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Insecticide susceptibility status and resistance mechanism of *Anopheles cracens* Sallum and Peyton and *Anopheles maculatus* Theobald (Family: Culicidae) from knowlesi malaria endemic areas in Peninsular Malaysia

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ABSTRACT

Objective: To assess the insecticide susceptibility status of *Anopheles cracens* (*An. cracens*) and *Anopheles maculatus* (*An. maculatus*) from knowlesi malaria endemic areas in Peninsular Malaysia towards DDT, malathion and deltamethrin and to determine the resistance mechanism involved. **Methods:** Adult and larval mosquitos were collected for surveillance. Susceptibility status of *Anopheles* was determined using the standard WHO adult bioassay, larval bioassay and biochemical enzyme assay. **Results:** WHO adult bioassay results indicated *An. cracens* collected from Kampung Sungai Ular, Pahang was resistant towards 4% DDT, while *An. maculatus* collected from Kampung Sokor, Kelantan and Kampung Sungai Lui, Selangor exhibited resistance towards 4% DDT. However, the enzyme activity profiles varied according to strains and species. The resistance ratio of larval bioassay, showed that all strains and species tested were susceptible to malathion and temephos. **Conclusions:** Since only a few anopheline strains exhibited low level of insecticide resistance towards malathion, DDT and temephos. These insecticides are still considered effective for vector control program towards *An. cracens* and *An. maculatus*.

1. Introduction

Anopheles mosquitoes are the main vectors that transmit human malaria which is a disease caused by the infections of the protozoan parasites such as *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae* and *Plasmodium ovale*. Malaria is one of the most important tropical diseases in the world. In 2015, 214 million cases of malaria were reported, along with an estimated 438 000 of malaria deaths globally[1]. Being the prominent vectors

of malaria, the *Anopheles* mosquitoes are widely distributed around the world, but only around 40 species are important vectors of malaria. *Anopheles balabacensis*, *Anopheles campestris*, *Anopheles epiroticus*, *Anopheles flavirostris*, *Anopheles latens*, *Anopheles letifer* and *Anopheles maculatus* (*An. maculatus*) are examples of *Anopheles* species that are responsible for spreading human malaria in Malaysia[2].

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Recently, human malaria cases caused by simian *Plasmodium knowlesi* (*P. knowlesi*) has been reported and found in most states of Peninsular Malaysia. *P. knowlesi* is a zoonotic malaria parasite of long tailed (*Macaca fascicularis*) and pig tailed (*Macaca nemestrina*) macaques. In South East Asia, *P. knowlesi* parasite was common and transmitted by mosquitoes of the *Anopheles leucosphyrus* group. In Peninsular Malaysia, *Anopheles cracens* (*An. cracens*), *Anopheles hackeri* and *Anopheles introlatus* have been shown to be the vector of *P. knowlesi* infection, while *Anopheles balabacensis* and *Anopheles latens* were identified as the vector in Sabah and Sarawak, respectively[3,4].

Vector control is an important component of the WHO Global Strategy for Malaria Control, for which its objective is to break the transmission of malaria parasite using indoor residual spraying or pyrethroid-impregnated materials. Malaria vector control activities in Malaysia focus mainly on the use of insecticide-treated bed nets and indoor residual spraying. Since 1998, deltamethrin wettable powder has replaced DDT as the main pyrethroid used in residual spraying, while permethrin was used to treat the bednets. Fogging with malathion technical grade is carried out as a supplementary measure in the event of outbreak to control malaria transmission. In addition, temephos (Abate®) is used as a larvacide for anti-larval activities[5,6].

Clearly, the control programme in Malaysia relies heavily on the chemical insecticides to curb the malaria situation. However, widespread resistance of vectors towards insecticides may limit the usage of insecticides and the effectiveness of insecticides in vector control programme. Due to these factors, resistance monitoring programme should be carried out constantly for early detection of the problem and rapid assimilation of information on the resistant mosquito population to strategies counter-measures. The aim of the current study was to assess the insecticide susceptibility status of *An. cracens* and *An. maculatus* from knowlesi malaria endemic areas in Peninsular Malaysia towards DDT, malathion and deltamethrin and to determine the resistance mechanism involved.

2. Materials and methods

2.1. Study sites

This study was carried out in Kampung Mela Kuala Lipis Pahang, Kampung Sungai Ular Balok Pahang, Lata Cemerung Dungun Terengganu, Kampung Sokor Tanah Merah Kelantan, Mersing Reserved Forest Johor, Kampung Sahom Kampar Perak, Gerik Hulu Perak and Kampung Sungai Lui Hulu Langat Selangor. Both Kampung Mela and Kampung Sungai Ular Pahang are rubber estate, oil palm plantation and fruit orchard areas. Meanwhile, Lata Cemerung is a camping site, Kampung Sokor is a gold mining site, Mersing Reserved Forest is a logging track, Kampung

Sahom is an aboriginal village, Gerik Hulu Perak is a forest near to the border of Thailand; and Kampung Sungai Lui is a village near to the border of Negeri Sembilan and Pahang. Figure 1 is the map of Peninsular Malaysia showing location of the 8 study sites selected. The area selection was based on knowlesi malaria cases reported for the year 2011 to 2014. In these study areas more than 80% of the villagers exploited their land for rubber and oil palm plantations and to a lesser extent for fruit orchards.



Figure 1. Location of 8 study sites in Peninsular Malaysia.

2.2. Adult surveillance

Adult mosquitoes were caught monthly from March 2014 to October 2015. The sampling was conducted by using human landing catches and CO₂-baited Centers for Disease Control light traps (CDC-LT) simultaneously. Outdoor collection of mosquitoes was done separately from the 8 sites, for 3 consecutive nights at each site from 18:00 to 6:00 h. Alive adult mosquito were brought back to the lab and blood fed by using mice for colonization purpose.

Human landing catches were conducted by 3 teams of 2 catchers per team. They were randomly assigned into 3 teams and the teams were also randomly assigned for different location within the same study site. All mosquitoes that landed on the bare legs were caught by using 50 mm × 19 mm glass vials which were subsequently plugged with cotton wool. CDC-LT baited with CO₂ were fixed randomly at the sampling sites. The CDC-LT was operated by four 1.5 V batteries which drive the suction fan and a 1.5 w bulb. The CO₂ was emitted from 0.5 kg dry ice placed in a 6 square inches ice box suspended adjacent and slightly above the light trap. Morphological identification of collected *Anopheles* mosquitoes was done by using standard taxonomy keys[7,8] at the site the following morning.

2.3. Establishment of *Anopheles* species colonization

A colony of *Anopheles* was established from wild-caught fully engorged adult female. After the engorged adult female was maintained for 4-5 days and/or until gravid in the insectarium, it was then placed in a screen-topped plastic cup for oviposition, which contains filter paper lined inside the cup and 25 mL of reverse osmosis water. The eggs attached to the moist side of the filter paper and/or floating on the water surface were rinsed and transferred to white plastic tray (25 cm × 36 cm × 6 cm) containing 1 500 mL reverse osmosis water with wet filter paper lining the inside. During the embryonation period, the eggs were exposed to a 40-watt light for warming the eggs until hatching. Once hatched the 1st instar larvae were transferred daily from the oviposition cup to white plastic tray (25 cm × 36 cm × 6 cm) containing 1 500 mL reverse osmosis water. Each tray can accommodate 80 1st instar larvae. Ground fish food was used as larval feed. After pupation, approximately 100 pupae were placed in a plastic cup (14.5 cm in diameter and 6 cm in depth) containing 150 mL of distilled water and were kept in 30 cm × 30 cm × 30 cm cage in the presence of 10% sucrose solution for adult emergence. The first generation of the colony was used for susceptibility test. The insectarium was maintained at (27±2) °C, 70%-80% relative humidity, and illumination from a fluorescent lighting was provided approximately for 12 h/day.

2.4. Susceptibility test of *Anopheles* mosquitoes

Susceptibility status of *Anopheles* mosquitoes was examined using three different bioassays, namely WHO adult bioassay, larval bioassay and biochemical enzyme assay.

2.4.1. Adult bioassay

WHO insecticide monitoring test kit comprised WHO test tubes (12.5 cm in length, 4.4 cm in diameter) and WHO impregnated papers (12 cm × 15 cm), were purchased from Vector Control Research Unit, School of Biological Sciences, Universiti Sains Malaysia, Penang, which was designated by WHO for the production of WHO insecticide test kits. The test tubes were used for exposing the mosquitoes to the insecticides at WHO recommended diagnostic concentration. In this study, the mosquitoes were subjected to 4.00% dichloro-diphenyl-trichloroethane (DDT), 5.00% malathion and 0.25% deltamethrin for 1 h. F1 generation of field-collected *Anopheles* species from all localities were used in the adult bioassay.

Adult bioassay was conducted according to WHO standard procedure^[9]. A total of 20 sugar-fed adult female mosquitoes aged 2-5 days old were transferred to the tube with green dot (holding tube) and kept isolated/aside for 1 h to acclimatize the mosquitoes. Mosquitoes were then transferred to the tube with

red dot (exposure tube) and exposed to insecticide impregnated paper. Each insecticide testing involved three replicates and 3 controls. During the exposure, knockdown time was recorded every five minutes for 1 h. After 1 h, mosquitoes were transferred to a fresh paper cups, fed with 10% sugar solution and mortality was recorded at 24 h post-exposure. According to WHO criteria for determining resistance, a mortality of 98% to 100% indicates susceptibility, 90% to 97% of mortality indicates possibility of resistance that require further confirmation and less than 90% of mortality indicates resistance^[9].

Data obtained during 1 h exposure time were subjected to Probit analysis (SPSS ver. 11.5) to compute the lethal time (LT₅₀) values. Resistance ratio (RR) was calculated by dividing the LT₅₀ value of the field strain to the susceptible strain, which was maintained at the Medical Entomology Unit, Institute for Medical Research. RR was determined as follow:

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

2.4.2. Larval bioassay

Larval susceptibility test was conducted according to WHO larval susceptibility bioassay^[10]. WHO supplied insecticides namely malathion (781.25 mg/L stock solution) and temephos (156.25 mg/L stock solution) were purchased from Vector Control Research Unit, School of Biological Sciences, Universiti Sains Malaysia, Penang. Malathion (3.125 mg/L) and temephos (0.250 mg/L) were tested at diagnostic dosages for larval mosquitoes. Various concentrations of malathion and temephos were prepared and used to determine the lethal concentration (LC₅₀). All the insecticide solutions were prepared in disposable paper cups of 300 mL capacity with triplicates. The cups were left undisturbed for 30 min after pipetting of the required insecticide into them. A total of 25 3rd instar larvae were then introduced into each cup. Larval mortality was recorded after 24 h post exposure. If the mortality of control larvae is between 5% and 20%, the mortalities of treated groups should be corrected according to Abbott's formula as shown below:

$$\text{Corrected mortality (\%)} = \frac{\% \text{mortality in test} - \% \text{mortality in control}}{100 - \% \text{mortality in control}} \times 100$$

LC₅₀ value was calculated by using Probit analysis software (Probit SOFTWARE Ltd) to determine the knockdown time of the mosquito. RR value was calculated by dividing the LT₅₀ value of the field strain to the susceptible strain. When RR value is less than 5, the field population is considered susceptible, when RR value is between 5 and 10 the field mosquitoes are considered to have moderate resistance, and when RR is more than 10, the field mosquitoes are highly resistant.

2.4.3. Biochemical enzyme assay

Biochemical assay of the resistance enzymes was conducted by using selected female adult field mosquitoes. Monooxygenases (MO) enzyme assay was performed according to Nazni *et al.*[11]. The colour intensities were measured at wavelength 630 nm by using an immunoassay reader (Dynatech, Model MR 5000). Non-specific esterase (NSE) enzyme microassay was conducted according to Lee[12] and the reaction was measured at wavelength 450 nm. Glutathione-S-transferase (GST) enzyme was conducted as described by Lee and Chong[13]. The enzyme reaction was read at 450 nm, while the acetylcholinesterase (AChE) microassay was conducted according to Brogdon *et al.*[14] and the enzyme activity was assessed at 410 nm.

Protein assay of mosquito was conducted according to the method of Bradford[15]. A standard curve was prepared by using bovine serum albumin as the protein standard (Bio-rad Protein Assay Kit) and the protein concentration of mosquito was determined based on this standard curve.

2.5. Statistical analysis

LT₅₀ and LC₅₀ values were determined by using Probit analysis. One-way ANOVA followed by Tukey test was used to determine the significant differences between groups.

3. Results

3.1. Entomological surveillance of *An. cracens* and *An. maculatus*

During the survey performed from March 2014 until October 2015, a total of 492 *Anopheles* female adults, which consisted of *An. cracens* (46.3%) and *An. maculatus* (53.7%) were collected. As for mosquito larvae, a total of 110 were collected and the majority of them were those of *An. cracens* (Table 1).

Table 1

Total number of adult and larva of *Anopheles* mosquitoes collected according to species and sites.

Locality	<i>An. cracens</i>		<i>An. maculatus</i>	
	Adult	Larva	Adult	Larva
Kg. Mela	46	4	46	0
Kg. Sg. Ular	46	4	18	0
Lata Cemerung	54	10	20	2
Kg. Sokor	27	50	69	0
Mersing	24	10	32	2
Kg. Sahom	28	18	35	0
Gerik	3	5	26	0
Kg. Sg. Lui	0	5	18	0
Total	228	106	264	4

3.2. Susceptibility test of *Anopheles* mosquitoes

3.2.1. WHO adult bioassay

An. cracens collected from Kampung Sg. Ular and Kampung Sahom showed possible resistance to 5% malathion. Strains of Kampung Mela, Kampung Sokor and Kampung Sahom showed a possible resistance to 4% DDT, while strain from Kampung Sg. Ular showed confirmed resistant towards 4% DDT. *An. cracens* of all 6 strains tested remained susceptible to 0.05% deltamethrin (Table 2).

In the case of *An. maculatus*, Kampung Sg. Lui strain was the only strain that showed a possible resistance to 5% malathion. There was also one strain which was the Kampung Sokor strain that showed suggested resistance towards 0.05% deltamethrin. All other strains tested remained susceptible to 5% malathion and 0.05% deltamethrin. Strain of Gerik showed a suggested resistance to 4% DDT, while Kampung Sokor and Kampung Sg. Lui indicated confirmed resistant to 4% DDT (Table 2).

With regards to RR and LT₅₀, the highest RR towards 5% malathion amongst the *An. cracens* was exhibited by Kampung Sahom strain (1.42) with LT₅₀ of 64.95 min which was found significantly higher when compared to other strains, except for

Table 2

Insecticide susceptibility of adult *Anopheles* mosquitoes from selected knowlesi malaria endemic areas in Peninsular Malaysia (1 h insecticide exposure).

<i>Anopheles</i> larvae	Location	Generation	5% Malathion		4% DDT		0.05% Deltamethrin	
			Mortality (%)	Status	Mortality (%)	Status	Mortality (%)	Status
<i>An. cracens</i>	Laboratory strain	F9	100.0	S	100.0	S	100.0	S
	Kg. Mela	F1	100.0	S	92.0	RS	100.0	S
	Kg. Sg. Ular	F1	96.0	RS	89.0	RC	100.0	S
	Kg. Sokor	F1	100.0	S	96.0	RS	100.0	S
	Lata Cemerung	F1	100.0	S	100.0	S	100.0	S
	Mersing	F3	100.0	S	100.0	S	100.0	S
	Kg. Sahom	F1	92.0	RS	96.0	RS	100.0	S
<i>An. maculatus</i>	Laboratory strain	F126	100.0	S	100.0	S	100.0	S
	Kg. Sokor	F1	100.0	S	89.0	RC	96.0	RS
	Mersing	F1	100.0	S	100.0	S	100.0	S
	Kg. Sahom	F1	100.0	S	98.0	S	100.0	S
	Gerik	F1	100.0	S	96.0	RS	100.0	S
	Kg. Sg. Lui	F1	92.0	RS	88.0	RC	100.0	S

S: susceptible; RS: suggested resistant; RC: confirmed resistant.

Table 3LT₅₀ values (minutes) of insecticide against adult *Anopheles* species from selected knowlesi malaria endemic areas in Peninsular Malaysia.

<i>Anopheles</i> larvae	Location	5.00% Malathion			4.00% DDT			0.05% Deltamethrin		
		LT ₅₀	Slope (b)	RR	LT ₅₀	Slope (b)	RR	LT ₅₀	Slope (b)	RR
<i>An. cracens</i>	Lab strain	45.82±3.45 ^b	4.07±0.55	-	49.92±8.43 ^b	9.86±0.77	-	61.92±6.74 ^c	3.67±1.18	-
	Kg. Mela	42.32±2.18 ^b	7.78±1.45	0.92	63.43±3.78 ^c	3.56±0.92	1.27	70.59±8.46 ^{cd}	7.86±0.74	1.14
	Kg. Sg. Ular	60.49±7.75 ^c	2.68±0.89	1.32	67.92±6.84 ^c	7.56±1.14	1.36	77.40±7.88 ^d	6.84±0.88	1.25
	Kg. Sokor	42.45±3.14 ^b	3.08±1.68	0.93	49.05±2.14 ^b	3.15±0.77	0.98	63.44±3.45 ^c	2.15±1.76	1.02
	L.Cemerung	40.32±1.89 ^{ab}	6.54±1.08	0.88	33.45±5.56 ^a	6.95±0.92	0.67	45.20±1.37 ^b	6.92±1.17	0.73
	Mersing	33.91±6.55 ^a	5.48±0.14	0.74	26.46±5.97 ^a	2.65±0.59	0.53	35.29±1.04 ^a	4.49±0.53	0.57
	Kg. Sahom	64.95±2.45 ^c	4.72±0.78	1.42	64.50±6.87 ^c	7.85±0.88	1.29	71.21±7.72 ^{cd}	2.67±0.95	1.15
<i>An. maculatus</i>	Lab strain	36.16±3.78 ^c	9.79±1.09	-	48.65±2.56 ^{cd}	3.57±0.76	-	65.18±6.48 ^b	3.68±0.79	-
	Kg. Sokor	26.03±3.24 ^{ab}	4.29±0.47	0.72	45.73±1.92 ^{bc}	6.76±1.23	0.94	36.50±9.04 ^a	5.98±1.11	0.56
	Mersing	23.87±1.76 ^a	6.68±1.22	0.66	39.89±1.87 ^{ab}	9.78±0.94	0.82	37.80±8.56 ^a	4.78±0.93	0.58
	Kg. Sahom	31.01±5.66 ^{abc}	6.15±0.87	0.86	56.43±5.93 ^{de}	5.69±0.46	1.16	86.04±1.46 ^c	8.44±0.97	1.32
	Gerik	29.65±8.95 ^{abc}	5.45±0.56	0.82	35.51±7.75 ^a	6.94±0.98	0.73	43.02±1.04 ^a	6.83±1.17	0.66
	Kg. Sg. Lui	33.27±6.43 ^{bc}	8.49±1.43	0.92	60.33±8.46 ^e	4.75±1.09	1.24	94.51±6.05 ^c	8.73±0.88	1.45

Values followed by different letters within a column for each species are significantly different (one-way ANOVA followed by Tukey test, $P < 0.05$).

Kampung Sg. Ular. *An. cracens* of Kampung Sg. Ular strain showed the highest RR towards 4% DDT (1.36) with LT₅₀ of 67.92 min which was found significantly higher than Kampung Sokor, Lata Cemerung and Mersing. This strain also showed highest RR towards 0.05% deltamethrin (1.25) with LT₅₀ of 77.40 min which was significantly higher than all other strains tested. As for *An. maculatus* it was the Kampung Sg. Lui strain that demonstrated the highest RR (0.92) towards 5% malathion with LT₅₀ of 33.27 min which was significantly higher only to that of Mersing strain. *An. maculatus* of Kampung Sg. Lui strain also was the strain that exhibited the highest RR towards 4% DDT (1.24; LT₅₀ of 60.33 min) and deltamethrin (1.45; LT₅₀ of 94.51 min) which was significantly higher than all others strain except Kampung Sahom strain (Table 3).

3.2.2. WHO larval bioassay

The larval bioassay study showed that for *An. cracens*, all strains remained susceptible to temephos. However, *An. cracens* of Lata Cemerung and Kampung Sahom strains demonstrated possible resistance towards malathion. Most *An. maculatus* strains were also

found susceptible to temephos but two strains, those of Kampung Sokor and Kampung Sahom showed possible resistance while Kampung Sg. Lui strain demonstrated confirmed resistance to malathion (Table 4).

Table 4Insecticide susceptibility test of *Anopheles* larvae from selected knowlesi malaria endemic areas in Peninsular Malaysia (24 h exposure).

<i>Anopheles</i> larvae	Location	Generation	Mortality (%)	
			Malathion (3.125 mg/L)	Temephos (0.250 mg/L)
<i>An. cracens</i>	Laboratory strain	F9	100.0	100.0
	Kg. Mela	F1	100.0	100.0
	Kg. Sg. Ular	F1	100.0	100.0
	Kg. Sokor	F1	100.0	100.0
	Lata Cemerung	F1	96.0	100.0
	Mersing	F3	100.0	100.0
	Kg. Sahom	F1	96.0	100.0
<i>An. maculatus</i>	Laboratory strain	F126	100.0	100.0
	Kg. Sokor	F1	96.0	100.0
	Mersing	F1	100.0	100.0
	Kg. Sahom	F1	94.0	100.0
	Gerik	F1	100.0	100.0
	Kg. Sg. Lui	F1	89.0	100.0

Table 5LC₅₀ values of insecticide against *Anopheles* larvae from selected knowlesi malaria endemic areas in Peninsular Malaysia (ng/L).

<i>Anopheles</i> larvae	Location	Malathion			Temephos		
		LC ₅₀	RR	Status	LC ₅₀	RR	Status
<i>An. cracens</i>	Lab strain	51.50	-	Susceptible	4.81	-	Susceptible
	Kg. Mela	86.10	1.7	Susceptible	7.37	1.7	Susceptible
	Kg. Sg. Ular	96.40	1.9	Susceptible	6.89	1.6	Susceptible
	Kg. Sokor	75.80	1.5	Susceptible	6.89	1.6	Susceptible
	Lata Cemerung	75.80	1.5	Susceptible	6.89	1.6	Susceptible
	Mersing	80.90	1.6	Susceptible	6.89	1.6	Susceptible
	Kg. Sahom	96.40	1.9	Susceptible	7.37	1.7	Susceptible
<i>An. maculatus</i>	Lab strain	44.30	-	Susceptible	4.28	-	Susceptible
	Kg. Sokor	75.44	1.8	Susceptible	6.57	1.6	Susceptible
	Mersing	66.60	1.6	Susceptible	6.57	1.6	Susceptible
	Kg. Sahom	71.10	1.7	Susceptible	6.99	1.7	Susceptible
	Gerik	71.00	1.7	Susceptible	6.57	1.6	Susceptible
	Kg. Sg. Lui	71.20	1.7	Susceptible	7.42	1.8	Susceptible

With regards to RR and LC₅₀, both species indicated a low RR (less than 5) for larvicidal activity. However, all strains exhibited relatively high LC₅₀ as compared to the laboratory strain, indicating that all field populations are possibly susceptible towards malathion and temephos (Table 5).

3.2.3. Biochemical enzyme assay

3.2.3.1. MO enzyme assay

The analysis of MO enzyme activity showed that, among all the strains of *An. cracens* mosquito tested, Kampung Sahom strain exhibited the significantly highest MO activity of 30.837 µmole product/min/µg protein which was significantly higher than all other strains with RR of 2.9 fold higher than RR of laboratory strain. *An. maculatus* of the same strain also showed the highest MO activity of 17.467 µmole product/min/µg protein. Its RR was 1.7 fold higher than that of the laboratory strain and significantly higher than strains of Kampung Sokor, Mersing and Gerik (Table 6).

Table 6

MO activity for *Anopheles* species from selected knowlesi malaria endemic areas in Peninsular Malaysia.

<i>Anopheles</i> larvae	Species/Strain	Mean±SE (µmole product/min/µg protein)	RR
<i>An. cracens</i>	Laboratory strain	10.641±1.480 ^a	-
	Kg. Mela	20.208±0.430 ^b	1.9
	Kg. Sg. Ular	22.350±0.390 ^b	2.1
	Kg. Sokor	9.574±0.300 ^a	0.9
	Lata Cemerung	9.570±0.250 ^a	0.9
	Mersing	7.443±1.340 ^a	0.7
	Kg. Sahom	30.837±3.900 ^c	2.9
	<i>An. maculatus</i>	Laboratory strain	10.275±0.350 ^b
	Kg. Sokor	7.193±0.300 ^{ab}	0.7
	Mersing	5.137±0.540 ^a	0.5
	Kg. Sahom	17.467±2.100 ^c	1.7
	Gerik	7.192±0.460 ^{ab}	0.7
	Kg. Sg. Lui	14.384±1.100 ^{bc}	1.4

Values followed by different letters within a column for each species are significantly different (one-way ANOVA followed by Tukey test, $P < 0.05$).

3.2.3.2. NSE enzyme assay

The analysis of NSE enzyme activity showed that *An. cracens* of Kampung Sahom strain demonstrated the highest activity (0.868 µmole α-naphthol/min/µg protein) which was significantly higher than all other strains and RR that was 2.1 folds higher than the RR of the laboratory strain. As for *An. maculatus*, the Kampung Sg. Lui strain was found to be the strain with the highest NSE enzyme activity of 0.800 µmole α-naphthol/min/µg protein and RR that was 1.8 folds higher than RR of the laboratory strain and significantly higher than all other strains (Table 7).

Table 7

NSE activity for *Anopheles* species from selected knowlesi malaria endemic areas in Peninsular Malaysia.

<i>Anopheles</i> larvae	Species/Strain	Mean±SE (µmole α-naphthol/min/µg protein)	RR
<i>An. cracens</i>	Laboratory strain	0.414±0.030 ^b	-
	Kg. Mela	0.496±0.010 ^b	1.2
	Kg. Sg. Ular	0.661±0.040 ^c	1.6
	Kg. Sokor	0.248±0.050 ^a	0.6
	Lata Cemerung	0.289±0.020 ^a	0.7
	Mersing	0.207±0.030 ^a	0.5
	Kg. Sahom	0.868±0.060 ^d	2.1
	<i>An. maculatus</i>	Laboratory strain	0.445±0.010 ^{bc}
	Kg. Sokor	0.356±0.070 ^{ab}	0.8
	Mersing	0.267±0.040 ^a	0.6
	Kg. Sahom	0.533±0.020 ^c	1.2
	Gerik	0.311±0.030 ^{ab}	0.7
	Kg. Sg. Lui	0.800±0.050 ^d	1.8

Values followed by different letters within a column for each species are significantly different (one-way ANOVA followed by Tukey test, $P < 0.05$).

3.2.3.3. GST enzyme assay

The analysis of GST enzyme activity showed that, all *An. cracens* and *An. maculatus* strains showed relatively low GST enzyme activity. In fact when compared to laboratory strain *An. cracens*, strains of Kampung Sokor, Lata Cemerung, and Mersing showed GST enzyme activity that were significantly lower than laboratory strain. As for *An. maculatus*, all strains demonstrated significantly lower GST enzyme activity than laboratory strain (Table 8).

Table 8

GST activity for *Anopheles* species from selected knowlesi malaria endemic areas in Peninsular Malaysia.

<i>Anopheles</i> larvae	Strains	Mean±SE (µmole CDNB/min/µg protein)	RR
<i>An. cracens</i>	Laboratory strain	0.598±0.080 ^c	-
	Kg. Mela	0.530±0.050 ^{bc}	0.9
	Kg. Sg. Ular	0.548±0.070 ^{bc}	0.9
	Kg. Sokor	0.479±0.110 ^b	0.8
	Lata Cemerung	0.350±0.050 ^a	0.6
	Mersing	0.349±0.030 ^a	0.6
	Kg. Sahom	0.528±0.090 ^{bc}	0.9
	<i>An. maculatus</i>	Laboratory strain	0.614±0.120 ^c
	Kg. Sokor	0.491±0.080 ^b	0.8
	Mersing	0.368±0.065 ^a	0.6
	Kg. Sahom	0.490±0.175 ^b	0.8
	Gerik	0.430±0.110 ^{ab}	0.7
	Kg. Sg. Lui	0.429±0.095 ^{ab}	0.7

Values followed by different letters within a column for each species are significantly different (one-way ANOVA followed by Tukey test, $P < 0.05$).

3.2.3.4. AChE enzyme assay

Comparing the findings to the laboratory strain, *An. cracens* of Kampung Sahom strain showed the highest AChE enzyme activity of 169.7% and RR which was 1.7 fold higher than that of the laboratory strain. *An. maculatus* of the same locality was also the strain that showed the highest AChE enzyme activity of 116.1% and RR which was 1.16 fold higher than that of the laboratory strain (Table 9).

Table 9

AChE activity for *Anopheles* species from selected knowlesi malaria endemic areas in Peninsular Malaysia.

<i>Anopheles</i> larvae	Strains	AChE activity (%)	RR
<i>An. cracens</i>	Laboratory strain	0.774	-
	Kg. Mela	148.400	1.480
	Kg. Sg. Ular	127.500	1.270
	Kg. Sokor	93.600	0.940
	Lata Cemerung	59.000	0.590
	Mersing	85.700	0.860
	Kg. Sahom	169.700	1.700
<i>An. maculatus</i>	Laboratory strain	0.986	-
	Kg. Sokor	79.600	0.800
	Mersing	70.100	0.700
	Kg. Sahom	116.100	1.160
	Gerik	100.700	1.010
	Kg. Sg. Lui	112.900	1.130

Based on analysis of all enzymes, it was found that *An. cracens* of Kampung Sahom strain exhibited the highest level of enzyme activity for MO, NSE and AchE. *An. maculatus* from the same locality also exhibited the highest enzyme activity for MO and AchE. The highest activity of NSE was shown by *An. maculatus* strain from Kampung Sg. Lui. As far as GST enzyme activity is concerned, all field strains of *An. cracens* and *An. maculatus* exhibited low level of GST enzyme activity as compared to laboratory strain.

4. Discussion

In this study, the insecticide susceptibility status and resistance mechanism of *An. cracens* and *An. maculatus* collected from knowlesi malaria endemic areas in Peninsular Malaysia were determined using the standard WHO adult bioassay, larval bioassay and biochemical enzyme assay.

Based on result of the WHO adult bioassay, it was clear that several *An. cracens* strains tested have developed some degree of resistance towards 5% malathion namely strain of Kg Ular in Pahang and Kg Sahom in Perak (“possible resistance”) and to 4% DDT namely strain of Kampung Sg. Mela in Pahang, Kampung Sokor in Kelantan dan also Kampung Sahom in Perak (also “possible resistance”). In addition, strain of Kampung Sg Ular in Pahang indicated “confirmed resistance” to 4% DDT. *An. maculatus* strain also demonstrated development of some degree of resistance not only to 5% malathion (“possible resistance” by Kampung. Sg. Lui strain in Selangor) and 4% DDT (“possible resistance” by Gerik strain in Perak, “confirmed resistance” by Kampung Sokor strain in Kelantan and Kampung Sg. Lui strain in Selangor) but to 0.05% deltamethrin as well (“possible resistance” by Kampung Sokor strain in Kelantan) which was not observed in the *An. cracens* strains tested during this study. With regards to the *An. maculatus* strain that demonstrated development of some degree of resistance to 4% DDT, for example the Kampung Sg. Lui strain in Selangor,

the mosquito was collected from rubber estate, oil palm plantation and fruit orchard where agricultural pesticides are being frequently used. Cross resistance may be one of the reasons accounting for the development of resistance towards DDT for this species in this particular location. Interestingly Rohani *et al.*[16] showed that the mortality of *An. maculatus* in three localities in Selangor, namely Sungai Congkak, Sungai Tamu and Kuang were 93.3%, 87.5% and 86.7% respectively indicating low resistance towards DDT has been detected much earlier in Selangor.

These results were also in line with the study done in Betau, Pahang whereby *An. maculatus* of Betau strain was reported as “confirmed resistance” towards DDT with mortality of 86.7% using WHO adult bioassay[17]. Similar study conducted in three other localities also in Pahang, namely Temerloh, Senderut and Pos Lenjang however were in contrast to these results[18].

Apart from *An. maculatus* of Kampung Sg Lui, Selangor, *An. maculatus* of Kg Sokor, Kelantan was also detected as “confirmed resistance” towards 4% DDT in the current study. These finding however were not in agreement with study conducted in the same state but involving different areas namely Gua Musang and Jeli[18]. In that particular study the same anopheline species exhibited only “possible resistance” towards 4% DDT with mortality of 90.7% and 96.7%, respectively. Nonetheless it is not impossible that the resistance status in Kelantan has changed from possible resistance to confirmed resistance.

It is also interesting to note that *An. maculatus* from Kampung Sokor, Kelantan was found having “possible resistance” (96% of mortality) against 0.05% deltamethrin. The fact that Kelantan is sharing its border with Thailand, this result was found in agreement to the study by Chaumeau *et al.*[19]. In that study *An. maculatus* collected from Thailand-Myanmar border also demonstrated “possible resistance” towards deltamethrin and permethrin (85% and 97% mortality respectively)[19]. In another study on *An. maculatus* from Northern Thailand 74% to 92% mortality towards methyl parathion was indicated[20]. These studies together with result obtained from the current study strongly suggest that resistance has developed in the area around the Thailand border.

In addition to susceptibility test, biochemical enzyme assay was also conducted in the adult and larval stages of these mosquitoes. Development of physiology resistance to insecticide is accounted for increased detoxification activity and over expression of enzyme. Chareonviriyaphap *et al.*[21] reportedly showed that oxidases are the major enzyme contributors to pyrethroid resistance in metabolic mechanism of resistance in *Anopheles minimus* of susceptible and resistant colonies in Thailand. This is in accordance with the current findings where “possible resistance” or “confirmed resistance” detected were associated with elevation of enzyme activity as shown by *An. cracens* of Kampung Sahom strain that exhibited highest level of enzyme activity for MO, NSE and AchE. Similarly *An. maculatus* of Kampung Sg. Lui strain

(having high MO and AchE enzyme activity).

Based on the evaluation criteria of WHO[22], it is concluded that most of the field strains tested remain susceptible towards DDT, malathion and deltamethrin, except strains from Kampung Sungai Ular (Pahang), Kampung Sg. Lui (Selangor) and Kampung Sokor (Kelantan). Further investigation is needed to confirm the insecticide susceptibility of *An. maculatus* of the neighbouring areas. On the other hand, this is the first report of insecticide susceptibility of *An. cracens* and biochemical detection of resistance mechanism in *An. maculatus* and *An. cracens* in Malaysia. The data will serve as important reference for further monitoring and planning of counter measures to ensure continued effectiveness of chemical insecticides used in malaria vector control.

Conflict of interest statement

We declare that we have no conflict of interest.

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