

Assessing the susceptibility status of *Aedes albopictus* on Penang Island using two different assays

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Abstract. Routine surveillance on resistant status of field mosquito populations is important to implement suitable strategies in order to prevent pest outbreaks. WHO test kit bioassay is the most frequent bioassay used to investigate the susceptibility status of field-collected mosquitoes, as it is relatively convenient to be carried out in the field. In contrast, the topical application of active ingredient is less popular in investigating the susceptibility status of mosquitoes. In this study, we assessed the susceptibility status of *Aedes albopictus* Skuse collected from two dengue hotspots on Penang Island: Sungai Dua and Persiaran Mayang Pasir. Two active ingredients: permethrin and deltamethrin, were used. WHO test kit bioassay showed that both wild strains collected were susceptible to the two active ingredients; while topical application assay showed that they were resistant. This indicated that WHO test kit bioassay less sensitive to low level of resistance compared to topical application assay. Hence, topical application is expected to be more indicative when used in a resistance surveillance programme.

INTRODUCTION

Aedes albopictus, also known as the Asian tiger mosquito, is an important vector of dengue (Boromisa *et al.*, 1987; Rigau-Pérez *et al.*, 1998; Gratz, 2004) and chikungunya (Gratz, 2004; Pialoux *et al.*, 2007; W.H.O., 2008). Although *Ae. albopictus* originated from Asia (Gratz, 2004), it has now widely spread to many parts of the world (Gratz, 2004; Benedict *et al.*, 2007). *Aedes albopictus* can be found close to human settlements, both indoor and outdoor (Chan *et al.*, 1971) in rural and urban areas (Rohani *et al.*, 2001). This greatly increases the chances of the mosquitoes coming in contact with humans, and hence, increases the chances of transmitting diseases.

The control of mosquito populations depends heavily on chemical applications. To date, major classes of insecticides used to control mosquitoes are pyrethroid, organophosphate, carbamate and DDT

(dichloro-diphenyl-trichloroethane) (Nauen, 2007). Over-dependence on chemical control has induced insecticide resistance (Hemingway, 2000; Rodriguez *et al.*, 2001; Casida & Quistad, 2004) and subsequently caused control failures. Resistant population of pests including mosquitoes has been reported in many corners of the world (I.C.M.R., 2002; Daaboub *et al.*, 2008; A.P.R.D., 2009a; 2009b; Loke *et al.*, 2010; Ranson *et al.*, 2010; Lima *et al.*, 2011).

A typical method used in measuring the resistance status of mosquitoes towards insecticides is by using the standard WHO diagnostic test kit assay for adulticides (Sathantriphop *et al.*, 2006; Duchon *et al.*, 2009; Ramphul *et al.*, 2009). The WHO diagnostic test kit utilises the usage of a single diagnostic dose to assess the susceptibility status of test samples. In this assay, both reference and wild strains are exposed to papers impregnated with the diagnostic dose of the insecticide for a fix

period of time in an exposure tube. Knockdown rate within that period and the 24 hour post-treatment mortality are recorded. Data from both reference and wild strains are compared to determine whether or not the wild population is resistant.

Another bioassay that could be employed to determine adult mosquito susceptibility status is via topical application (Duchon *et al.*, 2009; W.H.O., 2009b). This method has been used for many other pests but it is not popular for mosquitoes (Wang *et al.*, 2004; Kristensen *et al.*, 2005; Puinean *et al.*, 2010;). Topical application is basically a dose-mortality assay, where adult mosquitoes are exposed to different concentrations of insecticide applied on their prothorax. Twenty-four hours mortality are used to determine sample's susceptibility status and is expressed as resistant ratio (RR) (W.H.O., 2009b).

The WHO diagnostic test kit provides a fast and convenient way to determine the susceptibility of field mosquitoes. However, its sensitivity is rather questionable. This is due to the fact that dosage used is double that of the dose that would kill 99.9% of individuals in a population (W.H.O., 2009a). Field populations that exhibit low resistance towards particular insecticides might be classified as susceptible when using a dose that is twice that of the dose that kills most individuals of a normal population. The actual susceptibility status of a wild population might be misled by the diagnostic dose and this can sabotage the implementation of preventative measures to intercept upcoming mosquito outbreaks as well as disease outbreaks. On the other hand, topical application, although tedious, would be more sensitive as dosages that cause a fix percentage of mortality are compared. In this study, we aimed to compare the sensitivity of the WHO diagnostic test assay and topical application on field-collected *Ae. albopictus*.

MATERIALS AND METHODS

Mosquitoes

Susceptible reference strain (LAB) of *Ae. albopictus* was from the Vector Control

Research Unit, Universiti Sains Malaysia, and has been reared in the insectarium of the Unit for more than 30 years without exposure to insecticides. Field mosquitoes were collected using the ovitrap technique, the cheapest and easiest method for collecting *Aedes* mosquitoes (Yap, 1975a). The ovitraps were coloured black since the colour is more attractive to mosquitoes (Yap, 1975b). Ovitrap were set in two dengue hotspots on Penang Island: the JKR Quarters in Sungai Dua (SG2), adjacent to the USM main campus, and along Persiaran Mayang Pasir (PMP) a residential area in the township of Bayan Baru. Eggs and larvae collected were reared to adults up to the F1 generation. Larvae were fed food made from a fine mixture of dog biscuit (frieskies), beef liver and milk powder in the ratio of 2:1:1 by weight. Adults were fed with 10% sucrose solution while a white mouse restrained in a wire cage was used for blood-feeding purpose. The F1 generation of the wild strain generation was used in all assays and only non blood-fed 3-5 day old female mosquitoes were used in each assay.

Insecticides

The most commonly used insecticides to control adult mosquitoes in Malaysia are malathion and permethrin. From our conversation with staff of Penang Vector Borne Disease Control Department, insecticides used in the above-mentioned study sites were permethrin and deltamethrin. Hence, these two insecticides were tested. Technical grade deltamethrin 99.8% (Sigma-Aldrich) and permethrin 98.0% (Sigma-Aldrich) were used in all assays.

WHO Test Kit Assay

The technical grade active ingredients (ai) were diluted in acetone and silicone oil to the desired concentrations. At least 5 concentrations were used for each ai, ranging from 0.006%-0.020% for deltamethrin and 0.040%-0.200% for permethrin. Whatman No 01 filter papers were impregnated with 2 ml of the diluted ai and air-dried for 24 hours (W.H.O., 2009a).

Twenty five susceptible LAB strain females were exposed to a series of doses of impregnated paper in exposure tubes for an

hour. Four replicates were conducted with controls exposed only to silicone oil. Mosquitoes were kept in holding tubes for 24 hours at $27\pm 2^{\circ}\text{C}$ and $80\pm 10\%$ relative humidity, and provided with 10% sucrose solution on cotton wool. Mortality was recorded 24 hours post-treatment and analysed. The LD 99 value obtained was doubled to obtain the diagnostic dose, which was 0.2% for deltamethrin and 0.7% for permethrin. The diagnostic dose-impregnated papers were prepared and the LAB, SG2 and PMP strains were exposed to them. The knockdown rate and 24 hours post-treatment mortality were recorded (W.H.O., 2009a; W.H.O., 2009c).

Topical Application

A series of concentration of permethrin and deltamethrin in acetone were prepared. Fifty female mosquitoes were weighed prior to the assay. The mosquitoes were anaesthetised using carbon dioxide for 10 seconds, and placed on ice covered with aluminium foil in place of a cold plate. This kept them immobile. An automated micropipette (Eppendorf) was used to deposit 0.1 ul of ai onto the pronotum of the mosquitoes. Controls were applied with 0.1 ul acetone instead (W.H.O., 2009b). The mosquitoes were kept in holding cups at $27\pm 2^{\circ}\text{C}$ and $80\pm 10\%$ relative humidity and provided with 10% sucrose solution on cotton wool. Mortality was recorded 24 hours post treatment. Four replicates were conducted for each concentration and control, and each replicate consisted of 25 mosquitoes unit.

Analysis

Controls with mortality between 5-20% were corrected using Abbott's formula while those which exceed 20% were discarded. Both mortality and knockdown rates were analysed by regression-probit using SPSS v 16.0 to obtain the LD values (for determination of diagnostic dose and for topical application) and KT values (knockdown rate by diagnostic dose).

RESULTS

The diagnostic dose of 0.2% deltamethrin and 0.7% permethrin were investigated against *Ae. albopictus*, and used in subsequent WHO test kit assay. Diagnostic doses for *Ae. aegypti* were not used because in preliminary studies (data not published), we found that they did not confer full mortality against the mosquito.

Bioassay using the WHO test kit with diagnostic doses obtained from our experiment, showed both the SG2 and PMP strains are susceptible to permethrin and deltamethrin (Tables 1 and 2). The SG2 strain showed 99% mortality both to permethrin and deltamethrin while PMP strain showed 100% mortality. The knockdown rates of both field strains were not significantly different from that of the susceptible strain, and the highest resistance ratio based on the knockdown rate was 1.34. No knockdown was observed in control for WHO test kit bioassay, and control mortality was less than 5%. On the other hand, bioassay using the topical application showed that the field-collected mosquitoes had low resistance towards both active ingredients (Tables 3 and 4). The PMP strain *Ae. albopictus* showed a higher resistance than the SG2 strain. The resistance ratio obtained using topical application ranged from 3.69 to 8.99. Control mortality for all tests was less than 5% and hence, not corrected with Abbott's formula. According to both bioassays, deltamethrin was shown to be a more lethal insecticide than permethrin, by having a higher knockdown and mortality with low doses compared with permethrin (Table 1-4).

DISCUSSION

Samples were collected from Sungai Dua and Persiaran Mayang Pasir, which were dengue hotspots on Penang island (pers comm). The number of *Ae. aegypti* collected was minimal. The susceptibility status of

Table 1. Susceptibility status of *Ae. albopictus* towards diagnostic dose of deltamethrin

Strain	KT50 (minutes) (95% C.L.)	KT95 (minutes) (95% C.L.)	Mortality (%)	RR50	RR95	Slope \pm SE	X^2 (d.f.)	P value
LAB	7.63 (7.29-7.98)	12.68 (11.74-14.02)	100	-	-	7.458 \pm 0.581	2.733 (8)	0.950
SG2	9.08 (8.72-9.46)	13.61 (12.65-15.01)	99	1.19	1.07	9.357 \pm 0.809	8.586 (8)	0.378
PMP	9.17 (8.34-10.11)	17.03 (14.99-20.07)	100	1.20	1.34	9.366 \pm 0.808	8.464 (8)	0.390

Table 2. Susceptibility status of *Ae. albopictus* towards diagnostic dose of permethrin

Strain	KT50 (minutes) (95% C.L.)	KT95 (minutes) (95% C.L.)	Mortality (%)	RR50	RR95	Slope \pm SE	X^2 (d.f.)	P value
LAB	22.69 (21.25-24.18)	38.62 (35.45-42.93)	100	-	-	7.118 \pm 0.130	2.345 (8)	0.965
SG2	22.19 (20.78-23.66)	38.21 (35.05-42.48)	99	0.98	1.00	6.970 \pm 0.490	9.720 (8)	0.285
PMP	21.92 (20.56-23.36)	39.21 (36.39-42.55)	100	0.97	1.02	6.246 \pm 0.420	5.443 (8)	0.709

Table 3. Susceptibility status of *Ae. albopictus* towards deltamethrin by topical application

Strain	LD50 (ng/mg) (95% C.L.)	LD95 (ng/mg) (95% C.L.)	RR50	RR95	Slope \pm SE	X^2 (d.f.)	P value
LAB	0.00082 (0.0063-0.0010)	0.00497 (0.0342-0.0869)	-	-	2.126 \pm 0.203	1.182 (3)	0.757
SG2	0.00302 (0.0238-0.0379)	0.01832 (0.1231-0.3361)	3.68	3.69	2.078 \pm 0.178	19.187 (4)	0.010
PMP	0.007374 (0.05616-0.0984)	0.03425 (0.2197-0.7121)	8.99	6.89	4.784 \pm 0.372	16.071 (4)	0.003

Table 4. Susceptibility status of *Ae. albopictus* towards permethrin by topical application

Strain	LD50 (ng/mg) (95% C.L.)	LD95 (ng/mg) (95% C.L.)	RR50	RR95	Slope \pm SE	X^2 (d.f.)	P value
LAB	0.02750 (0.2070-0.3500)	0.0907 (0.6537-1.6126)	-	-	4.384 \pm 0.395	0.626 (3)	0.890
SG2	0.13880 (1.0847-1.7978)	0.45800 (3.1498-9.0101)	5.05	5.05	2.783 \pm 0.211	22.424 (4)	0.000
PMP	0.21204 (1.7245-2.3529)	0.72339 (5.4898-10.7029)	7.71	7.98	2.904 \pm 0.270	4.384 (3)	0.223

Ae. albopictus from these two areas were investigated, where bioassay using the topical application indicated the presence of resistance whereas bioassay using the WHO test kit did not show any resistance. Important factors in the control and prevention of mosquito-borne diseases include reducing the vector abundance to decrease the chances of infectious bites, and this is achieved by using insecticides (Gratz, 2004; Eisen *et al.*, 2009). Failure of insecticides to control mosquito population due to resistance can contribute to a higher disease transmission rate (Eisen *et al.*, 2009), and this was the factor that jeopardised malaria control in Africa (Hemingway & Ranson, 2000; Enayati & Hemingway, 2010).

Although many works have been done to assess the susceptibility status of mosquitoes, exact threshold of resistance level that would cause control failure and disease outbreak has not been established. Even if indicated as resistant in susceptibility test, it does not necessarily mean field control has fail. Based on several works, Gratz (2004) reviewed that in areas where *Ae. aegypti* is absent or minimal, *Ae. albopictus* serves as the primary vector of dengue fever. Although we did not attempt to determine the presence of dengue virus from the mosquitoes collected, *Ae. albopictus* may be the more probable vector because *Ae. aegypti* collection was minimal. Hence, the dengue outbreak in the two study sites may be due to the failure to control *Ae. albopictus* because of resistance.

The WHO test kit is the standard and popular tool for assessing the susceptibility status of mosquitoes in the field (Sathantriphop *et al.*, 2006; Duchon *et al.*, 2009; Ramphul *et al.*, 2009). Compared to topical application, it is convenient and easy to handle, fast and doesn't require special equipment such as a chill plate and carbon dioxide tank which might not be appropriate for use in the field. Topical application requires a large number of sample which field collection are often not able to provide unless the samples are reared and their progenies used for the bioassay. This requires time and rapid diagnosis of susceptibility status is hence not possible. Handling mosquitoes for

topical application also requires extra care because they are very fragile even when using the finest forceps.

However, with the WHO test kit the amount of active ingredient the mosquitoes are in contact with in the exposure tube is not constant. From our observation, when held in the exposure tubes, most of the mosquitoes tend to rest on the netting area of the tube, possibly due to the irritancy nature of the ai (Chareonviriyaphap *et al.*, 2006; Diabate *et al.*, 2006; Mongkalagoon *et al.*, 2009). Hence, some will get in contact with the insecticides more than others, affecting the outcome of the bioassay. On the other hand, topical application ensures mosquitoes are exposed to a constant amount of ai. The dose used in WHO test kit bioassay is two times the dose that will kill 99.9% of a normal population (W.H.O., 2009a). Therefore it is effective against mosquitoes with low level of resistance, thus categorising them as susceptible. This would seriously affect the sensitivity of the assay because high resistance level is required to be detected by this method (Coleman & Hemingway, 2007).

Based on our results, we conclude that topical application is a more sensitive and more indicative bioassay than the WHO test kit. It should be used in routine insecticide resistance surveillance to predict possible outbreak so that a rapid control response can be employed.

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