Pyrethroid Resistance Status of *Aedes (Stegomyia) Aegypti (Linneaus)* from Dengue Endemic Areas in Peninsular Malaysia

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ABSTRACT

Vector control is still the principal method to control dengue and chemical insecticides, especially the pyrethroids such as permethrin are the forerunners of mosquito control agent. Intensive and extensive use of pyrethroids often result in resistance, thereby hampering control efforts. The present study was conducted to evaluate the susceptible status of *Aedes aegypti*, the primary vector of dengue against permethrin. A nationwide mosquito sampling via ovitrapping was conducted in 12 dengue hotspots across 5 states in Peninsular Malaysia. Field collected *Aedes* eggs were hatched and reared until L3 larval and further identified it species. Adult F0 *Aedes aegypti* were reared until F1 progeny and the female were used in adult assay, performed according to World Health Organization (WHO) protocol as to determine the resistance level. The laboratory strain maintained for more than 1000 generations that were susceptible to permethrin served as the control strain. Evaluation of resistance ratio was assessed by comparing the knockdown rate with laboratory susceptible strain. In this present study, 70% of*Ae. aegypti* population from dengue hotspots was highly resistance to permethrin. The study clearly demonstrated that widespread of permethrin resistant *Ae. aegypti* in Malaysian mosquito's population, indicating the need of implementing an efficient pyrethroid resistance management.

KEYWORDS: *Aedes aegypti*, resistance, permethrin, insecticides

INTRODUCTION

Aedes aegypti is the important arthropod vector which is capable of transmitting disease such as dengue, chikungunya and yellow fever. The burden of mosquitoe-borne disease has affected millions of people globally[1]. In Malaysia, dengue has become an important killer disease, recorded with continuously increasing of dengue incidence rate (IR) every year, as of 2015, we have recorded the highest IR ever with 396 cases in 100,000 population[2]. Despite of many ongoing efforts to control dengue, controlling the mosquitoes is facing tremendous challenges; moreover the absence of a highly effective tetravalent dengue vaccine and specific antiviral treatment have limited the preventive measure for dengue control which is more likely to depends on the use of insecticide.

Ae.aegypti prefer to breed in man-made containers[3]. Uncontrolled and abundance of man-made containers in urban environment has promoted the breeding and enhanced the disease transmission. Usage of chemical insecticides for larviciding and adulticiding are the two major activities for mosquito control programmes. The chemical insecticide classified under the class of organophosphate namely known as temephos is the common larvicide used to control larval stage; while pyrethroids group are usedto control adult mosquitoes by indoor residual spraying (IRS), outdoor residual spraying (ORS), thermalfogging and in Ultra-Low Volume (ULV) application[4]. In Malaysia, this photostable compound has replaced the usage of dichlorodiphenyltrichloroethane (DDT) since 1999. Retracking the usage of pyrethroids in Malaysia, bioresmethrin has been used as the earliest pyrethroid to encounter the first nationwide dengue outbreak in 1973. They were then re-introduced in dengue control program as adulticide which replaced malathion because of it odourless characteristic, effective knockdown and low toxicity to human[5]. Up to date, there are varieties of pyrethroids available in the market and they are widely used in most countries in dengue operational spray and agriculture[6].

It is very unfortunate if mosquitoes develop resistance towards pyrethroids because this event leads to the failure of vector control strategy. Currently, the issue on pyrethroids resistance has been much debated. Many studies reported that pyrethroid resistance were already widespread in *Ae. Aegypti*e specially in most of the Asean countries

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including Malaysia.[7,8,9,10,11,12,13] The development of pyrethroids resistance were believed due to the intensive and indiscriminate use of the insecticides. Those that survived from selection pressure were further sustained and build up in their next generation that is more tolerant to the insecticides.[14,15] Two modes of resistance that are significantly contribute for the development of pyrethroids resistance involves elevation of metabolic detoxification enzyme and genetic mutation in insecticidal target site receptor or also defined as knockdown resistance (kdr).

Previous studies on the pyrethroids status in *Aedes* mosquitoes caught in certain areas of dengue localities in Malaysia were found to develop resistance to pyrethroids.[11,13,16] Lack of information about the resistance status of dengue primary vector especially in the dengue hotspot area has prompted us to conduct this study by expanding mosquito sampling. The evidence-based data of permethrin susceptibility status in *Aedes aegypti* from this current study could be utilized to minimize the spread of pyrethroids resistance which could threaten the effectiveness of dengue control strategy. Besides, such information is important for the implementation of insecticide resistance management for the dengue control program.

MATERIALS AND METHODS

Study sites

Ae. aegypti eggs were collected from dengue endemic localities across 5 states in Peninsular Malaysia from September 2015 until May 2016 (Table 1).

Table 1. Study site coordinates

LOCALITIES	Coordinates
SELANGOR	
Seksyen 5, Bandar Rinching (S5, BR)	2°55' 45.1'' N 101° 51'40.0''E
Taman Sungai Jelok, Kajang (TSJ)	2°59'34.9''N 101°48'06''E
Ridzuan Condominium, Petaling Jaya (RC)	3°04'45.0''N101° 36'20.2''E
S15, Bandar Baru Bangi (S15,BBB)	2°55'44.1''N 101°46'01.2''E
Pangsapuri Sri Meranti, Cheras (PSM)	3°11' 28.3'' N 101° 36'00.8''E
MELAKA	
Taman Tasik Utama (TTU)	2°16' 37.5'' N 102° 17'03.6''E
Pangsapuri Taman Tasik Utama (PTTU)	2°16' 04.2'' N 102° 16'48.3''E
Kampung Sungai Putat (KSP)	2°15' 01.56'' N 102° 15'51.51''E
KEDAH	
Flat Paya Nahu (PN)	5°38'45.6''N 100°31'02.1''E
KUALA PERLIS	
Taman Seri Bayu (TSB)	6°24'03.1'N 100°08'22.7''E
JOHOR	
Taman Dahlia (TD)	1°30'40.7''N 103°41'42.3''E
Flat Camar (FC)	1°30'05.3''N 103°40'46.5''E

Mosquito sampling

F1 progeny of *Ae. aegypti* colonies from fields collected mosquitoes were used for bioassay test. The laboratory strain was use as the susceptibility strain. This strain was originated from Selangor generations; free from exposure to any insecticide. It has been maintained for more than F1000 in the insectarium of the Institute for Medical Research (IMR), Kuala Lumpur, Malaysia. The field and laboratory strains were reared in different rooms under a controlled condition at 29°C ± 2°C and 68±5% relative humidity.

Placement and collection of field strain

The standard ovitrapping method was employed according to Lee[17]. A total of 30 ovitraps were placed indoor and outdoor, respectively, in 15 pre-selected houses. Ovitraps were collected after five days of ovitrapping period. All household members were notified with the purpose of placing ovitrap as well as the time and date to be collected in order to maximize the number of ovitraps collected. These ovitraps were brought back to the laboratory and the contents inside each ovitrap were poured into individual plastic containers which were topped up with fresh water. The containers were kept covered to prevent other mosquitoes from depositing inside the containers. All hatched larvae at the third instar stage were identified and pooled into a container based on locality collected, then were bred into the adult stage as zero filial generation (F0).

Colonization of *Ae. aegypti*

F0 *Ae aegypti* was reared in cages and blood-fed at day four so as to obtain eggs. These eggs were hatched and reared until they reached the adult stage of F1 generation. Repeated blood-feeding was performed to obtain another batch of F1 eggs in order to increase the number of the mosquitoes. All *Ae. aegypti* were supplied ad libitum with 10% sucrose solution.

Insecticide

Permethrin impregnated papers at the WHO designated diagnostic concentration of 0.75% were bought from the WHO center VSCRU, USM. Vector Control Research Unit, Universiti Sains Malaysia, Penang. This Unit was designated by the WHO Pesticide Evaluation Scheme (WHOPES) to produce the standard WHO Insecticide Test Kits; inclusive of the insecticide impregnated papers.

WHO adult bioassay

Bioassay of adult mosquitoes was performed by employing the standard WHO susceptibility test.[20] A total of 150 sugar-fed adult female mosquitoes aged five days old were used. Four replicates consisted of 15 adult mosquitoes per replicate were exposed to the permethrin impregnated paper at the 0.75% diagnostic dosage. Other four replicates of the same number of adult female mosquitoes were exposed to the paper impregnated with 1ml of ethanol which served asblank control. The respective exposure period was one hour as recommended by the WHO.

The cumulative knockdown counts were recorded every one minute within the exposure period, or until 90% knockdown was observed. After the exposure period, all the tested mosquitoes (live and knocked down) were transferred into clean paper cups and supplied with 10% sugar solution in cotton balls. After a 24-h recovery period, the adult mortality was recorded.

Analysis of adult 24-h bioassay

The mean percentage of adult mortality was determined. If the control mortality fell between 5% and 20%, the percentage mortality was corrected by using the Abbot's formula[18]. No correction was required if the control mortality was <5% and if the control mortality was >20%, the experiment was repeated. To determine resistance, the following criteria were used: 98-100% mortality –susceptible, 90-97% mortality – incipient resistance that needs confirmation, <90% - resistant[19].

Analysis of knockdown resistance ratio

The cumulative knockdown data were subjected to probit analysis[19] by using IBM SPPS Statistics version 19.0 (IBM Corporation, U.S) to obtain 50% knockdown time. The resistance ratio was determined by comparing with the LT_{50} of field strain with susceptible laboratory strain.

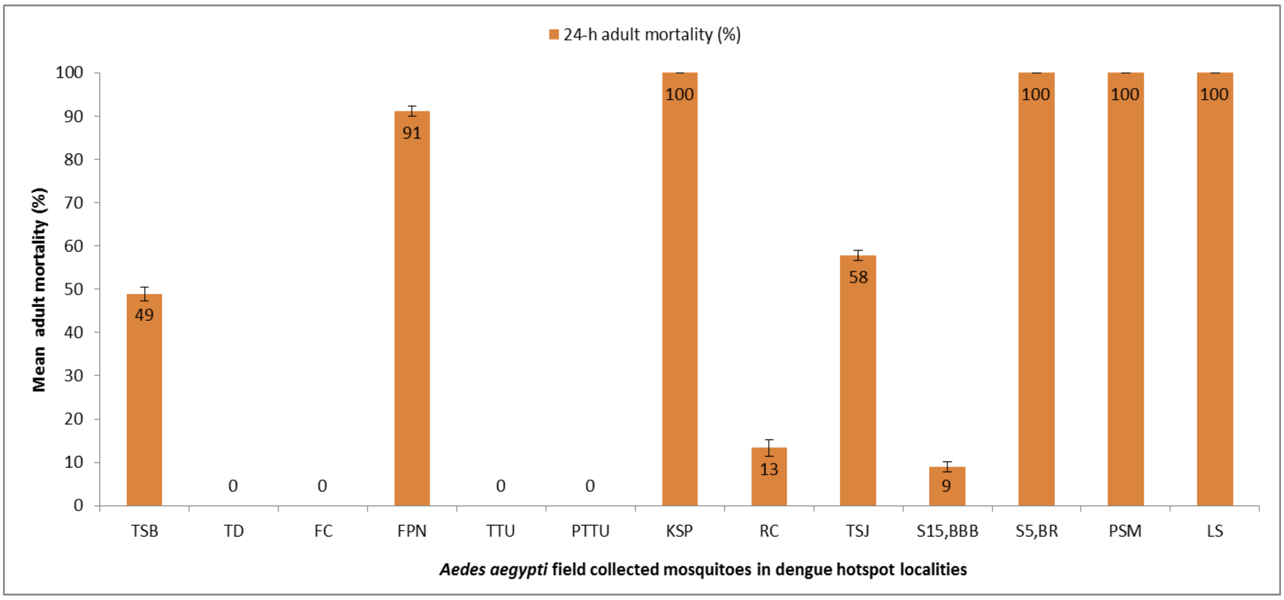

Fig. 1. Comparative 24-h adult mortality of *Aedes aegypti*, F1 generation to permethrin at 0.75% diagnostic concentration. (*TSB, Taman Seri Bayu; TD, Taman Dahlia; FC, Flat Camar; FPN, Flat Paya Nahu; TTU, Taman Tasik Utama; PTTU, Pangsapuri Taman Tasik Utama; KSP, Kampung Sungai Putat; RC, Ridzuan Condominium; TSJ, Taman Sungai Jelok; S15, BBB, S15, Bandar Baru Bangi; S5, Bandar Rinching; PSM, Pangsapuri Seri Meranti; LS, Laboratory strain)*

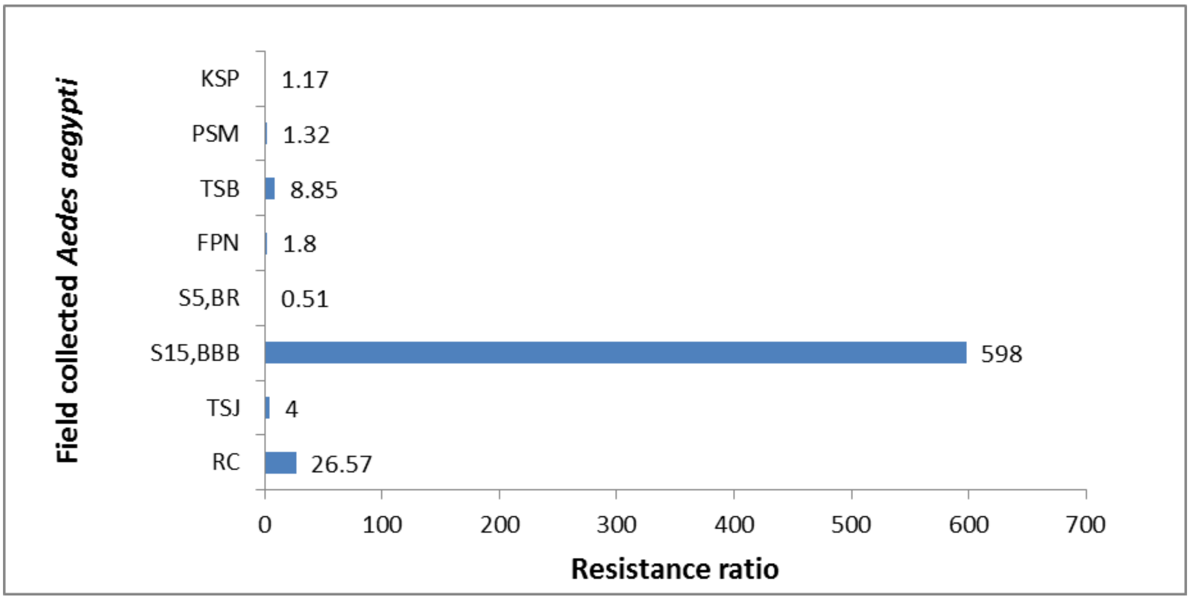

Fig. 2. Knockdown resistance ratio of field collected *Aedes aegypti* within an hour exposure to permethrin. (KSP, Kampung Sungai Putat; PSM, Pangsapuri Seri Meranti; TSB, Taman Seri Bayu; FPN, Flat Paya Nahu; S5, Bandar Rinching; S15, BBB, S15, Bandar Baru Bangi; TSJ, Taman Sungai Jelok; RC, Ridzuan Condominium).

Table 2 Knockdown time 50 (KD_{50}), confidence interval and regression analysis of field collected *Ae. Aegypti* to 0.75% permethrin at one hour exposure period.

States	Collection sites	KD_{50} (min)	95% CI (min - max)	Regression line
Selangor	**Ridzuan Condominium (RC)**	293.26	114.77 - 12446.39	Y= 1.16X-5.25
	Taman Sungai Jelok (TSJ)	44.56	40.37 - 50.20	Y= 2.34X-11.72
	S15, Bandar Baru Bangi (S15,BBB)	6613.06	N.A.	Y= 0.6X-6.93
	S5,Bandar Rinching (S5,BR)	5.7	4.86 - 6.50	Y= 3.21X - 9.49
	Pangsapuri Seri Meranti (PSM)	14.72	13.46 - 14.98	Y=9.03X-10.88
Melaka	**Taman Tasik Utama (TTU)**	NA	NA	NA
	Pangsapuri Taman Tasik Utama (PTTU)	NA	NA	NA
	Kampung Sungai Putat (KSP)	13.15	Y=8.38X - 9.37	12.34 - 13.85
Kedah	**Flat Paya Nahu (FPN)**	20.28	18.75 - 21.72	Y=4.96X- 15.22
Kuala Perlis	**Taman Seri Bayu (TSB)**	98.66	78.65 - 145.61	Y=2.54X-8.64
Johor	**Taman Dahlia (TD)**	NA	NA	NA
	Flat Camar (FC)	NA	NA	NA
Susceptible strain		11.15	10.17 - 12.10	Y= 4.21X-13.10

NA. Absence of knockdown was observed in Ae. aegypti collected in Johor (TD and FC) and Melaka (TTU and PTTU), indicating presence of high permethrin resistance.

Adult Susceptible Status

In our study, we have employed diagnostic concentration of permethrin based on WHO adult assay guideline for *Anopheline* which was 0.75%.[20] This concentration was much more higher than the recommended dose for *Aedes* mosquitoes by WHO that released early this year which was only 0.25%.[24]Having said that, adult *Ae. Aegypti* collected from 9 out of 12 localities were resistant to permethrin. *Ae. aegypti* that recorded with a complete adult mortality were from Seksyen 5, Bandar Rinching (S5, BR), Pangsapuri Sri Meranti (PSM) from Selangor state, and Kampung Sungai Putat (KSP) from Melaka state (Fig.1.)

It was clearly shows that *Ae. aegypti* from Taman Tasik Utama (TTU) and Pangsapuri Taman Tasik (PTTU) from Melaka state;and Taman Dahlia (TD) and Flat Camar (FC) from Johor state were highly resistance to permethrin, as complete absence of knockdown and 24 h adult mortality were recorded. Absence of knockdown in mosquitoes samples from dengue hotspot areas (TTU, PTTU, TD and FC) within the exposure time indicates a possible of cross resistance against the permethrin (Fig.2.).

Adult survivability for *Ae. aegypti* from Seksyen 15, Bandar Baru Bangi (S15, BBB); Ridzuan Condominium(RC); Taman Sungai Jelok (TSJ), Taman Seri Bayu (TSB) and Flat Paya Nahu (PN) ranged from 9% to 91% at 24 h post exposure indicated development of permethrin resistant strains (Fig. 1.).

DISCUSSION

Dengue fever and severe dengue are serious mosquito-borne diseases in sub-tropic and tropical countries[1]. The main option for dengue control relies on the usage of chemical insecticide to control and/or prevent the disease transmission. However, the threat on the effectiveness of mosquito control is the development of insecticide resistance. Thus, in this study, we conducted a comprehensive mosquito sampling across 5 states in Peninsular Malaysia. Evaluation of the resistance levels are focused on the adult assays, while elucidation of resistance profiles will be studied further.

Based on adult bioassay, the degree of knockdown resistance was corresponded with the level of resistance. For instance, *Ae. aegypti* from S15, BBB exhibited high mortality rate to permethrin also showed high knockdown rate which was almost reached 600 folds when compared with laboratory strain (Fig. 2.& Table 1). Based on these findings, it is worth noted that Malaysian *Ae. aegypti* found to be resistance to permethrin were highly localized and confined to particular areas and not affecting the insecticidal efficacy in other areas reportedly with the absence of pyrethroid resistance. Here, we suggest a large scale and consistence insecticide resistance monitoring in dengue hotspot areas in order to prevent the spread of pyrethroid resistance.

Studies pertaining to the pyrethroids resistance have been conducted all over the world as most of the dengue endemic countries nowadays widely use pyrethroids in their vector control program. Review on the pyrethroids resistance showed that mosquito resistance to pyrethroids was already widespread[7]. However, there was a paucity of information on the resistance status of Malaysian *Ae. aegypti* especially from dengue endemic localities available in the literature, as most of the resistance studies are focusing in *Ae. albopictus* and *Culex quinquefasciatus*.[21,22] The permethrin resistant of adult *Ae. aegypti* mosquitoes from dengue endemic areas were previously reported in Seksyen 7, Shah alam (Selangor)[11];Taman Melati, Vista Angkasa and Desa Tasik (Kuala Lumpur)[13]; Bukit Jambul and Permatang Damar Laut (Penang); Taman Desa Tasik and Sungai Besi (Kuala Lumpur); Bandar Baru Kubang Kerian and Jalan Telipo (Kota Bahru, Kelantan); Taman Century and Taman Gembira (Johor Bahru).[12]

The are many suggested alternatives to encounter pyrethroidresistance. The most often application strategies for prevention of pyrethroids resistance are rotational replacement of other insecticides with different mode of actions and/or alternative non-insecticidal application such as *Bacillus thuringiensis* H-14 (*Bti*).[23] As noted, pyrethroids resistance involved two general mechanisms; there are increased of mixed-function oxidases (MFO) activities and mutation in pyrethroid-sodium channel receptor. At present, synergist piperonylbutoxide (PBO) has been proven to overcome metabolic oxidases detoxification by inhibiting the oxidases in mosquitoes.[13]Observation of the highly resistant *Ae. aegypti* mosquitoes due to MFO that were colonized in the laboratory until F4 generation indicated reversion of MFO activities (IMR unpublished data). This suggest that metabolic enzyme could be reversed if selection pressure to the respective insecticides are removed.

CONCLUSION

Permethrin is one of the insecticide categorized under the class of pyrethroids. The evaluation of permethrin in this present study provided a current evidence-based report of permethrin resistance status in Malaysian *Ae. aegypti* collected from dengue hotspots across three states in the Peninsular Malaysia. This comprehensive monitoring demonstrated a widespread of permethrin resistant in *Aedes aegypti* mosquitoes.The existence of pyrethroid resistance in primary dengue vector is an alarming issue and needed to be countered. A good insecticide resistance management will be the vital factor in preventing and countering the spread of insecticide resistance. Establishing a centralized and good data management of resistance status of the dengue vector are crucially indeed for a successful resistance management program.

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