Tropical Biomedicine 31(4): 670–679 (2014)

Bioassay and biochemical studies of the status of pirimiphos-methyl and cypermethrin resistance in *Aedes* (*Stegomyia*) *aegypti* and *Aedes* (*Stegomyia*) *albopictus* (Diptera: Culicidae) in Singapore

Lee, R.M.L.[1], Choong, C.T.H.[1,2], Goh, B.P.L.[2], Ng, L.C.[1] and Lam-Phua, S.G.[1*]
[1]Environmental Health Institute, National Environment Agency, 11 Biopolis Way #06-05/08, Helios Block, Singapore 138667
[2]National Institute of Education, Nanyang Technological University, 50 Nanyang Avenue, Singapore 638798
[*]Corresponding author email: phua_sai_gek@nea.gov.sg
Received 11 December 2013; received in revised form 14 April 2014; accepted 21 April 2014

Abstract. *Aedes* (*Stegomyia*) *aegypti* (Linnaeus) and *Ae.* (*Stegomyia*) *albopictus* (Skuse) were sampled from five regions of Singapore (Central, North East, North West, South East and South West) and tested with diagnostic concentrations of the technical grade insecticides, pirimiphos-methyl and cypermethrin. Biochemical assays were performed on the same populations of *Ae. aegypti* and *Ae. albopictus* to determine activities of detoxifying enzymes, including non-specific esterase (EST), monooxygenase (MFO) and acetylcholinesterase (AChE). The diagnostic test showed that all *Ae. aegypti* populations were susceptible to pirimiphos-methyl (mortality = 99 to 100%), but resistant to cypermethrin (mortality = 11 to 76%). Resistance to pirimiphos-methyl was observed in all *Ae. albopictus* populations (mortality = 49 to 74%) while cypermethrin resistance was detected in most *Ae. albopictus* populations (mortality = 40 to 75%), except those from Central (mortality = 86%) and South East (mortality = 94%) showing incipient resistance. The biochemical assays showed that there was significant enhancement ($P < 0.001$) of MFO activity in pyrethroid-resistant *Ae. albopictus* populations and most *Ae. aegypti* populations. The biochemical assay results suggested that AChE could play a role in pirimiphos-methyl resistance of *Ae. albopictus* in South West, South East and North East regions. The small but significant increase in EST activities in *Ae. aegypti* from all regions suggest that it may play a role in the observed cypermethrin resistance.

INTRODUCTION

Dengue fever and chikungunya are of public health concern in Singapore. Several serious dengue outbreaks in Singapore were reported in 1973 (1187 cases), 1997 (4300 cases), 2005 (14 209 cases), 2007 (8826 cases) and 2013 (22 077 cases) (Goh, 1998; Ministry of Health Singapore, 2013). At this juncture, the number of chikungunya cases increased from a total of 60 cases in the last three years (between 2010 and 2012) to 1059 cases in 2013 (Ministry of Health Singapore, 2013). *Aedes aegypti* and *Ae. albopictus* are the vectors of Dengue and Chikungunya viruses in Singapore. *Aedes aegypti* is the primary dengue vector that dwells closely with humans in the urban environment, while *Ae. albopictus* is the secondary dengue vector that dwells mainly outdoors (Goh, 1998). *Ae. albopictus* is a major vector for Chikungunya virus transmission (Ng and Hapuarachchi, 2010). Singapore's mosquito control program has been maintained as an integrated program that includes environmental management and source reduction through public education and enforcement. Following the outbreak in 2005, Singapore's vector control programme has been enhanced. Two key novel features have been incorporated, namely intersectoral and interagency collaboration; and a decision support system

that is built on 4 cornerstones - case, virus, entomological surveillance and ecological information (Ng and Vythilingam, 2012). Use of chemicals for adulticiding has been restricted to the management of difficult clusters of dengue and chikungunya.

However, chemical control such as adulticiding and larviciding may result in the development of insecticide resistance in the mosquito populations. In the 1970s, organophosphates (temephos and malathion), chlorinated hydrocarbon (DDT) and pyrethroid (bioresmethrin) were used for mosquito population control in Singapore (Ong *et al.*, 1979; Tan *et al.*, 1998). *Aedes aegypti* and *Ae. albopictus* were susceptible to malathion (mortality = 100% at 5% dosage) (Ong *et al.*, 1979). However, the use of malathion was gradually phased out in the early 1980s because of its unpleasant smell. Bioresmethrin, a pyrethroid insecticide, replaced malathion (Lai *et al.*, 2001). In the 1980s, permethrin was introduced to replace bioresmethrin and was used for 20 years until permethrin resistance was detected in 1991. An organophosphate, pirimiphos-methyl was introduced in 1993 for use in the *Aedes* control programme (Tan *et al.*, 1998). Since then, the susceptibility of *Aedes* mosquitoes to pirimiphos-methyl has been regularly monitored. Lai *et al.* (2001) reported that both *Ae. aegypti* and *Ae. albopictus* populations in Singapore were susceptible to pirimiphos-methyl (RR_{50} = 1.5 and 1.4, respectively), but resistance to permethrin was persistent (RR_{50} = 12.9 and 1.8, respectively). Resistance to pyrethroid in *Ae. aegypti* has been found in other countries, including Thailand and Indonesia (Astari and Ahmad, 2005; Paeporn *et al.*, 2004). In addition, *Ae. aegypti* in some areas, such as the north central and north eastern part of Thailand, and seven localities of Colombia were also reported to be resistant to organophosphate (Fonseca-Gonzalez *et al.*, 2010; Pethuan *et al.*, 2007). In central Africa, *Ae. aegypti* from Libreville and *Ae. albopictus* from Buea and Yaoundé were found to be resistant to DDT, and incipient resistance to deltamethrin was also observed in *Ae. albopictus* from Yaoundé (Kamgang *et al.*, 2011).

To date, cypermethrin is the most frequently used pyrethroid by the pest control industry in Singapore, but the susceptibility of *Aedes* to the chemical has not been evaluated locally. Besides, it is important to understand the mechanisms that are responsible for insecticide resistance among the *Ae. aegypti* and *Ae. albopictus* by biochemical assays.

The objective of this study is to determine the susceptibility status of field populations of *Ae. aegypti* and *Ae. albopictus* to pirimiphos-methyl, which is used by dengue control programme for cluster management and cypermethrin, one of the most widely used chemical by the pest control industry. Bioassay and biochemical assays were performed on the adult field populations.

MATERIALS AND METHODS

Mosquitoes

Long-established susceptible laboratory strains (S-Lab) of *Ae. aegypti* and *Ae. albopictus* were used as reference strains for WHO bioassays and a control for biochemical assays. Field *Ae. aegypti* and *Ae. albopictus* larvae collected from five regions (Central, North East, North West, South East and South West) (Figure 1) of Singapore between 2004 to 2007 (Table 1) were reared to adults. These larvae were collected by National Environment Agency officers during routine house-to-house inspections and from routine ovitrap monitoring. The collected larvae were pooled and reared in one colony, according to their respective regions. The first generation (F1) of adults were used for diagnostic and biochemical assays. Approximately 1800 eggs were hatched and reared to adults for each test. All test populations were maintained at a daily temperature of 24.5 ± 0.5°C, and a relative humidity of 74 ± 4%. All the test adults were fed with 10% sugar solution which contained vitamin B complex during the rearing period.

Experimental design

The study followed the following procedures: (1) baseline susceptibility and the diagnostic

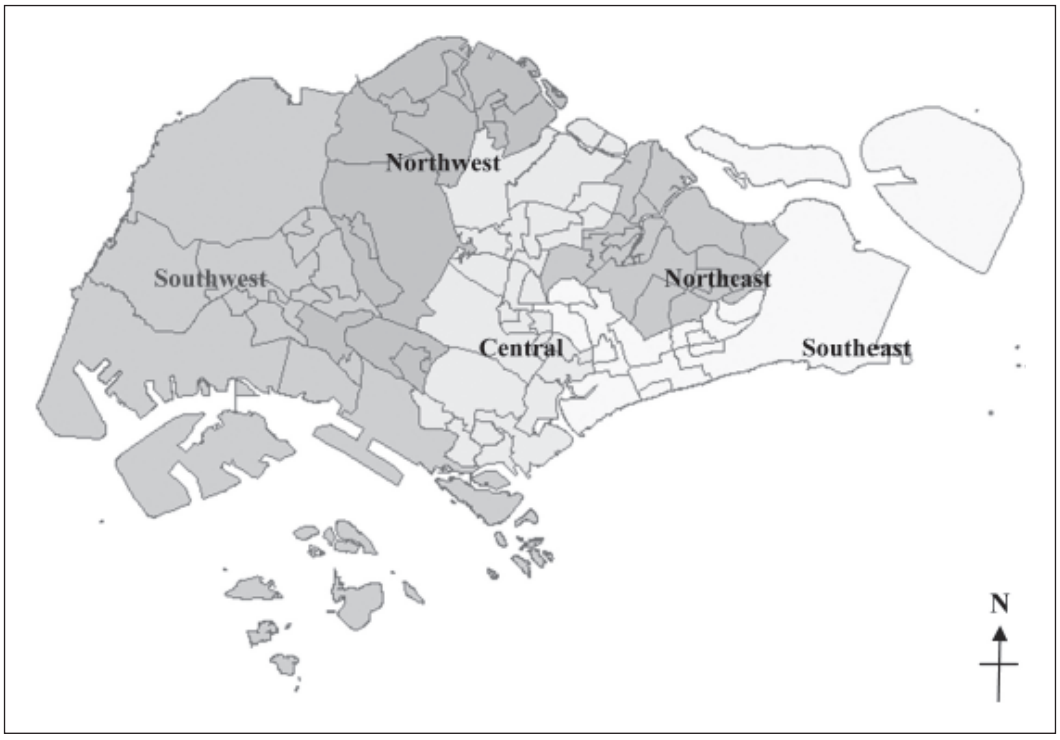

Figure 1. Map of Singapore showing the five regions where *Aedes* larvae were collected for the study.

Table 1. Larval collection period for the diagnostic tests of the insecticides, 2004–2007

Region	Pirimiphos-methyl		Cypermethrin	
	Ae. aegypti	*Ae. albopictus*	*Ae. aegypti*	*Ae. albopictus*
C	Jun - Jul 2004	Jun - Jul 2004	Mar - May 2007	Mar - Apr 2007
NE	Aug 2004	Jul 2004	May - Jun 2007	Mar - May 2007
NW	Aug 2004	Aug-04	Dec 2006 - May 2007	Nov 2006 - Apr 2007
SE	Feb - Mar 2004	Feb - Mar 2004	Jun - Jul 2007	Feb 2007
SW	Sep 2004	Aug 2004	Jan - Feb 2007	Dec 2006 - Mar 2007

concentration of each insecticide were determined by exposure of S-Lab strains of *Ae. aegypti* and *Ae. albopictus* to each insecticide, (2) F1 *Ae. aegypti* and *Ae. albopictus* from the five different regions were tested using this diagnostic concentrations to determine susceptibility status, (3) same batch of the mosquitoes were subjected to biochemical assays. Figure 2 illustrates the experimental design of the study.

Insecticides

Technical grade of pirimiphos-methyl (91.1% w/w; Syngenta-Muenchwilen, Münchwilen, Switzerland) and cypermethrin (92% w/w; Asiatic Agricultural Industries Pte Ltd, Singapore) were used. A series of concentrations of these two insecticides were prepared and impregnated on filter papers following the procedure as stated in Lai *et al.* (2001). Briefly, a sheet of filter paper (Whatman no. 1, 15 x 12 cm) was placed on

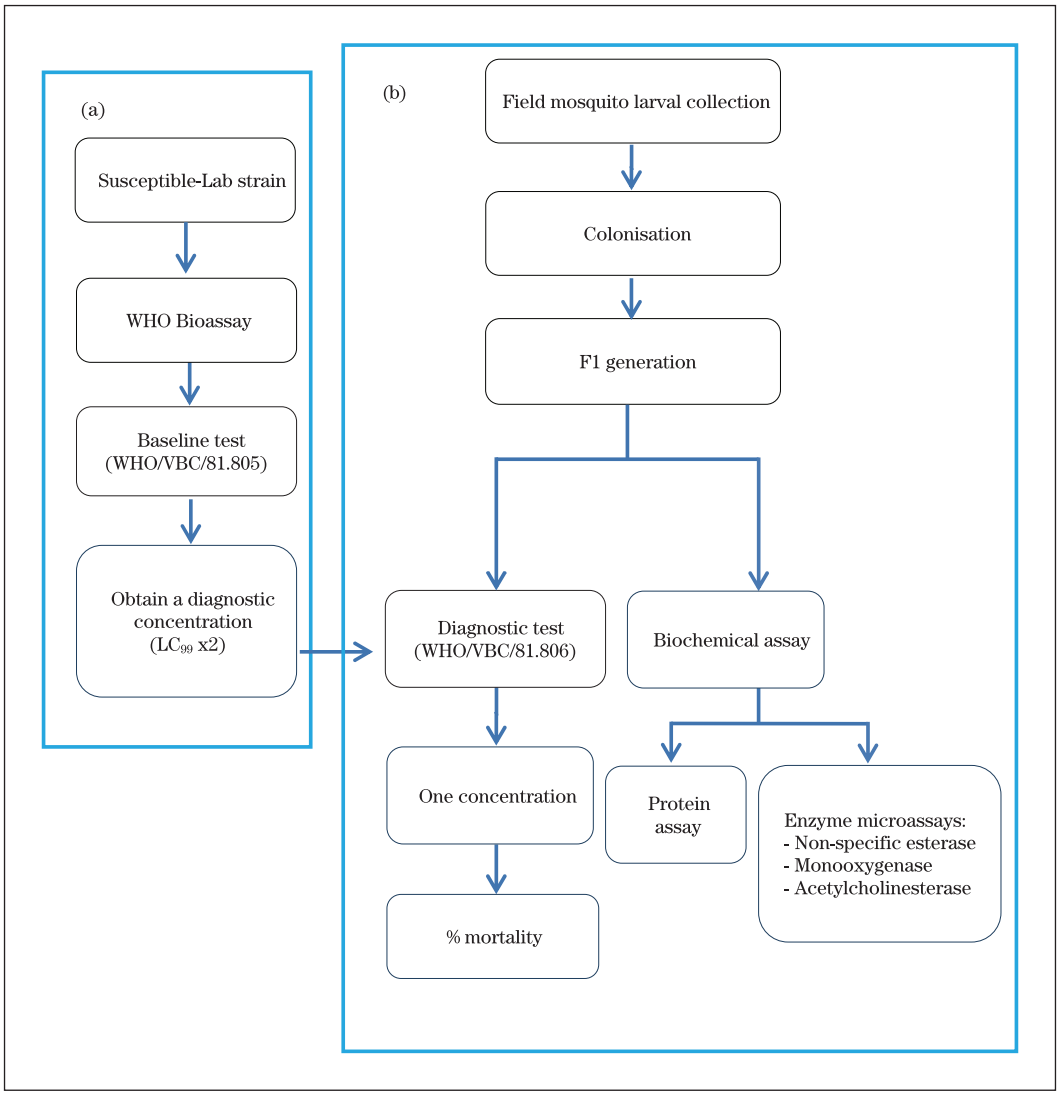

Figure 2. Experimental design of the study. (a) To obtain a diagnostic concentration from Susceptible-Lab strain by WHO bioassay. (b) To perform diagnostic test and biochemical assays on field mosquito adults.

a stainless steel tray (19.8 x 13.3 cm). Three ml of each concentration was introduced onto the paper via fast circular motion from middle of the paper circulating out. The insecticide impregnated paper was dried for one hour and wrapped in aluminium foil with both end folded and sealed with masking tapes. The sealed paper was then put into zip-lock bag (Seow Khim Polythelene Co Pte Ltd (SKP), Singapore). The control paper was coated with only acetone.

WHO Adult Bioassay

WHO Base-line test

Each test consisted of one control and a series of five concentrations of the insecticide. There were four replicates for each concentration, including control. Twenty-five 3-10 days old sugar fed female mosquitoes per replicate were exposed to each concentration of insecticide. After exposure, the mosquitoes were transferred to the holding tubes. The exposed mosquitoes were

fed with ten percent sugar solution with vitamin B complex. Mortality was recorded 24 hours after exposure. In order to establish diagnostic concentration, mortality that ranged from 0 to 100% was used to generate LC_{50} and LC_{99} values according to WHO guidelines, WHO/VBC/81.805 (W.H.O, 1981b).

Diagnostic concentration tests

The diagnostic concentrations of pirimiphos-methyl and cypermethrin for both field strain *Ae. aegypti* and *Ae. albopictus* were obtained from the susceptibility baseline test of the respective S-Lab strains (described earlier on). A summary of the diagnostic concentrations was presented in Table 2. The diagnostic test was conducted according to WHO guidelines, WHO/VBC/81.806 (W.H.O., 1981a). Each test consisted of four replicates for control and the diagnostic concentration of each insecticide.

Biochemical assay

Rapid enzyme kits (Lee, 1990) developed by the Institute for Medical Research (IMR), Kuala Lumpur were used. The protocol used for this enzyme assay followed the technique recommended by WHO (W.H.O., 1998b) with some modifications. Expression or elevations of enzymes corresponding to insecticide resistance were quantified using ELISA reader (Basic-Tecan, Sunrise). Details of the protein assay, non-specific esterase (EST), monooxygenase (MFO) and insensitive AChE isozymes (AChE) assays are shown in Figure 3. As the enzyme assay measures the activity of non-specific esterase, monooxygenase and insensitive AChE isozymes, a higher value of optical density indicates a higher level of tolerance/ resistance to the insecticide. Briefly, individual F1 adult mosquitoes (between 4-11 days old) were put on ice and homogenised in 1 ml of phosphate buffered saline solution, pH 7.2. A 100 or 200 µl portion of the homogenate, depending on the enzyme assay, was then transferred into the well of microassay plates. Duplicates of the homogenate were prepared for each mosquito homogenate. A control was also prepared using phosphate buffered saline solution. Forty mosquitoes were processed for each enzyme. For AChE assay, two reactions were prepared for each sample. While one reaction was allowed to progress, the other was inhibited using 0.1 µl of propoxur (inhibitor) followed by adding of DTNB.

Protein assay was conducted to detect the differences in size among individuals that might require correction factors for the enzyme assays. Mosquitoes were also weighed in groups of 40 using a weighing balance (XT 220A, Precisa) to nearest milligram, to determine the correlation between weight, protein content and enzyme activities. The total soluble protein content of mosquitoes was determined by using the Bio-Rad Protein Assay Kit© (Bio-Rad, California, U.S.A.). Bovine serum albumins of concentrations ranging from 0 to 0.1 µg/µl were used to generate standard curve, in order to determine absolute protein concentration.

Data Analyses

All statistical analyses were performed using SPSS15.0 (SPSS Inc). Diagnostic

Table 2. Diagnostic concentration (%) of pirimiphos-methyl and cypermethrin based on S-Lab strain

Species	Insecticides	No. tested	LC_{50} (95%CL) (%)	LC_{99} (95%CL) (%)	Diagnostic concentration (%)
Aedes aegypti	Pirimiphos-methyl	1800	0.0320 (0.0308-0.0331)	0.07558 (0.0706-0.0819)	0.1512
	Cypermethrin	2800	0.1014 (0.0762-0.1351)	1.4298 (0.5806-3.5622)	2.8600
Aedes albopictus	Pirimiphos-methyl	1800	0.0098 (0.0089-0.0107)	0.01589 (0.0125-0.0202)	0.0318
	Cypermethrin	1720	0.0416 (0.0344-0.0503)	0.6156 (0.3527-1.0932)	1.2310

LC: Lethal concentrations
CL: Confidence limits
Diagnostic concentration: LC_{99} x 2

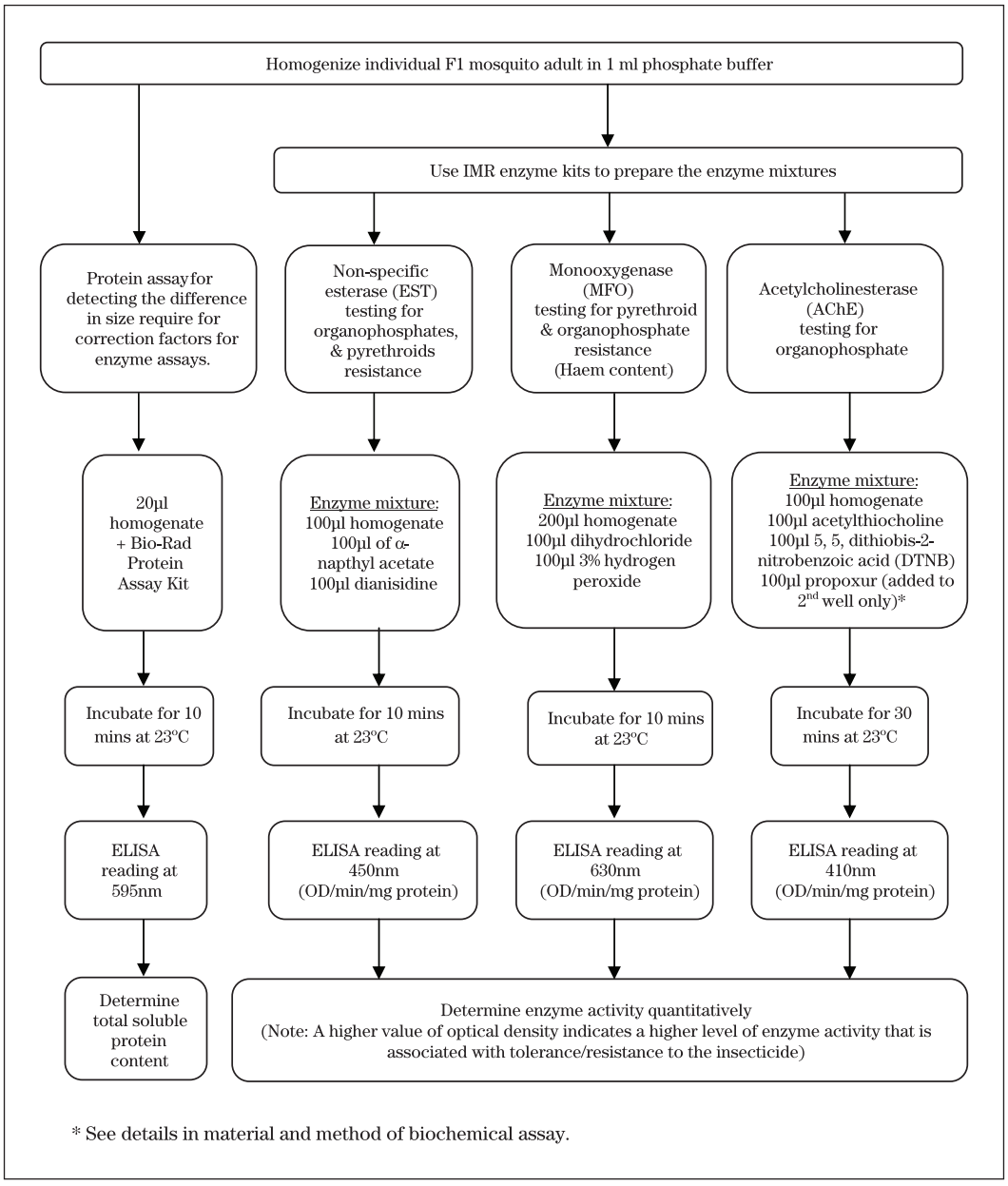

Figure 3. Flow chart of biochemical assay.

concentration for each insecticide is derived from doubling the lethal concentration that kills 99% of the susceptible population tested (LC_{99} x 2). The interpretation of results for diagnostic concentrations tests was based on the WHO recommendation (W.H.O., 1998a). The mosquito population is considered susceptible if the mortality is 98-100%, resistant if the mortality is <80% and further tests needed if the mortality ranged between 80-97% as this suggests possible (or incipient) resistance.

All the data from each strain were pooled, and the mean and standard deviation were calculated. The data were compared with S-lab strain for significant different. Data for enzymes activity were presented as Mean ± SD. Regression analysis was conducted on the protein levels measured from the mosquito homogenates and the weight of the

insects. This was conducted to differentiate if weight measurement could be used as a basis for comparisons of mosquito populations collected from the field, as well as determine the quality of laboratory-reared strains. One-way Analysis of Variance (ANOVA) of weight-to-weight comparison showed that there was no significant difference in weights among all the field mosquitoes that were colonised to F1 generation in the insectary ($P > 0.05$). The results indicated a high consistency in the sizes of both field and S-Lab strains of mosquitoes reared. ANOVA was applied to determine the differences of mosquito weights between field and S-Lab strains. It was also used to determine if there was any difference in enzyme activities among field and S-Lab strains. All data were tested for normality (through graphical representation of residuals and Kolmogorov-Smirnov test). This was followed by determination of homogeneity of variances (Levene test) prior to ANOVA analysis. No transformation was applied on the raw data for mosquito weights. Hypothesis of equal variances is assumed based on Levene test for equality of variances at $P > 0.05$. Transformation (square root) was applied on the enzyme activity raw data to fulfil the assumptions of ANOVA (Zar, 1999). Tukey's HSD (Honestly Significant Difference) test was used for the subsequent *post-hoc* multiple comparisons (Zar, 1999), and statistical significance was assumed at $P < 0.05$.

RESULTS

Diagnostic Tests

A total of 6451 *Ae. aegypti* and 7566 *Ae. albopictus* from the five regions were tested against pirimiphos-methyl and cypermethrin for diagnostic concentration tests. The mortalities rate of *Ae. aegypti* and *Ae. albopictus* to these two insecticides are shown in Tables 3 and 4, respectively. The diagnostic test results showed that all the *Ae. aegypti* populations were susceptible to pirimiphos-methyl (mortality = 98.6 to 100%) but were resistant to cypermethrin (mortality = 11.0 to 75.6%). Resistance to pirimiphos-methyl was observed in all the *Ae. albopictus* populations (mortality = 49.1 to 73.9%). On the other hand, *Ae. albopictus* populations of Central and South East regions (mortality = 86.1 and 94.3%, respectively) exhibited incipient resistant to cypermethrin, while *Ae. albopictus* of North East, North West and South West (mortality = 40.0, 66.8 and 74.5%, respectively) exhibited resistance.

Biochemical Tests

Enzyme Activities

The mean (±SD) values of enzyme activities for each insecticide and region are shown in Tables 3 and 4. In order to determine if the observed insecticide resistance of local *Ae. aegypti* and *Ae. albopictus* populations are due to elevated enzyme levels, 40 adults of each species from the five regions were individually assayed for AChE, EST, and MFO enzyme activities. A significant increase in EST activity in *Ae. aegypti* of all the five regions, and MFO activity in *Ae. aegypti* of Central, South East and South West regions, except North East and North West regions ($P < 0.001$) was observed (Table 3). This showed that *Ae. aegypti* populations were heterogeneous towards MFO activity. It was also observed that there was a significant but small increase in AChE activity in *Ae. aegypti* populations of South East and South West regions. This corroborated with the low level of resistance ratios (RR_{50}= 1.1, data not shown) for pirimiphos-methyl.

Table 4 showed that *Ae. albopictus* in all five regions exhibited elevation of MFO activity and was significantly different from the S-lab strain ($P < 0.001$). It was observed that the higher the level of MFO activity, the lower the mortality in cypermethrin treated *Ae. albopictus* strains. A significant increase in EST activity was found among *Ae. albopictus* in North East and North West regions, where they were also resistant to cypermethrin and pirimiphos-methyl. There was also a significant increase in AChE activity in pirimiphos-methyl resistant *Ae. albopictus*, except Central and North West regions. This showed that the *Ae. albopictus* populations were heterogeneous towards AChE activity.

Table 3. Mortality rate and mean value of enzyme activities of adult *Aedes aegypti* mosquito populations collected across Singapore

Strains	Mean % Mortality[1] ± SD		Esterase[3] (OD/min/mg protein)	Monooxygenase[3] (OD/min/mg protein)	AChE[3] (OD/min/mg protein)
	Organophosphate	Pyrethroid			
	0.15% pirimiphos-methyl	2.86% cypermethrin	Mean ± SD	Mean ± SD	Mean ± SD
S-Lab[2]	–	–	0.009 ± 0.002	0.197 ± 0.029	0.0047 ± 0.001
Central	100 (700)	23.5 (596)	0.014 ± 0.007*	0.282 ± 0.046*	0.0067 ± 0.001
North East	100 (400)	75.6 (599)	0.010 ± 0.001*	0.221 ± 0.035	0.0055 ± 0.002
North West	100 (399)	33.3 (578)	0.013 ± 0.003*	0.241 ± 0.036	0.0065 ± 0.001
South East	99.9 (1621)	11.0 (589)	0.022 ± 0.003*	0.384 ± 0.062*	0.0073 ± 0.002*
South West	98.6 (396)	32.7 (573)	0.013 ± 0.003*	0.252 ± 0.052*	0.0074 ± 0.001*

( ) Number of mosquitoes tested
[1] Mean % mortality at diagnostic concentration of each insecticide
[2] S-Lab is a long established susceptible laboratory strain tested
[3] Total of 40 mosquitoes were processed for each enzyme per strain. Two replicates for each enzyme reaction and one for control.
* Value significantly different from the *Ae. aegypti* S-Lab strain ($P < 0.001$)

Table 4. Mortality rate and mean value of enzyme activities of adult *Aedes albopictus* mosquito populations collected across Singapore

Strains	Mean % mortality[1] ± SD		Esterase[3] (OD/min/mg protein)	Monooxygenase[3] (OD/ mg protein)	AChE[3] (OD/min/mg protein)
	Organophosphate	Pyrethroid			
	0.03% pirimiphos-methyl	1.23% cypermethrin	Mean ± SD	Mean ± SD	Mean ± SD
S-Lab[2]	–	–	0.012 ± 0.004	0.164 ± 0.033	0.005 ± 0.001
Central	73.50 (1991)	86.10 (579)	0.014 ± 0.004	0.235 ± 0.042*	0.007 ± 0.001
North East	66.30 (400)	40.00 (590)	0.018 ± 0.005*	0.285 ± 0.048*	0.008 ± 0.001*
North West	73.90 (399)	66.80 (593)	0.017 ± 0.004*	0.276 ± 0.090*	0.006 ± 0.002
South East	53.30 (1456)	94.30 (592)	0.013 ± 0.003	0.221 ± 0.050*	0.008 ± 0.001*
South West	49.10 (387)	74.50 (579)	0.016 ± 0.009	0.260 ± 0.038*	0.008 ± 0.001*

( ) Number of mosquitoes tested
[1] Mean % mortality at diagnostic concentration of each insecticide
[2] S-Lab is a long established susceptible laboratory strain tested
[3] Total of 40 mosquitoes were processed for each enzyme per strain. Two replicates for each enzyme reaction and one for control.
* Value significantly different from the *Ae. albopictus* S-Lab strain ($P < 0.001$)

DISCUSSION

This study clearly showed that *Ae. aegypti* adult populations across Singapore were highly resistant to cypermethrin with mortality of 11.0 – 75.6% and RR_{50} of 47.9 – 76.8 (data not shown). *Aedes aegypti* populations are still susceptible to pirimiphos-methyl but *Ae. albopictus* exhibited low level of resistance. Ranson *et al.* (2010) reported that resistance to organophosphate (temephos) and pyrethroids is widespread in *Ae. aegypti* in countries such as Thailand, India, Caribbean Island and Brazil, and has also been reported in *Ae. albopictus*. For examples, resistance to cypermethrin in adult *Ae. aegypti* has been reported in Indonesia (Astari and Ahmad, 2005) and Brazil (Lima *et al.*, 2011). The resistance of adult *Ae. albopictus* to cypermethrin was also reported in Pakistan (Khan *et al.*, 2011). Adult *Aedes albopictus* in Singapore have shown greater tolerance to pirimiphos-methyl as compared to that in the past 15 years. In this study, its RR_{50} of 2.1 (data not shown) was more than 0.5 times the RR_{50} of 1.4 in 1998 (Lai *et al.*, 2001). Whereas, the RR_{50} of 1.1 in some *Ae. aegypti* populations were less than the RR_{50} of 1.5 in 1998. This demonstrated that *Ae. aegypti* population in Singapore is still susceptible to pirimiphos-methyl. This could be due to the long term implementation of dengue control strategy that judiciously uses

pirimiphos-methyl for indoor misting at dengue cluster areas only. Moreover, the current application concentration (2.43%) used in the field is also higher than 0.03% of the diagnostic concentration of pirimiphos-methyl to *Ae. albopictus*. Therefore, pirimiphos-methyl is still an effective insecticide for vector control.

Besides knowing the resistance or susceptibility status, it is also important to understand the mechanisms responsible for insecticide resistance in *Ae. aegypti* and *Ae. albopictus*. The elevated MFO and EST observed in insecticide resistant strains may be linked to pyrethroid resistance (Verhaeghen *et al.*, 2009). However, synergism and kdr-type studies will have to be conducted in order to verify the role of elevated MFO and EST levels in pyrethroid resistance. *Aedes aegypti* population in the South East and South West regions showed small but significant enhancement of AChE activities, though the populations were still susceptible to pirimiphos methyl. This suggests that resistance might be developing.

The *Ae. albopictus* populations in all the five regions showed significantly high MFO activity. It was observed that *Ae. albopictus* in North East and North West regions that exhibited significant high level of MFO activity also had significant high level of EST. Verhaeghen *et al.* (2009) reported that MFO, in general, can mediate resistance to all classes of insecticides. This suggests that MFO could be the predominant enzyme responsible for pyrethroid resistance in *Ae. albopictus.* There was also significantly high level of AChE in pirimiphos-methyl resistant *Ae. albopictus* in North East, South East and South West, suggesting that AChE could play a role in organophosphate resistance in *Ae albopictus*.

This study showed that the metabolic mechanisms may be involved in pyrethroid and organophosphate resistance in *Ae. aegypti* and *Ae. albopictus* populations. However, metabolic mechanisms alone do not seem to explain fully the elevated resistance levels to the insecticide. Further studies with synergists are needed in order to provide additional information on metabolic mediated resistance mechanisms.

Acknowledgements. To Mr Png Ah Bah, Mdm Foo Siew Yoong and Ms Pang Sook Cheng for the identification of field mosquito samples from five regions, to Dr Chong Chee Seng, Dr David Lee and Ms Agnes Koou for their comments on the manuscript, to the members of staff in the Environmental Health Department for their continual support in surveys and collection of field mosquito samples in their regions, to Nanyang Polytechnic students (Ms Heng Wei Yun, Mr S. Kiri Shankar, Mr Li Kang Yang, Mr Aldred Goh and Mr Law Keng Koon) for their laboratory assistance, and to M/S Syngenta-Muenchwilen, Switzerland and M/S Asiatic Agricultural Industries Pte Ltd, Singapore, for their supply of technical grade pirimiphos-methyl and cypermethrin, respectively.

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