide resistance among Malaysian *Aedes albopictus* Skuse larvae based on revised diagnostic doses of larvicides

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ABSTRACT

The susceptibility levels of Malaysian *Aedes albopictus* larvae sampled from several agricultural, fogging-free residential and dengue prone residential areas against different larvicides were evaluated using revised diagnostic doses derived from the $2xLC_{99}$ values of the reference strain. Upon 24-hour recovery period of WHO larval bioassay, incipient resistance was observed among *Ae. albopictus* larvae from rubber estates against fenitrothion (96.67% mortality) and permethin (97.00% mortality) while *Ae. albopictus* larvae from rice cultivation areas were moderately resistant to fenthion (94.33% mortality). *Aedes albopictus* larvae from dengue prone residential areas developed moderate to high resistance against dichlorodiphenyltrichloroethane (DDT), fenitrothion, fenthion, propoxur and permethrin (79.67% – 97.33% mortality). Moderate to high resistance were also demonstrated among all populations of *Ae. albopictus* larvae against temephos and chlorpyrifos (63.00% – 97.67% mortality). Except for *Ae. albopictus* larvae from oil palm plantations, all *Ae. albopictus* larval populations were also highly resistant to bendiocarb (65.67% – 89.67% mortality). Cross resistance between larvicides from similar and different insecticide classes were also revealed in this study. The use of revised diagnostic doses established from the local reference strain could reduce the possibility of underestimation or overestimation of the insecticide susceptibility status of field strain populations.

Keywords: *Aedes albopictus*; diagnostic doses; larvicides; larval bioassay; Malaysia.

INTRODUCTION

Aedes albopictus Skuse is an important mosquito species that is involved in the transmission of dengue, yellow fever, chikungunya and Zika virus in many countries including Malaysia. Although the vectorial capacity of *Ae. albopictus* in conveying arboviruses is poorer than the principal vector of these diseases which is *Ae. aegypti* (Hussain *et al*., 2018), it has acquired greater public health concerns due to the fact now that *Ae. albopictus* is more urbanized than *Ae. aegypti* in adapting themselves to diverse breeding habitats (Wan-Norafikah *et al*., 2018). *Aedes albopictus* larvae are commonly discovered in natural breeding sites such as in plant axils (Ceretti-Junior *et al*., 2016) and also in man-made receptacles like unused tires and flower pots (Villena *et al*., 2017; Wan-Norafikah *et al*., 2017).

Among numerous control approaches of mosquito vectors, source removal has been proven to be the most effective tool in diminishing the mosquito populations. However, the conduct of source removal is labour demanding and costly (Unlu *et al*., 2016). Furthermore, the awareness level of the communities on the importance of source removal and their participation in such activities are still poor. Hence, the chemical control using adulticides and larvicides has been preferred as another practice in vector control strategies. Larviciding is a complementary method of mosquito larval control especially when source removal is not feasible (Koou *et al*., 2014). Nevertheless, heavy dependence and multifarious use of chemical insecticides have prompted a tremendous challenge in the management of vector control. A continuous use of chemical insecticides could result in the insecticide resistance development in mosquito vectors in which subsequently leading to failures in vector control strategies (Messenger *et al*., 2017).

Insecticide resistance development among mosquito vectors are not only induced by the use of insecticides in public health, but also by extensive use of pesticides in agriculture (Ghorbani *et al*., 2018) in which some of them possess similar modes of action with insecticides of public health. Nevertheless, many previous studies on insecticide susceptibility performed in Malaysia have been focusing only on mosquito larvae and/or adult mosquitoes collected from residential areas in urban and suburban areas particularly with reported dengue or chikungunya cases. Furthermore, the recommended diagnostic dosages of larvicides for *Aedes* larvae by World Health Organization (WHO) (1992) only covers both organochlorines and organophosphates. These generalized diagnostic dosages of larvicides may not be accurate to be applied in all areas as each *Aedes* population from different areas experienced various levels of insecticide exposures from both public health and agricultural activities which influenced the susceptibility status of

these mosquito populations against each larvicide. Consequently, an establishment of revised diagnostic dosages of these larvicides is needed for *Aedes* laboratory reference strain in order to obtain the double LC_{99} values of these larvicides which could then be used as the revised diagnostic dosages to reveal the susceptibility status of *Aedes* field populations against these larvicides as defined by WHO. Hence, this study aims to establish the diagnostic dosage of larvicides for *Ae. albopictus* reference strain larvae and consequently to determine the susceptibility status of *Ae. albopictus* field strains larvae from fogging-free agricultural and residential areas as well as from dengue prone residential areas against the revised diagnostic dosages of larvicides.

MATERIALS AND METHODS

Study Areas

Aedes albopictus field populations were collected from a total of fifteen study areas throughout Peninsular Malaysia. These study areas were comprised of human dwellings within the agricultural and residential areas that were free from any vector control activities and also residential areas with recurrent vector control activities due to dengue cases reported to the Ministry of Health Malaysia (Table 1). The agricultural areas were represented by three oil palm plantations, rubber estates, and rice cultivation areas each with consistent use of agricultural pesticides for crop pest management. Oil palm plantations, rubber estates and rice cultivation areas were selected for this research work following their importance in Malaysian industry and have been named as the top most widely planted industrial crops in Malaysia (Department of Agriculture Peninsular Malaysia, 2015). All the experimental results for each study area were first analysed individually and then as groups according to their types of area.

Mosquito Samples

Sixteen populations of *Ae. albopictus* were used for this study which were the laboratory strain and fifteen field strains. *Aedes albopictus* laboratory strain (F69) represented the reference strain of this study and was initially captured from Selangor, Malaysia and has been maintained in the insectarium of the Institute for Medical Research (IMR), Kuala Lumpur, Malaysia for more than ten years. *Aedes albopictus* laboratory strain is free from any past exposure to insecticides.

Table 1. Geographical description of study areas

State	District	Study areas	Geographical description (coordinates and elevation)
Agricultural area : Oil palm plantations			
Johor	Kota Tinggi	University of Malaya Oil Palm Research Plantation, Jementah **(Kota Tinggi OP)**	02˚01.727'N, 103˚51.924'E; 28 m
Selangor	Klang	Jalan Paip Kiri, Meru **(Klang OP)**	03˚09.201'N, 101˚27.535'E; 5 m
Pahang	Temerloh	Taman Paya Pulai **(Temerloh OP)**	03˚27.642'N, 102˚28.098'E; 42 m
Agricultural area : Rice cultivation areas			
Selangor	Kuala Selangor	Parit 3, Ban 3, Tanjung Karang **(Kuala Selangor PD)**	03˚29.770'N, 101˚09.288'E; -25 m
Kedah	Kulim	Kg. Terat Batu, Mukim Sidam Kanan **(Kulim PD)**	05˚32.741'N, 100˚32.350'E; 9 m
Negeri Sembilan	Kuala Pilah	Kg. Padang Lebar Terachi, Tanjong Ipoh **(Kuala Pilah PD)**	02˚44.520'N, 102˚07.787'E; 81 m
Agricultural area : Rubber estates			
Selangor	Sungai Buloh	Sungai Pelong **(Sungai Buloh RB)**	03˚12.549'N, 101˚32.436'E; 39 m
Pahang	Temerloh	Taman Jaya 8 **(Temerloh RB)**	03˚27.423'N, 102˚27.638'E; 43 m
Johor	Kota Tinggi	Malaysian Rubber Board, Desaru **(Kota Tinggi RB)**	01˚33.844'N, 104˚14.267'E; 23 m
Residential area : Fogging-free residential areas			
Selangor	Shah Alam	Alam Nusantara, Setia Alam **(Shah Alam FF)**	03˚06.692'N, 101˚28.134'E; 34 m
Kedah	Padang Serai	Taman Serai Wangi, Mukim Kulim **(Padang Serai FF)**	05˚31.301'N, 100˚32.673'E; 3 m
Pahang	Temerloh	Taman Seberang Temerloh **(Temerloh FF)**	03˚26.985'N, 102˚26.743'E; 19 m
Residential area : Dengue prone residential areas			
Johor	Kota Tinggi	Felda Air Tawar 2 **(Kota Tinggi DEN)**	01˚40.552'N, 104˚01.340'E; 5 m
Selangor	Shah Alam	Kg. Padang Jawa, Seksyen 17 **(Shah Alam DEN)**	03˚03.000'N, 101˚29.200'E; 1 m
Federal Territory of Kuala Lumpur	Cheras	Kg. Cheras Baru **(Cheras DEN)**	03˚06.630'N, 101˚45.101'E; 89 m

Kg. = Kampung.

Meanwhile, *Ae. albopictus* field populations were obtained from fifteen study areas using ovitraps. An ovitrap surveillance was conducted once for five consecutive days in each study area. Standardized ovitraps as defined by Lee (1992) which were filled with 10% hay infusion water (Reiter *et al*., 1991) were deployed in each study area. All ovitraps were utilized by following the guidelines of Ministry of Health Malaysia (1997) and placed randomly indoors and outdoors, close to human dwellings. Ovitraps were collected and transported back to the laboratory after five days of placement.

Mosquito Colonization

In the laboratory, the contents of recovered ovitraps from the field were poured into individual covered plastic containers and topped up with dechlorinated water. The liver powder and small pieces of partially-cooked cow liver were added into each container for larval feeding. All hatched larvae (F0) were reared and later morphologically identified at fourth instar larvae using standard taxonomic keys by Division of Medical Entomology (2000a, 2000b) and Jeffery *et al*. (2012). Only *Ae. albopictus* larvae from all study areas were further colonized to adulthood in the insectarium to produce their offsprings (F1). The late third (3^{rd}) instar larvae of *Ae. albopictus* (F1) were then utilized in the testing.

All populations of *Ae. albopictus* including the reference strain were handled in the same manner through all manipulations and free from any insecticide exposure. The temperature of the insectarium was maintained at 27 ± 2°C and 75 ± 10% relative humidity (R.H.).

Larvicides

Fifteen larvicides were utilized in this study which included the organochlorines DDT and dieldrin; the organophosphates malathion, fenitrothion, fenthion, temephos, chlorpyrifos and bromophos; the carbamates propoxur and bendiocarb; as well as the pyrethroids permethrin, deltamethrin, lambdacyhalothrin, cyfluthrin and etofenprox. These larvicides were supplied as 0.25 g/ 50 ml solution per bottle from the WHO Collaborating Centre; Vector Control Research Unit (VCRU) in Universiti Sains Malaysia (USM), Penang, Malaysia.

WHO Larval Bioassay

Both the establishment of revised diagnostic dosage of larvicides for reference strain larvae and the determination of susceptibility of field strains larvae against established revised diagnostic dosage of larvicides were performed using the WHO larval bioassay method. The WHO larval bioassay was carried out by following the WHO standard procedure of larvicide testing (WHO, 2016).

For the establishment of revised diagnostic dosage of larvicides for the reference strain larvae, 250 ml of test mixture consisting of an appropriate volume of the larvicide diluted in dechlorinated tap water was prepared in a paper cup and allowed to mix together for at least an hour. A wide range of concentrations of each larvicide was prepared and tested. A narrower range of tested concentrations of each larvicide that caused 15%, 35%, 50%, 65% and 85% mortality at 24 hours post-exposure was used to estimate lethal concentrations values (LC_{50}, LC_{95} and LC_{99}). Twenty five (25) healthy late third instar larvae were introduced into each paper cup. Four (4) replicates were employed for each concentration of each larvicide. The control set comprising of 1 ml of absolute ethanol in 249 ml dechlorinated tap water per paper cup was also prepared in 4 replicates with similar stage and number of larvae.

Larval mortality percentage was recorded after 24 hours of exposure by calculating both moribund and dead larvae. Larvae were probed with a needle in the siphon or cervical region and considered dead if they failed to move, whereas, larvae that were incapable to appear at the water surface or not showing any sign of diving behaviour when the water was disturbed were treated as moribund larvae.

Upon obtaining the lethal $concentration_{99}$ (LC_{99}) of each larvicide for the reference strain, the susceptibility of *Ae. albopictus* field populations larvae against these established revised diagnostic dosage of larvicides were determined. Late third instar larvae of *Ae. albopictus* of all field populations were subjected to WHO larval bioassay which was performed in the same manner and conditions as described above. The susceptibility status of all *Ae. albopictus* field populations were evaluated by exposing them to double value of lethal $concentration_{99}$ (LC_{99}) of each larvicide tested on the reference strain. The larval mortality percentage was determined similarly as previously defined.

Data Analysis

The mortality percentage results for all concentrations of each larvicide that caused 15%, 35%, 50%, 65% and 85% mortality among *Ae. albopictus* reference strain at 24 hours post-treatment were used to generate the regression line of probit analysis. Lethal concentrations values (LC_{50}, LC_{95} and LC_{99}) of the reference strain were attained from the regression line constructed. Discriminating lethal dosages of larvicides for *Ae. albopictus* field populations larvae were values of twice the calculated lethal $concentration_{99}$ ($2xLC_{99}$) of the reference strain.

Mortality percentage of each *Ae. albopictus* field population upon exposures to all larvicides at revised diagnostic dosages ($2xLC_{99}$) was determined by calculating the number of dead and moribund larvae at 24 hours post-treatment. According to WHO (2016), larval bioassay of the respective larvicide was discarded and repeated when more than 10% of the larvae of control population pupated during the testing. If the mortality of control population was between 5% and 20%, the mortality percentage of the field population was corrected using Abbott's formula (1925) as follows:

$$\frac{\%\ Test\ Mortality - \%\ Control\ Mortality}{100 - \%\ Control\ Mortality} \times 100$$

Results with control mortalities that exceeded 20% were recorded but not analysed. The reliability of the data acquired affects the accuracy of results interpretation. The susceptibility status of each *Ae. albopictus* population based on their mortality percentages was classified according to the guidelines by WHO (2016): 98–100% mortality signified susceptibility; 90–97% mortality showed moderate or incipient resistance which has been confirmed by additional bioassay testings performed; and < 90% mortality confirmed the existence of high resistance.

Subsequently, Normality Test using Shapiro-Wilk test was carried out to validate that the data of mortality percentage for *Ae. albopictus* larval populations against revised diagnostic dosages ($2xLC_{99}$) of larvicides were normally distributed. One-way ANOVA and Post Hoc Test were then performed to determine any significant difference between populations from different types of area exposed to each larvicide. The correlation test using Pearson Correlation Test was also conducted to ascertain any significant cross resistance between two larvicides based on the data of mortality percentage of *Ae. albopictus* larval populations against revised diagnostic dosages ($2xLC_{99}$). The significant correlation value (r) of more than 0.4 ($r > 0.4$, $P \leq 0.05$) indicated a significant cross resistance between two tested larvicides. The significant correlation value (r) of more than 0.8 ($r > 0.8$, $P \leq 0.05$) implied a significantly strong cross resistance between two tested larvicides.

The probit analysis to generate the lethal concentration regression line of each larvicide for *Ae. albopictus* reference strain, the calculation of mortality percentage, Normality Test, One-way ANOVA, Post Hoc Test and the Pearson Correlation Test were performed using the computer-aided statistical programme (IBM SPSS Statistics version 23.0). All levels of statistical significance were determined at P = 0.05.

RESULTS AND DISCUSSION

The list of WHO recommended diagnostic dosages of larvicides only consisted of both organochlorines and organophosphates. Hence, an attempt has been carried out to determine the diagnostic dosages of larvicides covering all main insecticide classes to obtain complete insecticide susceptibility data and observe for any cross resistance occurred.

Initially, *Ae. albopictus* reference strain larvae were exposed to a range of concentrations for each larvicide which caused mortality between 5% and 95% at 24 hours post-exposure. The LC_{50} and LC_{99} values were generated from the regression lines constructed through the probit analysis based on these results of mortality percentages of *Ae. albopictus* reference strain at 24 hours post-exposure to each larvicide (Table 2). The revised diagnostic dosage ($2xLC_{99}$) values were then acquired from those LC_{99} values (Table 2). In comparison between the $2xLC_{99}$ values calculated with the WHO recommended dosages for organochlorines and organophosphates listed in Table 2, the $2xLC_{99}$ values generated were more diverse and higher than the WHO recommended dosages, except for the fenthion. According to Macoris *et al*. (2005), if the WHO recommended diagnostic dosage is lower than the revised diagnostic dosage and being used in the resistance monitoring testing, an overestimation of resistance among the field mosquito larval populations is highly possible. In contrast, if the WHO recommended diagnostic dose is higher than the revised diagnostic dose and being applied on the field mosquito larval populations, there is a chance of underestimating the resistance in these populations.

These revised diagnostic dosage ($2xLC_{99}$) values obtained for all classes of larvicides were then applied in the WHO larval bioassays involving all *Ae. albopictus* larval populations. *Aedes albopictus* larvae from all different types of area were found to be susceptible against both DDT and dieldrin except for *Ae. albopictus* larvae from dengue prone residential areas which demonstrated moderate resistance against DDT (Table 3). For organophosphates, *Ae. albopictus* larvae from all types of area were susceptible to both malathion and bromophos. The susceptibility against fenitrothion was also displayed in *Ae. albopictus* larvae from most types of area except for *Ae. albopictus* larvae from rubber estates and dengue prone residential areas that were resistant to fenitrothion. As for fenthion, *Ae. albopictus* larvae from oil palm plantations and rubber estates were susceptible against this larvicide, but moderate resistance was detected in *Ae. albopictus* larvae from rice cultivation areas and fogging-free residential areas while high resistance was demonstrated in *Ae. albopictus* larvae of dengue prone residential areas. Subsequently, moderate resistance against temephos was exhibited in *Ae. albopictus* larvae from oil palm plantations, rice cultivation areas and rubber estates, whereas *Ae. albopictus* larvae from both fogging-free residential areas and dengue prone residential areas were highly resistant to temephos. Furthermore, only *Ae. albopictus* larvae from oil palm plantations were moderately resistant to chlorpyrifos while the rest of the populations developed high resistance against the same larvicide.

In addition, mixed level of resistance was observed in *Ae. albopictus* larvae from different types of area against propoxur (Table 4). *Aedes albopictus* larvae from both oil palm plantations and dengue prone residential areas were the most susceptible and highly resistant against propoxur, respectively, while the rest of *Ae. albopictus* populations were moderately resistant to the same larvicide. In contrast, only *Ae. albopictus* larvae from oil palm plantations were susceptible to bendiocarb while other populations of *Ae. albopictus* larvae were highly resistant to bendiocarb. As for pyrethroids, incipient resistance was detected only in *Ae. albopictus* larvae from rubber estates and dengue prone residential areas against permethrin while susceptible status was achieved for the rest of the populations against all pyrethroids tested.

Results obtained from the Normality Test validated that data of mortality percentage of *Ae. albopictus* larval populations from different types of area against revised diagnostic dosages were normally distributed ($P > 0.05$). In terms of differences in the mortality percentages at 24 hours post-treatment of each larvicide between all *Ae. albopictus* larval field populations, One-way ANOVA revealed that significant differences were demonstrated in the selection of malathion, fenitrothion, fenthion, bromophos, propoxur, bendiocarb, permethrin, lambdacyhalothrin, cyfluthrin and etofenprox ($P \leq 0.05$). However, the Post Hoc Tukey HSD Test showed significant differences in the susceptibility status of *Ae. albopictus* larvae collected from agricultural and non-agricultural areas only for DDT, fenitrothion, fenthion, temephos, chlorpyrifos, carbamates, bendiocarb and permethrin exposures ($P \leq 0.05$).

The correlation analysis using the Pearson Correlation Test was also performed to determine any cross resistance between two tested larvicides using the percent mortality of *Ae. albopictus* larvae at revised diagnostic dosages ($2xLC_{99}$). Cross resistance between intraclass larvicides was demonstrated in organochlorines, organophosphates and carbamates (Table 5). Cross resistance was detected between DDT and dieldrin for organochlorines ($r = 0.514$, $P = 0.042$). Cross resistance within organophosphates was also exhibited among fenitrothion with fenthion ($r = 0.756$, $P = 0.001$) and temephos ($r = 0.646$, $P = 0.007$); fenthion with temephos ($r = 0.770$, $P = 0.000$) and chlorpyrifos ($r = 0.589$, $P = 0.016$); as well as temephos with chlorpyrifos ($r = 0.589$, $P = 0.016$). In carbamates, cross resistance was also displayed between propoxur and bendiocarb ($r = 0.789$, $P = 0.000$). Cross resistance among larvicides of pyrethroids was either not achieved or not able to be determined due to complete mortalities observed at 24 hours post-treatment.

Cross resistance between interclass larvicides was also exhibited among DDT with permethrin ($r = 0.615$, $P = 0.011$) and deltamethrin ($r = 0.641$, $P = 0.007$) as well as dieldrin with deltamethrin ($r = 0.554$, $P = 0.026$). Cross resistance was also displayed among fenitrothion with propoxur ($r = 0.720$, $P = 0.002$), bendiocarb ($r = 0.654$, $P = 0.006$) and permethrin ($r = 0.818$, $P = 0.000$) as well as fenthion with propoxur ($r = 0.928$, $P = 0.000$), bendiocarb ($r = 0.719$, $P = 0.002$) and permethrin ($r = 0.713$, $P = 0.002$). Moreover, temephos was cross resistant with propoxur ($r = 0.835$, $P = 0.000$), bendiocarb ($r = 0.723$, $P = 0.002$) and permethrin ($r = 0.609$, $P = 0.012$). Meanwhile, chlorpyrifos was cross resistant with propoxur ($r = 0.649$, $P = 0.007$) and bendiocarb ($r = 0.661$, $P = 0.005$). Cross resistance was also demonstrated between propoxur and permethrin ($r = 0.667$, $P = 0.005$) as well as between bendiocarb and permethrin ($r = 0.504$, $P = 0.047$).

Overall, diversified level of susceptibility was presented by *Ae. albopictus* larvae from different types of agricultural and residential areas against each larvicide at revised diagnostic doses established from the reference strain of the same species. These results indirectly revealed the miscellaneous history and frequency of insecticide exposures in different types of area which thereby suggesting different effective larvicides to be used at each of these study areas. Findings of this study showed the suitability of malathion and bromophos as the larvicides of choice for all types of area. The utilization of both fenitrothion and fenthion as larvicides were still acceptable in several agricultural areas but definitely not recommended for the use in dengue prone residential areas. Meanwhile, the plan of employing either temephos or chlorpyrifos in any of the study areas needs to be carefully determined since moderate to high resistance were recorded against both larvicides among all larval populations. On the other hand, regardless of the susceptibility status exhibited among almost all *Ae. albopictus* larval populations against both DDT and dieldrin, both larvicides were still not to be selected as the larvicides of choice for all study areas as their use in local vector control strategies had already been prohibited.

Table 2. Lethal concentration values at 50% (LC_{50}) and 99% (LC_{99}) for *Aedes albopictus* reference strain and revised diagnostic dosage ($2xLC_{99}$) values calculated as compared to WHO diagnostic dosages

Classes of Insecticides	Insecticides	LC_{50} (mg/L) 95% C.L.	LC_{99} (mg/L) 95% C.L.	Regression Line	Revised diagnostic dosage, $2xLC_{99}$ (mg/L)	WHO diagnostic dosage (mg/L)
Organochlorines	DDT	0.2160 (0.2090-0.2240)	0.4190 (0.3760-0.4910)	Y = 8.066x+5.370	0.8384	0.012
	Dieldrin	0.0820 (0.0790-0.0850)	0.1730 (0.1530-0.2070)	Y = 7.204x+7.816	0.3460	0.050
Organophosphates	Malathion	0.1610 (0.1390-0.1850)	2.5170 (1.5970-4.8550)	Y = 1.947x+1.546	5.0340	0.125
	Fenitrothion	0.0180 (0.0180-0.0190)	0.0270 (0.0250-0.0290)	Y = 13.786x+23.973	0.0540	0.020
	Fenthion	0.0050 (0.0050-0.0060)	0.0090 (0.0090-0.0110)	Y = 9.768x+22.141	0.0180	0.025
	Temephos	0.0180 (0.0180-0.0190)	0.0330 (0.0300-0.0380)	Y = 9.031x+15.721	0.0660	0.012
	Chlorpyrifos	0.0040 (0.0040-0.0050)	0.0080 (0.0070-0.0090)	Y = 8.925x+20.987	0.0160	0.012
	Bromophos	0.0510 (0.0490-0.0530)	0.1170 (0.1020-0.1420)	Y = 6.475x+8.370	0.2340	0.050
Carbamates	Propoxur	1.2590 (1.0780-1.4600)	2.4400 (1.8530-8.6600)	Y = 8.097x-0.811	4.8800	-
	Bendiocarb	0.7580 (0.6410-0.9070)	2.0380 (1.3980-7.2940)	Y = 5.413x+0.652	4.0760	-
Pyrethroids	Permethrin	0.0200 (0.0200-0.0210)	0.0290 (0.0280-0.0320)	Y = 14.461x+24.487	0.0580	-
	Deltamethrin	0.0090 (0.0080-0.0090)	0.0230 (0.0200-0.0290)	Y = 5.635x+11.570	0.0460	-
	Lambdacyhalothrin	0.0100 (0.0080-0.0130)	0.0440 (0.0250-0.2440)	Y = 3.675x+7.325	0.0880	-
	Cyfluthrin	0.0120 (0.0110-0.0120)	0.0370 (0.0310-0.0490)	Y = 4.595x+8.899	0.0740	-
	Etofenprox	0.0290 (0.0270-0.0300)	0.0760 (0.0650-0.0960)	Y = 5.484x+8.467	0.1520	-

C.L. = Confidence Limit

Regression Line generated from probit analysis using the mortality percentages of *Ae. albopictus* reference strain at 24 hours post-exposure.

Table 3. Percent mortality of *Aedes albopictus* larvae from different types of area against revised diagnostic dosage of larvicides (2xLC99) for organochlorines and organophosphates larval bioassay at 24 hours post-treatment

Types of area	Insecticides	Organochlorines		Organophosphates					
	Study areas	DDT 0.8384 mg/L	Dieldrin 0.3460 mg/L	Malathion 5.0340 mg/L	Fenitrothion 0.0540 mg/L	Fenthion 0.0180 mg/L	Temephos 0.0660 mg/L	Chlorpyrifos 0.0160 mg/L	Bromophos 0.2340 mg/L
Reference	Laboratory	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00
Oil palm plantations	Kota Tinggi OP	^{S}98.67 ± 0.88	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00	^{S}98.00 ± 1.53^{a}	^{S}98.67 ± 0.67^{a}	^{M}96.33 ± 3.67	^{M}95.67 ± 4.33^{a}	^{S}100.00 ± 0.00
	Klang OP								
	Temerloh OP								
Rice cultivation areas	Kuala Selangor PD	^{S}98.33 ± 0.33^{b}	^{S}99.33 ± 0.67	^{S}100.00 ± 0.00	^{S}98.67 ± 0.88^{b}	^{M}94.33 ± 3.48^{b}	^{M}97.67 ± 1.86^{b}	^{R}74.67 ± 10.74	^{S}100.00 ± 0.00
	Kulim PD								
	Kuala Pilah PD								
Rubber estates	Sungai Buloh RB	^{S}98.67 ± 0.88	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00	^{M}96.67 ± 0.88^{c}	^{S}99.00 ± 0.00^{c}	^{M}95.00 ± 2.08^{c}	^{R}84.00 ± 8.08	^{S}100.00 ± 0.00
	Temerloh RB								
	Kota Tinggi RB								
Fogging-free residential areas	Shah Alam FF	^{S}100.00 ± 0.00^{b}	^{S}99.00 ± 1.00	^{S}100.00 ± 0.00	^{S}99.33 ± 0.33^{cd}	^{M}94.00 ± 3.06^{cd}	^{R}89.33 ± 5.49	^{R}83.33 ± 4.37^{d}	^{S}100.00 ± 0.00
	Padang Serai FF								
	Temerloh FF								
Dengue prone residential areas	Kota Tinggi DEN	^{M}97.33 ± 1.76	^{S}98.67 ± 1.33	^{S}100.00 ± 0.00	^{R}88.67 ± 2.40^{abcd}	^{R}83.33 ± 1.76^{abcd}	^{R}84.00 ± 2.52^{bc}	^{R}63.00 ± 11.00^{ad}	^{S}100.00 ± 0.00
	Shah Alam DEN								
	Cheras DEN								
One way ANOVA		F = 0.891 df = 15 P = 0.522	F = 0.476 df = 15 P = 0.786	F = 0.000 df = 15 P = 0.000	F = 8.227 df = 15 P = 0.003	F = 6.893 df = 15 P = 0.005	F = 2.560 df = 15 P = 0.097	F = 2.100 df = 15 P = 0.149	F = 0.000 df = 15 P = 0.000

Percent mortality after 24 h (%) = Mean of mortality for larvae + Standard Error (S.E.)

S = susceptible, M = incipient / moderate / probable resistance, R = confirmed / high resistance as determined by WHO (2016).

Percent mortality followed by different letter indicated significant difference between one another (P ≤ 0.05) (Post Hoc Tukey HSD Test): ^{a} = Significantly different with oil palm plantations population, ^{b} = Significantly different with rice cultivation areas population, ^{c} = Significantly different with rubber estates population, ^{d} = Significantly different with fogging-free residential areas population.

Table 4. Percent mortality of *Aedes albopictus* larvae from different types of area against revised diagnostic dosage of larvicides (2xLC_{99}) for carbamates and pyrethroids larval bioassay at 24 hours post-treatment

Types of area	Insecticides	Carbamates		Pyrethroids				
	Study areas	Propoxur 4.8800 mg/L	Bendiocarb 4.0760 mg/L	Permethrin 0.0580 mg/L	Deltamethrin 0.0460 mg/L	Lambdacyhalothrin 0.0880 mg/L	Cyfluthrin 0.0740 mg/L	Etofenprox 0.1520 mg/L
Reference	Laboratory	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00
Oil palm plantations	Kota Tinggi OP	^{S}99.67 ± 0.33^{a}	^{S}99.33 ± 0.67^{a}	^{S}99.67 ± 0.33^{a}	^{S}99.33 ± 0.67	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00
	Klang OP							
	Temerloh OP							
Rice cultivation areas	Kuala Selangor PD	^{M}92.67 ± 5.33^{a}	^{R}84.67 ± 2.85^{a}	^{S}100.00 ± 0.00^{b}	^{S}99.67 ± 0.33	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00
	Kulim PD							
	Kuala Pilah PD							
Rubber estates	Sungai Buloh RB	^{M}95.67 ± 2.33^{c}	^{R}89.67 ± 7.84	^{M}97.00 ± 1.73	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00
	Temerloh RB							
	Kota Tinggi RB							
Fogging-free residential areas	Shah Alam FF	^{M}92.33 ± 5.36	^{R}83.00 ± 8.19	^{S}99.33 ± 0.67^{d}	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00
	Padang Serai FF							
	Temerloh FF							
Dengue prone residential areas	Kota Tinggi DEN	^{R}79.67 ± 2.73^{ac}	^{R}65.67 ± 7.06^{a}	^{M}91.00 ± 2.52^{abd}	^{S}98.33 ± 1.20	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00	^{S}100.00 ± 0.00
	Shah Alam DEN							
	Cheras DEN							
One way ANOVA		F = 3.487 df = 15 P = 0.044	F = 3.636 df = 15 P = 0.039	F = 5.953 df = 15 P = 0.008	F = 1.000 df = 15 P = 0.465	F = 0.000 df = 15 P = 0.000	F = 0.000 df = 15 P = 0.000	F = 0.000 df = 15 P = 0.000

Percent mortality after 24 h (%) = Mean of mortality for larvae + Standard Error (S.E.).
S = susceptible, M = incipient / moderate / probable resistance, R = confirmed / high resistance as determined by WHO (2016).
Percent mortality followed by different letter indicated significant difference between one another (P ≤ 0.05) (Post Hoc Tukey HSD Test): [a] = Significantly different with oil palm plantations population, [b] = Significantly different with rice cultivation areas population, [c] = Significantly different with rubber estates population, [d] = Significantly different with fogging-free residential areas population.

Table 5. Correlation of percent mortality of *Aedes albopictus* larvae at 24 hours post-treatment of revised diagnostic dosage of larvicides ($2xLC_{99}$)

Insecticides		OC		OP						CARB		PY			
		DDT 0.8384 mg/L	Dieldrin 0.3460 mg/L	Malathion 5.0340 mg/L	Fenitrothion 0.0540 mg/L	Fenthion 0.0180 mg/L	Temephos 0.0660 mg/L	Chlorpyrifos 0.0160 mg/L	Bromophos 0.2340 mg/L	Propoxur 4.8800 mg/L	Bendiocarb 4.0760 mg/L	Permethrin 0.0580 mg/L	Deltamethrin 0.0460 mg/L	Lambdacyhalothrin 0.0880 mg/L	Cyfluthrin 0.0740 mg/L
OC	Dieldrin 0.3460 mg/L	r = 0.514 P = 0.042													
OP	Malathion 5.0340 mg/L	N.D.	N.D.												
	Fenitrothion 0.0540 mg/L	r = 0.291 P = 0.273	r = 0.006 P = 0.983	N.D.											
	Fenthion 0.0180 mg/L	r = 0.368 P = 0.160	r = 0.271 P = 0.310	N.D.	r = 0.756 P = 0.001										
	Temephos 0.0660 mg/L	r = 0.065 P = 0.812	r = 0.112 P = 0.679	N.D.	r = 0.646 P = 0.007	r = 0.770 P = 0.000									
	Chlorpyrifos 0.0160 mg/L	r = 0.192 P = 0.476	r = 0.273 P = 0.306	N.D.	r = 0.437 P = 0.091	r = 0.589 P = 0.016	r = 0.589 P = 0.016								
	Bromophos 0.2340 mg/L	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.							
CARB	Propoxur 4.8800 mg/L	r = 0.240 P = 0.371	r = 0.069 P = 0.799	N.D.	r = 0.720 P = 0.002	r = 0.928 P = 0.000	r = 0.835 P = 0.000	r = 0.649 P = 0.007	N.D.						
	Bendiocarb 4.0760 mg/L	r = -0.002 P = 0.993	r = 0.156 P = 0.564	N.D.	r = 0.654 P = 0.006	r = 0.719 P = 0.002	r = 0.723 P = 0.002	r = 0.661 P = 0.005	N.D.	r = 0.789 P = 0.000					
PY	Permethrin 0.0580 mg/L	r = 0.615 P = 0.011	r = 0.294 P = 0.270	N.D.	r = 0.818 P = 0.000	r = 0.713 P = 0.002	r = 0.609 P = 0.012	r = 0.395 P = 0.129	N.D.	r = 0.667 P = 0.005	r = 0.504 P = 0.047				
	Deltamethrin 0.0460 mg/L	r = 0.641 P = 0.007	r = 0.554 P = 0.026	N.D.	r = 0.367 P = 0.162	r = 0.438 P = 0.090	r = 0.281 P = 0.292	r = 0.230 P = 0.392	N.D.	r = 0.291 P = 0.274	r = 0.124 P = 0.648	r = 0.468 P = 0.068			
	Lambdacyhalothrin 0.0880 mg/L	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.		
	Cyfluthrin 0.0740 mg/L	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	
	Etofenprox 0.1520 mg/L	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

Cross resistance between two larvicides (Pearson Correlation Test) based on the correlation of percent mortality at 24 hours post-treatment for two tested larvicides: r > 0.4 = Correlated (Two tested larvicides showed cross resistance between one another); r > 0.8 = Highly correlated (Two tested larvicides showed strong cross resistance between one another).
N.D. = Not Determined due to 100% mortality achieved for either one of the insecticide tested for correlation.
OC = Organochlorines; OP = Organophosphates; CARB = Carbamates; PY = Pyrethroids.
P ≤ 0.05 = Significant.

As reported by Wan-Norafikah *et al*. (2021), the same *Ae. albopictus* larval populations had been previously exposed to similar organochlorine and organophosphate larvicides at WHO recommended dosages. These dosages are more generalized to all *Aedes* populations regardless of their geographical and climatic backgrounds. In comparison between the WHO larval bioassay results using WHO recommended dosages by Wan-Norafikah *et al*. (2021) and the WHO larval bioassay results at revised diagnostic dosages from the present study, moderate to high resistance were exhibited among *Ae. albopictus* larvae from almost all types of area against organochlorines and organophosphates in the earlier study. Contrarily, dissimilar patterns of susceptibility among these larval populations against all classes of larvicides were demonstrated in the present study in which high susceptibility had been observed among *Ae. albopictus* larvae from various types of area against certain larvicides while some of them were either moderately or highly resistant to the rest of the larvicides. Overturned findings were also observed for certain organochlorine and organophosphate larvicides tested at both WHO recommended dosages and the revised diagnostic dosage ($2xLC_{99}$) values. As such, for bromophos, *Ae. albopictus* larvae from all types of area were classified as resistant when subjected to WHO recommended dose of 0.050 mg/L. However, this scenario was to the contrary when all these populations were categorized as susceptible to bromophos at 0.2340 mg/L of the revised diagnostic dosage ($2xLC_{99}$). Similar situation was observed for the susceptibility testings of these *Ae. albopictus* larvae against malathion and DDT. Hence, instead of using the WHO recommended doses or the revised diagnostic dosage ($2'LC_{99}$) values calculated from the LC_{99} of the reference strain only, it is strongly suggested that individual regression line, LC_{50} and LC_{99} values to be determined for each mosquito larval field population. These data which will be more specific to one particular population will allow the local health authorities to precisely verify the susceptibility status of each mosquito species population from that particular area and assist them in the selection of the most suitable larvicide to be applied at the respective locality.

Nevertheless, instead of determining and using the revised diagnostic dosage ($2xLC_{99}$) values as suggested by the WHO, most preceding studies by researchers in other parts of the world had been applying their own LC_{50} values in the larval bioassays conducted which also displayed various susceptibility status among their *Aedes* larval populations against different larvicides. For instance, fifteen field populations of *Ae. albopictus* larvae collected in Italy had been exposed to temephos at LC_{50} values determined between 0.0026 and 0.0085 mg/L which were even much lower than the WHO recommended dose for temephos (0.012 mg/L) (Romi *et al*., 2003). In southern India, *Ae. albopictus* immatures collected from two international airports were exposed to LC_{50} values of temephos (0.020 mg/L), fenthion (0.05 mg/L), malathion (1.0 mg/L) and fenitrothion (0.06 mg/L) (Sharma *et al*., 2004). In southern China, the LC_{50} values obtained against deltamethrin for six strains of *Ae. albopictus* larvae ranged between 0.011 and 0.038 mg/L (Li *et al*., 2018). Meanwhile, larval bioassays conducted by Ishak *et al*. (2015) in Malaysia showed higher LC_{50} for temephos in *Ae. albopictus* from Penang (0.020 mg/L) and Kuala Lumpur (0.015 mg/L) as compared to *Ae. aegypti* from similar study sites (0.006 – 0.008 mg/L). Two other studies in China also applied their own LC_{50} values in the larval bioassays carried out in which some of their larval populations showed resistant to pyrethroids like deltamethrin, beta-cypermethrin and permethrin as well as organochlorines, carbamates and organophosphates (Chen *et al*., 2016; Yiguan *et al*., 2017). The inconsistency among researchers on the use of either the WHO recommended doses, the self-determined LC_{50} values and the revised diagnostic dosage ($2xLC_{99}$) values suggested by the WHO in the larval bioassays has made the comparison between these findings to be more challenging.

Up till now, only two accessible former studies reported on the revised diagnostic dosages of larvicides using their reference strain of either *Ae. aegypti* or *Ae. albopictus* larvae but only covered between two and three common larvicides. Hence, the present study is the first attempt of establishing revised diagnostic doses of all classes of larvicides using the local reference strain of *Ae. albopictus* larvae. In Brazil, Macoris *et al*. (2005) reported that the revised diagnostic doses of fenitrothion, malathion and temephos for their *Ae. aegypti* Rockefeller strain were 0.0100 mg/L, 0.200 mg/L and 0.0080 mg/L, respectively, in which all these concentrations were much lower than the diagnostic doses of similar larvicides obtained in the current study. On the other hand, Rahim *et al*. (2016) performed almost similar larval bioassays to determine the discriminating diagnostic doses of temephos and malathion for *Ae. albopictus* susceptible strain reared at the Vector Control Research Unit (VCRU), Universiti Sains Malaysia (USM), Penang, Malaysia. They reported that the revised diagnostic doses of temephos and malathion for their reference strain were 0.020 mg/L and 0.200 mg/L, respectively. Their revised diagnostic dose of temephos was similar to the previous WHO recommended diagnostic dose of temephos while their revised discriminating diagnostic dose of malathion was higher than WHO recommended diagnostic dose of malathion but lower than the revised diagnostic dose of malathion obtained in the present study. All their five field strains collected from Penang showed either incipient resistance or high resistance against both larvicides. Rahim *et al*. (2017) also displayed revised diagnostic doses of malathion, permethrin and deltamethrin for Malaysian *Ae. albopictus* adults which were either much lower (for malathion) or much higher (for permethrin and deltamethrin) than the WHO recommended doses for *Ae. aegypti* adults. These results indicate the differences and significance of attaining the local diagnostic dosages in order to accurately determine the susceptibility status of local mosquito populations against insecticides. In fact, these diagnostic dosages should be species specific as the resistance development in both *Ae. aegypti* and *Ae. albopictus* populations seemed to be vastly different. Nevertheless, the process of obtaining the revised diagnostic dosages for all commonly used insecticides is time-consuming, labour intensive and requires a large number of mosquito samples.

Additionally, the differences in the diagnostic dosages could be due to genetical backgrounds of the mosquito populations (Lee *et al*., 1997). Moreover, since the diagnostic dose is closely related to sensitivity and specificity, the decrease of diagnostic dose could indicate an escalation of sensitivity but with the possibility of picking up either the susceptible strain or the resistant strain (Macoris *et al*., 2005).

Temephos is the preferred larvicide in the Malaysian vector control strategies. The operational dose of temephos for larviciding activity in Malaysia is 1 mg/L (Chen *et al*., 2005). Even though all field strains employed in the current study showed either incipient resistance or high resistance against temephos at $2'LC_{99}$ value of 0.0660 mg/L, the percentage mortality demonstrated by all these populations was at least 84%. Thus, it is expected that total mortality could be achieved in these field populations if temephos is applied at these study areas at operational dose of 1 mg/L. However, environmental parameters such as rain could also diminish the effectiveness of the insecticides (Rahim *et al*., 2016). Not only that, the migration of either susceptible or resistant mosquitoes could also affect the proportion of susceptible and resistant individuals in the field populations (Lee *et al*., 1997) which will indirectly influence the efficacy of the insecticides.

Organophosphate glyphosate, malathion, chlorpyrifos and propoxur as well as pyrethroid alphacypermethrin, cypermethrin and lambdacyhalothrin have been applied at various dosages and consistency in all agricultural sites selected for this study to control the agricultural pests like the bagworms and the brown plant hoppers. The application of these insecticides for agricultural

management purposes could be the reason of resistance detection in outdoor vectors; *Ae. albopictus* larvae from these agricultural areas against some of these insecticides although no mosquito vector control activity has ever been carried out in these localities.

As the revised diagnostic doses of larvicides for the susceptibility testing of field strain larvae are established from the reference strain of the same species, it is crucial to ensure that the susceptibility status of the local reference strain against all insecticides are maintained at the maximum levels in order to sustain its reference status. Furthermore, the establishment of local diagnostic dosages based on our own Malaysian reference strain is important in order to obtain a more reliable, significant and convincing findings on the susceptibility status of local mosquito vectors against all commonly used insecticides. The susceptibility level of the reference strain should be utilized as a guidance or an indication in the bioassay performed upon the field strain mosquitoes (Macoris *et al*., 2005). Even though results of the susceptibility tests obtained for field populations based on the $2xLC_{99}$ values of the local reference strain will only be useful to one particular country where the testing were conducted, these findings will still be comparable with reports of susceptibility testing from other countries that follow the same techniques suggested by the WHO. Hence, special attention and efforts should be given to ensure that the local laboratory strain used as a reference strain in the study is well-maintained in the laboratory for many generations with no compromise on any insecticide selection either purposely or unintentionally. Continuous monitoring on the susceptibility of the reference strain against all insecticides should be carried out to prevent the resistance development against any insecticides and thus, maintaining its status as a dependable reference strain in all mosquito studies. Researchers in other laboratories across the world also utilized several well-recognized laboratory susceptible strains such as New Orleans (NO) strain, Bora Bora strain or Rockefeller strain of *Ae. aegypti* as the reference strain of their studies. However, not all laboratories including entomology laboratories in Malaysia have access to these foreign laboratory susceptible strains which require various import procedures and legislations. Furthermore, the reference strain of *Ae. albopictus* used in this study originated from the Medical Entomology Unit, Institute for Medical Research (IMR) Malaysia. The Institute for Medical Research (IMR) is the research and diagnostic centre of the Ministry of Health (MOH) Malaysia in which all decisions on insecticides to be employed or any other approaches to be performed in the local vector control activities will be based on the research findings by researchers of IMR. Moreover, the use of local laboratory susceptible strains in determining the diagnostic dosages of insecticides before being compared with the field populations of the same species will reduce the differences between these strains to obtain more accurate data since all strains possess relatively similar genetical backgrounds (Lee *et al*., 1997). Hence, the employment of local laboratory susceptible strain especially from IMR, Malaysia remains the best option for now.

Meanwhile, cross resistance between larvicides from the same insecticide class was exhibited in organochlorines, organophosphates and carbamates, whereas the cross resistance between larvicides from different insecticide classes involved all four classes tested in this study. Cross resistance among larvicides from the same and different insecticide classes are not solely due to vector control activities since not all larvicides tested were employed in Malaysian public health, but also because of their extensive application in the agricultural practice. Hence, it is crucial for the local health authorities to ensure that only larvicides that were not involved in the cross resistance detected to be used in these study localities to diminish and prevent the breeding of *Ae. albopictus* larvae. Nevertheless, the gap between the laboratory findings and the decision making in the field that will verify the operational efficacy is still hard to be fulfilled as there are many other factors and limitation to be investigated and considered.

In essence, findings of this work showed inconsistent trends of susceptibility were presented among *Ae. albopictus* larval populations upon selection to all classes of larvicides at revised diagnostic dosages established from the local reference strain of *Ae. albopictus* larvae. Significant differences in the susceptibility levels of *Ae. albopictus* larvae from dengue prone residential areas as compared to agricultural areas were also observed against fenitrothion, fenthion, temephos, propoxur and permethrin. Consequently, larvicides for mosquito control that should be utilized in each type of area are diversified since different *Ae. albopictus* population possessed various susceptibility levels against each larvicide. Therefore, fruitful discussion, understanding and collaborating actions between all relevant agencies are essential to assure the effectiveness of the local vector control operations conducted.

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Declaration of Competing Interest

The authors declare that this research is an original work. It has not been published elsewhere. The authors have read and approved the manuscript. All authors have no conflicts of interest with respect to this study, authorship and publication.

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