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Status of pyrethroid resistance in *Aedes (Stegomyia) aegypti* (Linneaus) from dengue hotspots in Klang Valley, Malaysia

Siti-Futri, F.F.[1,2*], Rosilawati, R.[1], Wan, K.L.[2], Cheong, Y.L.[1], Nazni, W.A.[1] and Lee, H.L.[1]
[1]Medical Entomology Unit, and WHO Collaborating Centre for Vectors, Institute for Medical Research, Malaysia
[2]Centre for Biotechnology and Functional Food, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, Malaysia
[*]Corresponding author e-mail: rosilawati@imr.gov.my
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Abstract. The continued absence of an effective and safe tetravalent dengue vaccine and the lack of specific anti-viral treatment have made mosquito vector control using chemical insecticides as the mainstream for dengue prevention and control. However, the long-term use of chemical insecticides may induce resistance. Hence detection of insecticide resistance in dengue vectors is crucially important in ensuring the insecticide-based intervention in dengue control program is still effective and reliable. In this study, the susceptibility status of *Aedes aegypti* from five selected dengue hotspots in Klang Valley, Malaysia against pyrethroids was determined by employing the World Health Organization (WHO) protocol of adult bioassay. Four types of pyrethroids were tested against adult female *Aedes aegypti* to determine the knockdown rate, post 24-h adult mortality and resistance ratio. All field-collected *Aedes aegypti* strains were resistant to the four pyrethroids tested, except for the Taman Sungai Jelok (TSJ) strain. Permethrin exhibited the lowest knockdown rate against *Aedes aegypti*, followed by deltamethrin, cyfluthrin and lambda-cyhalothrin. This present study indicated the widespread of pyrethroid resistance in *Aedes aegypti* in Klang Valley, indicating the needs of implementing alternative measures in vector control program. The data in this study can be utilised as an input for insecticide resistance management of *Aedes aegypti* in Malaysia.

INTRODUCTION

Mosquitoes are notable blood sucking insect that cause many human diseases globally (WHO, 2011; Smith *et al*., 2016), and among the Culicidae, *Aedes aegypti* is recognised as the deadliest species that act as a vector of many serious diseases, including dengue, Zika and Chikungunya. This indoor breeding mosquito feeds at day time, and is anthropophilic. They are predominantly found in subtropical and tropical countries, dominating urban and suburban areas. They inhabit domestic man-made containers and breed in abundance in indoor than outdoor environment (García-Rejón *et al*., 2011). Equatorial climate with hot, rainy and humid environment has promoted mosquito growth, hence making this species to be easily found in country like Malaysia.

Intensive and indiscriminate use of insecticides on a large scale and continuation of using the same class of insecticides to control mosquito represent the major causative reasons for the development of insecticide resistance (WHO, 1957; Hemingway *et al*., 2004; Ranson *et al*., 2010). For these reasons, insecticides exert an intense selection pressure to mosquito populations and subsequently support the sustainability of their next generation that is fit enough to be well tolerated against contacted insecticide, and might result in zero-insecticidal effects (Brown, 1986).

At present, an effective and safe tetravalent dengue vaccine and specific anti-viral treatment of dengue are still unavailable, thereby making vector control intervention using chemical insecticides as an important frontline tool for dengue prevention (WHO, 2011). Unfortunately, the success of this intervention may be jeopardised if mosquitoes develop resistance to insecticides.

At present, Malaysia has a persistent number of dengue cases annually, resulting in increasing number of dengue hotspots especially in the Selangor state, with large amounts of insecticides being used to control dengue vectors (Nazni *et al.*, 2005; Wan-Norafikah *et al.*, 2012). Historically, malathion was used to control dengue vectors from 1974 onwards until 1996. Because of its disadvantages such as oily residue on the sprayed surface and strong odour that may be aversive to human smell, this organophosphate insecticide was then replaced by pyrethroids (Teng & Singh, 2001).

Despite the availability of many types of public health insecticides, pyrethroids are still the most widely used insecticides particularly in dengue endemic countries, including Malaysia. This is because pyrethroids tend to be more potent, last longer in the environment and less toxic to mammalian. However, lately, several studies have demonstrated that *Aedes aegypti* in certain areas has showed reduced susceptibility to pyrethroids (Wan-Norafikah *et al.*, 2012; Ishak *et al.*, 2015; Rosilawati *et al.*, 2017).

In view of the possible widespread pyrethroid resistance across Malaysia, a proactive resistance monitoring program evaluating the susceptibility status of the *Aedes aegypti* vector was initiated. Here, we have selected five dengue hotspots in Klang Valley and determined the susceptibility of *Aedes aegypti* to four different pyrethroids that have been widely used in dengue control in Malaysia. The findings in this study would be valuable in determining the pyrethroid resistance status of *Aedes aegypti* particularly in dengue hotspot settings, and for the purpose of resistance management to ensure the continued effectiveness of dengue control using insecticides.

MATERIALS AND METHODS

Study area

Mosquitoes were sampled by ovitrapping in five selected dengue hotspots in Klang Valley; Seksyen 3 (S3), Seksyen 8 (S8), Taman Sungai Jelok (TSK), Ridzuan Condominium (RC) and Taman Sri Kuching (TSK) (Figure 1). Klang Valley is a region centered in Kuala Lumpur and adjoining cities five districts in Selangor state. It consists of separate cities and suburbans area, which were integrated by a highly developed road network and expanding public transit system. The selection of localities were based on the number of dengue cases reported by the Ministry of Health (MOH) in 2017. According to the MOH's guidelines, dengue hotspot is defined as a locality that is reported to have dengue outbreak continuously up to 32 days. Whenever cases are reported, vector control via space spray techniques will be conducted within seven days of the reported dengue cases, and the second application will follows after seven to ten days (Tham, 1993). The type of building, environmental conditions and surrounding vegetation were noted in each locality (Table 1).

Mosquito field collection and colonisation

The ovitrapping method employed in the present study was described by Lee (1992). Designed to trap *Aedes* mosquitoes, ovitrap is a black coloured cylindrical container filled with water that acts as an ideal location for female *Aedes* mosquitoes to lay eggs. A total of 30 ovitraps were used for one site and placed for 5 days. For localities with landed houses, ovitrapping were conducted involving 15 randomly selected houses, where one ovitrap was placed indoor and outdoor, respectively per house. While for localities with high rise buildings, 30 ovitraps were placed randomly in the buildings. Placement of ovitraps for 5 days duration of in human dwelling has no adverse effect to human and this study does not involve

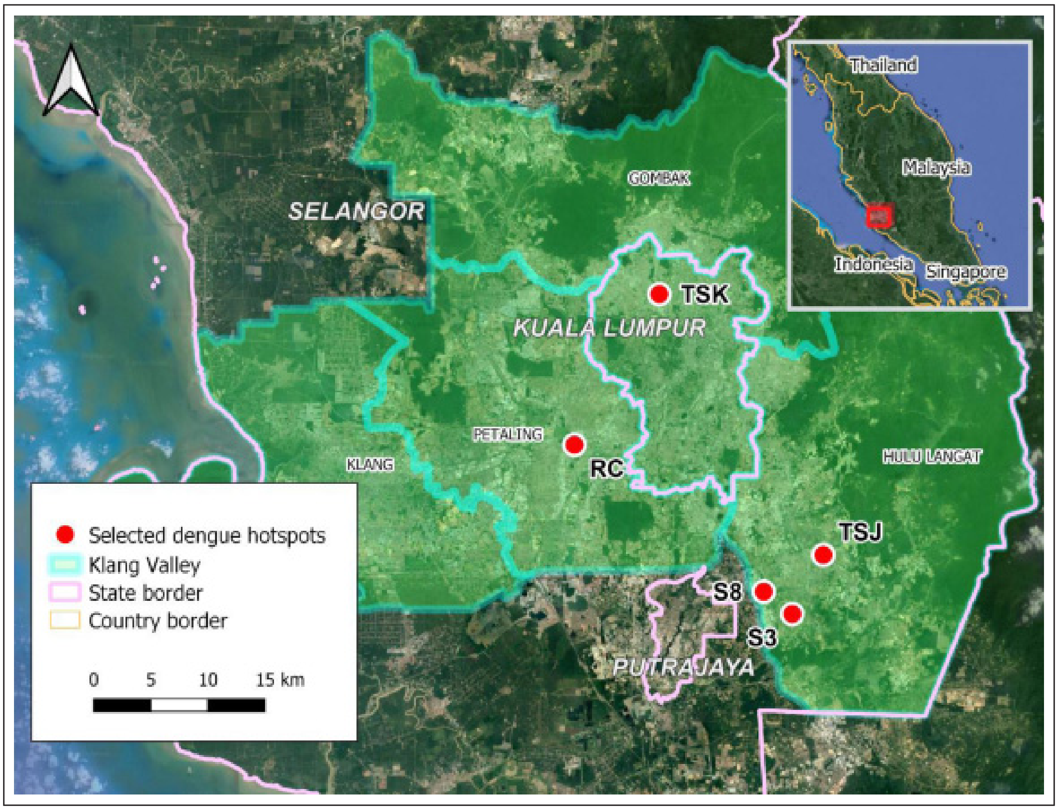

Figure 1. Locations of the five selected dengue hotspots in Klang Valley.

Table 1. Locality and sampling conducted in each locality

Locality Code	Locality	Sampling month	GPS coordinates	Type of building	Environmental condition and surrounding vegetation
S3	Seksyen 3, Bandar Baru Bangi	March 2017	N 2° 56' 21.076", E 101° 45' 54.045"	Landed	Located 4 km from Bandar Baru Bangi. Near to a lake and a public playground. Moderate vegetation.
S8	Seksyen 8, Bandar Baru Bangi	May 2017	N 2° 57' 50.148", E 101° 45' 17.964"	Landed	Located in Bandar Baru Bangi. Crowded with business centres and housing areas. Moderate vegetation.
TSJ	Taman Sungai Jelok, Kajang	July 2017	N 2° 59' 34.044", E 101° 48' 6.731"	Landed	Located in Kajang. Suburban area surrounded with dense vegetation.
RC	Ridzuan Condominium, Petaling Jaya	September 2017	N 3° 4' 42.888", E 101° 36' 22.391"	High rise	Located in Sunway City. Urban area surrounded with crowded buildings and residential areas, with moderate vegetation.
TSK	Taman Sri Kuching, Kuala Lumpur	December 2017	N 3° 11' 49.985", E 101° 40' 23.826"	High rise	Apartment with good ventilations. Located 6 km from Kuala Lumpur city centre with few construction areas nearby. Moderate vegetation.

participation of sample collection from human. The Ministry of Health's Medical Research and Ethic Committee (MREC) has approved the study protocol and granted ethical clearance with exemption of written and oral informed consent (Ethical approval: NMRR-18-12-39653).

All eggs collected from the ovitraps were brought back to the laboratory for hatching. The larvae were fed with ox liver and species identification was performed once the L_3 larvae has developed according to Rueda (2004) pictorial keys for mosquito identification. *Aedes aegypti* F_1 adults were then used in evaluating their resistance status against pyrethroids.

Reference mosquito strain

The IMR-laboratory strain (IMR-LS), which has been maintained in the insectarium of IMR for more than 1000 generations and fully susceptible to all classes of insecticides served as the reference strain.

WHO Adult Bioassay

The World Health Organization (WHO) of adult assay protocol was employed to evaluate the resistance status of mosquito against diagnostic concentration of type I pyrethroid (0.25% permethrin) and type II pyrethroids (0.05% deltamethrin, 0.15% cyfluthrin and 0.05% lambda-cyhalothrin) (2016b). Each assay consists of four replicates of treated tubes (with respective impregnated paper) and two replicates of control tubes (with pyrethroid control paper). A total of 25 sugar-fed female adult *Aedes aegypti* aged 3 to 7 days were introduced into each tube and observed for knockdown up to one hour; knockdown was scored if mosquito was incapable of flying or standstill. Scoring via cumulative knockdown count at every one minute interval within the exposure period was recorded. The mosquitoes were immediately transferred into holding tube and 10% sugar solution was provided for a 24-hour recovery period, and subsequently mortality was recorded.

Interpretation of mosquito susceptibility status

Evaluation of resistance was based on 24-hour percentage mortality and resistance ratio (RR) value. In accordance to the criteria set by the WHO, adult mortality with 98-100% mortality indicates susceptibility, 90-97% mortality indicates incipient resistance that needs confirmation and mortality less than 90% indicates resistance population. Meanwhile, the 50% knockdown time (KT_{50}) was determined by Probit analysis (Finney, 1971). Subsequently, the RR value was then computed to monitor the evolution of pyrethroid resistance in the tested *Aedes aegypti* population. The following formula was used to evaluate the RR value:

$$\text{Resistance Ratio (RR)} = \frac{KT_{50} \text{ of field strain}}{KT_{50} \text{ of laboratory susceptible strain}}$$

An RR value of less than 1 (<1) is considered as susceptible, an RR values of less than 5 (<5) indicates low resistance, an RR value of 5 – 10 indicates moderate resistance and an RR value of more than 10 (>10) indicates highly resistant to insecticide (WHO, 2016b).

RESULTS

As the control mortality was less than 5%, adult mortality of all field-collected *Aedes aegypti* strains was not adjusted according to Abbott's formula (WHO, 2016b). The findings from the WHO adult assays indicated that the *Aedes aegypti* population in four localities, namely S3, S8, RC and TSK were found to be associated with pyrethroid resistance. The results also showed that the type I pyrethroid (permethrin) has lost its effectiveness against all field-collected *Aedes aegypti* i.e. the S3, RC and TSK strains were recorded with complete absence of adult mortality, whilst the S8 and TSJ strains exhibited 19% and 15% adult mortality post 24-hour exposure, respectively. On the other hands, the adult mortality of field-collected *Aedes aegypti* strains against type II pyrethroids demonstrated a variable resistance pattern with a high degree of cross-resistance, whereby the S3, S8, RC and TSK strains were not affected by type II pyrethroids (deltamethrin, cyfluthrin and lambda-cyhalothrin). On the contrary, a cross resistance pattern was observed in the TSJ strain, in which this strain was fully susceptible to both deltamethrin and cyfluthrin, but exhibited resistance to both permethrin and lambda-cyhalothrin (Figure 2).

These findings are reinforced by the evidence that the adult mortality of S3, S8, RC and TSK strains were shown to exhibit adult mortality of less than 90%, ranging from 17% to 82% against deltamethrin, 34% to 63% against cyfluthrin, and 6% to 89% against lambda-cyhalothrin. In accordance to the criteria set by the WHO, adult mortality with less than 90% indicates resistance population (WHO, 2016b). It appeared that *Aedes aegypti* strains from these localities were also resistant to type II pyrethroids, suggesting that all three insecticides i.e. deltamethrin, cyfluthrin and lambda-cyhalothrin were no longer effective to control dengue vector in these areas.

In addition, knockdown resistance (KT_{50}) data supported the findings of the resistance status in all field-collected strains. As the mosquito populations expressed lower susceptibility to the insecticides, almost all strains were still alive with zero knockdown effects during one hour of exposure to permethrin, indicating highly resistant against permethrin. While for deltamethrin, cyfluthrin and lambda-cyhalothrin, the KT_{50} and resistance ratio values were varied (Table 2). All strains showed resistance level ranging from 1.01 – 6.85 folds resistance to deltamethrin, 1.12 – 7.56 folds resistance to cyfluthrin and 1.25 – 287.74 folds resistance to lambda-cyhalothrin (Table 2).

In summary, the variability of adult mortality values and resistance ratios of *Aedes aegypti* from five dengue hotspots to type I (permethrin) and type II (deltamethrin, cyfluthrin and lambda-cyhalothrin) pyrethroids was an evidence of cross-resistance mechanism. Overall, type I pyrethroid (permethrin) exhibited the lowest adulticidal effects with the absence of knockdown effects against all five field-collected *Aedes aegypti* compared to type II pyrethroids, indicating that permethrin as well as deltamethrin, cyfluthrin, and lambdacyhalothrin were no longer effective in combating *Aedes aegypti* in these five dengue endemic areas.

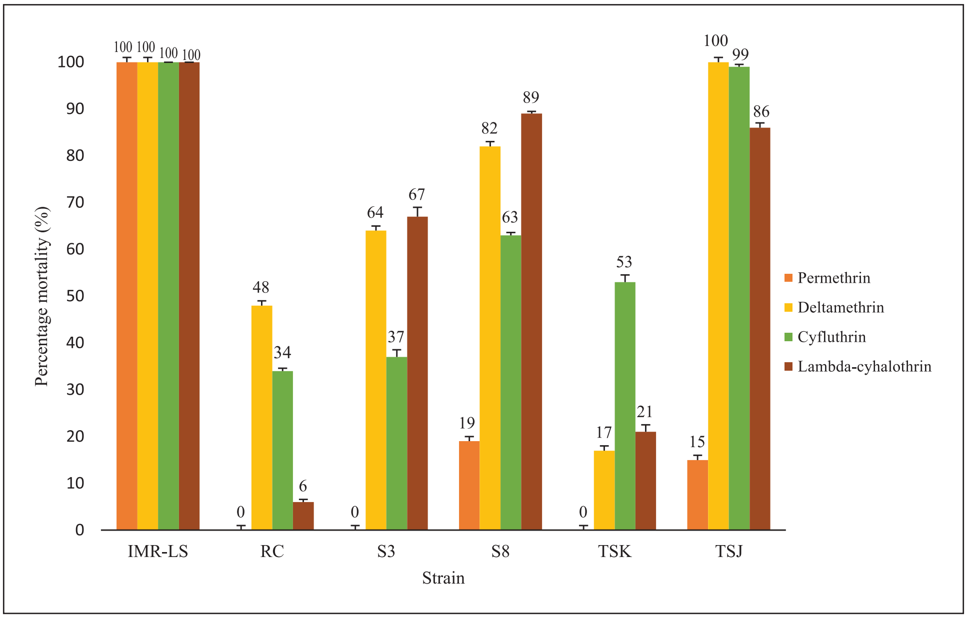

Figure 2. Susceptibility status of *Aedes aegypti* collected in Klang Valley against pyrethroids (IMR-LS, IMR Laboratory strain; RC, Ridzuan Condominium; S3, Seksyen 3; S8, Seksyen 8; TSK, Taman Sri Kuching; TSJ, Taman Sungai Jelok).

Table 2. Comparative knockdown time (KT_{50}) and resistance ratio (RR) of *Aedes aegypti* collected in Klang Valley against pyrethroids

Strain	(n)	Permethrin		Deltamethrin		Cyfluthrin		Lambda-cyhalothrin	
		KT_{50}	RR	KT_{50}	RR	KT_{50}	RR	KT_{50}	RR
IMR-LS	100	25.62	–	18.10	–	17.05	–	28.29	–
RC	100	NA	NA	69.05	3.82	87.91	5.16	8141.26	287.74
S3	100	NA	NA	NA	NA	128.83	7.56	188.66	6.67
S8	100	325.68	12.7	41.2	2.28	43.41	2.55	35.44	1.25
TSK	100	NA	NA	123.92	6.85	114.66	6.73	124.58	4.40
TSJ	100	68.15	2.66	18.30	1.01	19.17	1.12	37.76	1.33

** A total of 100 mosquitoes were tested against each insecticide.*
KT, Knockdown time; NA, not determined by probit analysis due to low knockdown rate (< 5%), indicating highly resistant strain against pyrethroid; RR, Resistance ratio.
(IMR-LS, IMR Laboratory strain; RC, Ridzuan Condominium; S3, Seksyen 3; S8, Seksyen 8; TSK, Taman Sri Kuching; TSJ, Taman Sungai Jelok).

DISCUSSION

In the past 10 years, an increasing number of dengue cases have been encountered globally. According to the WHO (2014), it is estimated that 2.5 billion people are at risk and mostly in tropical and subtropical countries, including Malaysia (Ahmad *et al.*, 2018), Thailand (Lauer *et al.*, 2017), Indonesia (Wahyono *et al.*, 2017), Singapore (Hii *et al.*, 2009) and Japan (Kutsuna *et al.*, 2015). In Malaysia, dengue cases showed an uprising trend from 6,543 cases with 28 deaths in 1995 to a high of 83,849 cases with 177 deaths in 2017, and Selangor has recorded the highest number of dengue cases among the 13 states in Malaysia, with 44,884 cases involving 59 deaths reported (MOH, 2017). Factors such as rapid urbanisation, population growth, heavy international travel and climate changes have been known to contribute to the global dengue burden and ideally promote dengue viral transmission by mosquito vectors (Gubler, 2011; Murray *et al.*, 2013; Ahmad *et al.*, 2018).

Chemical insecticides are the mainstream for dengue control due to the lack of an effective and safe tetravalent dengue vaccine (WHO, 2016a). The current control of dengue relies on the use of adulticides, which are mostly pyrethroid compounds. In Malaysia, pyrethroids have been used for almost 30 years, replacing organophosphates such malathion and fenitrothion (Teng & Singh, 2001). Pyrethroid insecticide is characterised as an odourless compound with high knockdown effect and toxic to many pests, and is widely used in agriculture and public health sectors. This photostable compound demonstrates low toxicity to human compared to DDT and organophosphates, and thus represents the most favourable choice of adulticides used in fogging and ultra-low volume applications in Malaysia as well as in many other countries. As a consequent, it will be detrimental if pyrethroids is no longer effective in killing adult vector mosquitoes as this could jeopardise the effectiveness of current dengue control programmes (Vythilingam *et al.*, 1992).

In the present study, we initiated a proactive action to evaluate pyrethroid resistance status of *Aedes aegypti* in dengue hotspots in the Klang Valley. According to the Ministry of Health Malaysia guidelines, a residential area is defined as a dengue hotspot if dengue cases are continuously reported within the control area over 32 days from the first reported case. Dengue control intervention is conducted via vector control in order to breach the cycle of dengue transmission to stop the spread of the disease. Generally, the health authority applies adulticides in infected areas within seven days of the reported dengue case via space spray techniques such as ultra-low volume spraying and thermal fogging, with the main aim of killing the infective adult *Aedes* mosquitoes carrying the dengue virus. The first fogging activity would be followed by the second application, which is seven to ten days later. The two treatment cycles are based on the life cycle of *Aedes* mosquitoes and extrinsic incubation period of the virus in the mosquito. The outbreak will be declared over once a 20 days transmission-free period is achieved (Tham, 1993). Nevertheless, the intense exposure of insecticide in dengue outbreak areas appears to significantly induce the development of insecticide resistance in *Aedes* vectors.

The present study on the level of resistance of *Aedes aegypti* in all five strains in comparison with the IMR-LS strain indicated varying level of resistance to pyrethroids. Permethrin (type I) resistance was observed in all strains with extremely low percentage of adult mortality (<19%), resulting in more than 12-folds resistance ratio. As for other insecticides i.e. deltamethrin, cyfluthrin and lambda-cyhalothrin (type II), the percentage of mortality and resistance ratio values recorded for all *Aedes aegypti* strains varied; only the TSJ strain showed high percentage of mortality of more than 99% against pyrethroids and low resistance ratio to deltamethrin and cyfluthrin at 1.01-fold and 1.12-fold respectively, indicating high susceptibility to these insecticides (Table 2).

Based on these findings, all five *Aedes aegypti* strains showed resistance to permethrin (type I) and were conferred with some degree of cross resistance to type II pyrethroids (deltamethrin, cyfluthrin and lambda-cyhalothrin), except for the TSJ strain, which was highly susceptible to deltamethrin and cyfluthrin. Type II pyrethroid contains the α-cyano-3-phenoxybenzyl group that is known to be more neurotoxic and exhibits higher killing effect compared to type I pyrethroid (Smith, 2016). Comparable ratios of resistance for type I and type II pyrethroids supported the cross resistance hypothesis. The widespread usage of permethrin has conferred cross resistance to other pyrethroids including those that are not commonly used in vector control programmes (Flores *et al.*, 2013).

The geographical pattern of all five dengue hotspots indicated that the residential areas where S3, S8, RC and TSK strains were collected are located in an urban area, whilst TSJ strain was collected from a sub-urban area. Since *Aedes aegypti* is a peri-domestic mosquito with high preference of breeding in man-made containers mostly found in urbanised areas, hence the risk of dengue outbreak in urban areas is high. High frequency of space spraying is thus often conducted to curb the continuous outbreaks, and such intensive application of insecticide would lead to resistance development in the target mosquitoes.

The study clearly indicated that pyrethroids resistance is widespread in dengue hotspots. However, these incidence of resistance is localised (Rosilawati *et al.*, 2017), and does not represent the overall resistance status of *Aedes aegypti* in dengue hotspots in the whole Klang Valley as this region which includes the adjoining Kuala Lumpur city centre and five districts of Petaling, Gombak, Klang, Hulu Langat and Kuala Langat is huge. Previously, widespread of pyrethroid resistance had been reported in Klang Valley, including Taman Melati, Vista Angkasa and Desa Tasik (Wan-Norafikah *et al.*, 2010), Shah Alam (Loke *et al.*, 2012), Kuala Lumpur (Ishak *et al.*, 2015), Bandar Baru Bangi, and Petaling Jaya (Rosilawati *et al.*, 2017) and Hulu Selangor, Kuala Selangor, Petaling and Gombak (Leong *et al.*, 2019).

These evidence based data in the present study indicate the challenges faced in the chemical control of dengue vectors. Thus, there is an urgent need of an immediate action in comprehensively evaluating the pyrethroid resistance in *Aedes* mosquito in all dengue hotspots in Klang Valley. Pyrethroid resistance management strategy should be implemented. Monitoring the status of pyrethroid resistance and implementation of appropriate counter measures to overcome pyrethroid resistance will be essential to ensure the effectiveness of dengue control programmes. Other than that, rotation of insecticides with different mode of action, proper insecticide dosing and integrating usage of piperonyl butoxide as a synergist would be good counter measures to overcome pyrethroid resistance. Overall, it is crucial to constantly monitor the resistance status of dengue vector in order to forestall increase in the resistance of *Aedes aegypti* against the commonly used insecticides in dengue control programmes.

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