PREVALENCE OF *PLASMODIUM FALCIPARUM KELCH13* POLYMORPHISMS IN MALAYSIA (2008 - 2017)

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Abstract. Artemisinin combination therapies (ACTs) are recommended by the World Health Organization for treatment of uncomplicated malaria caused by *Plasmodium falciparum*. However, artemisinin resistance in the Greater Mekong Subregion was detected in 2008 and has since spread to the other parts of the region. Mutations in the propeller domain of *P. falciparum* kelch-13 protein (pfk13) serve as molecular markers for partial artemisinin resistance (delayed parasite clearance). Prevalence of *pfk13* propellar domain mutations/substitutions in 125 archived diagnostic blood samples in Malaysia from 2008 to 2017 were determined by nested-PCR and direct sequencing. Pfk13, C580Y and P553L mutations, previously confirmed and validated as markers for artemisinin resistance, were found in two samples; N537I and A675V mutations, classified as candidates/associated with delayed parasite clearance, in three samples; and eight novel substitutions, with seven sequences containing two amino acid changes. These findings constitute baseline data and further investigations are needed to correlate amino acid changes present in *pfk13* propellar domain with delayed parasite clearance, *in vitro* and *ex vivo* parasite artemisinin sensitivity.

Keywords: *Plasmodium falciparum*, artemisinin resistance, genotyping, *kelch13* mutation, Malaysia

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INTRODUCTION

There is a long history of malaria from the beginning of parasite species identification, parasite life cycle, discovery of antimalarials, deployment of antimalarial drugs, mechanism of antimalarial action, and up to evolution of resistance (Bruce-

Chwatt, 1962; Coatney *et al*, 1971; Bruce-Chwatt, 1981), and yet it is still a worldwide burden with 219 million cases and estimated deaths of 435,000 in 2017 (WHO, 2018a). Malaria parasites responsible for human malaria are *Plasmodium falciparum* (most virulent), *P. knowlesi* (most recent discovery) *P. malariae*, *P. ovale* and *P. vivax* (Chin *et al*, 1965; Garnham, 1966; Coatney *et al*, 1971; Singh *et al*, 2004).

Human malaria parasites, particularly *P. falciparum*, consistently evolve resistance to widely used antimalarials, with the first report in 1910 against quinine (da Silva and Benchimol, 2014; Duru *et al*, 2016), followed in 1960 against chloroquine (Young and Moore, 1961; Montgomery and Eyles, 1963) and resistance phenotypes have since spread to almost all regions that are endemic for *P. falciparum* infection including Malaysia (Zhao *et al*, 2019; He *et al*, 2019; Wongsrichanalai and Meshnick, 2008; Severini and Menegon, 2015; Norahmad *et al*, 2016). Currently, chloroquine is no longer used in treating *P. falciparum* infection and recently artemisinin combination therapy (ACT) has been recommended as first-line drug treatment for falciparum malaria (WHO, 2015; WHO, 2018a).

ACT is a combination of artemisinin (or a number of analogs), a fast-acting, but rapidly detoxified, sesquiterpene lactone containing an endoperoxide 1,2,4-trioxane ring, responsible for its mechanism of action, together with another anti-malarial drug of a different class that has a slower elimination profile (WHO, 2015; WHO, 2018a; WHO, 2018b). WHO (2015) recommends the following ACTs to treat uncomplicated falciparum malaria based on their effects on *P. falciparum* local strains: artemether-lumefantrine, artesunate-amodiaquine, artesunate-mefloquine, artesunate-sulfadoxine-pyrimethamine, dihydroartemisinin-piperaquine and Pyronaridine- artesunate fixed-dose combination (Pyramax®; WHO, 2017). For the time being, ACT appears to be the most effective anti-malarial treatment for *P. falciparum* infection because there is no other alternatives to match its efficacy (WHO, 2015).

However, *P. falciparum* resistance (delayed parasite clearance) to artemisinin and its partner drugs has been reported in countries of the Greater Mekong Subregion (GMS), namely, Cambodia (Amaratunga *et al*, 2012; Ashley *et al*, 2014), China (Huang *et al*, 2015), Laos (Iwagami *et al*, 2018), Myanmar (Kyaw *et al*, 2013; Ashley *et al*, 2014; Tun *et al*, 2015), Thailand (Phyo *et al*, 2012; Ashley *et al*, 2014; Imwong *et al*, 2015), and Vietnam (Hien *et al*, 2012; Ashley *et al*, 2014; Thriemer *et al*, 2014; Thuy-Nhien *et al*, 2017). Hence, the efficacy of artemisinin and its partner drugs in neighboring countries of GMS, including Malaysia, needs to be monitored even though there have been no reports of delayed parasite clearance pertaining to the use of ACTs thus far (WHO, 2018b).

Throughout history, it had been apparent that one of the major challenges facing application of anti-malarial drugs to eradicate malaria stems from emergence of drug resistance in parasites (Menard and Dondorp, 2017). Hence, collecting data for monitoring of drug resistance and its distribution is an urgent need (Fairhurst, 2015; Fairhurst and Dondorp, 2016). At present, specific *P. falciparum* genes associated with development of resistance to anti-malarial drugs have been identified to carry point mutation. Polymorphisms in propeller domain of *P. falciparum kelch13* (*pfk13*) have been identified in artemisinin-related parasite delayed clearance (Ariey *et al*, 2014). These include F446I, N458Y,

Y493H, C580Y, M476I, R539T, I543T, P553L, and R561H (Ariey *et al*, 2014; Straimer *et al*, 2015; WHO, 2018b; Zaw *et al*, 2017). Identification of other *pfk13* single nucleotide polymorphisms (SNPs) will inevitably be reported but these should be accompanied by demonstration of change(s) in parasite phenotype related to artemisinin resistance.

Malaysia in 2013 adopted use of ACTs as the preferred treatment for uncomplicated malaria infection for all plasmodium species (Ministry of Health Malaysia, 2013), but it is worrisome that this resistance may have started to manifest in the country. Norahmad *et al* (2016) addressed issues on drug resistance genes in Sabah, in particular *P. falciparum* multidrug resistance gene (*pfmdr*) and chloroquine transporter gene (*pfcrt*), but failed to detect polymorphisms in *pfk13* propeller region, but this might be due to insufficient sample size and study area as the survey is only limited to Kota Marudu and Kalabakan district, representing only two of the 25 districts in Sabah state (Malaysian Borneo) of Malaysia.

In Malaysia, artemisinin resistance or the distribution of mutations in its associated or identified markers is still not well investigated. Thus, further research is warranted to determine the current status of artemisinin resistance by continuously surveying the prevalence of molecular markers so as to realize Malaysia's aim to eliminate human malaria by the year 2020 (Ministry of Health Malaysia, 2013). The study determined the distribution of artemisinin drug resistance *pfk13* propellar domain SNPs among confirmed malaria-positive samples in Malaysia.

MATERIAL AND METHODS

Study design and sample collection

The prevalence study was conducted based on the availability of samples collected and related information. Samples were selected from archived diagnostic samples collected by the Parasitology Unit, Institute for Medical Research (IMR) from all states in Malaysia from 2008 to 2017. Selection was based on species-specific identification by nested-PCR (Singh *et al*, 1999; Singh *et al*, 2004) and only samples with mono-infection with *P. falciparum* were chosen for this study. Samples were either anticoagulated venous blood and/or dried blood spot (DBS) on filter paper of *P. falciparum* confirmed cases.

Approval for this study was obtained from the Medical Research and Ethics Committee, Ministry of Health Malaysia (Ref no. NMRR-17-757-35548 dated 27 July 2017, Ref (12) KKM/NIH-SEC/P17-898).

Amplification of *pfk13* fragment and sequencing

DNA was isolated from blood samples using a spin column protocol (QIAamp Blood Mini® Kit; Qiagen, Hilden, Germany) and QIAamp® DNA Mini Kit (Qiagen) for venous blood and DBS samples respectively. Genotyping of *pfk13* propeller domain was carried by a nested-PCR protocol previously described by Talundzic *et al* (2015a) and modified for quantitative (q)PCR (Rubio JM, unpublish protocol). Reaction mixture contained 50-200 ng DNA, 2-4 mM dNTPs, 0.1-0.3 μM primers Pfa-k13-3-2PCR/F (5′-GCCTTGTTGAAAGAAGCAGA-3′) and Pfa-k13-2-2PCR/R (5′-GCCAAGCTGCCATTCATTTG-3′), 50 mM $MgSO_4$, 2X Hotsplit polymerase buffer (Biotools, Madrid, Spain), 4 nM EvaGreen® dye (Biotium, Hayward, CA, USA) and distilled H_2O to a final volume of 20 μl. Thermocycling was

performed using a Rotor-Gene® Q MDx (Qiagen, Germany) as follows: 95ºC for 10 minutes, followed by 45 cycles of 95ºC for 15 seconds, 56ºC for 15 seconds and 70ºC for 60 seconds. Specificity of amplification was demonstrated by a single melting temperature of 0.5ºC. Amplicons were then purified using a QIAquick PCR purification kit (Qiagen) and directly sequenced (Apical Scientific Sdn Bhd, Selangor, Malaysia). Positive controls were DNA extracted from six *P. falciparum* strains with known *pfk13* alleles (kindly provided by Prof. Didier Menard from the Institut Pasteur du Cambodge, Cambodia). Sequences were analyzed with BLAST program (http://blast.ncbi.nlm.nih.gov/). Multiple nucleotide sequence alignments and analysis were performed using a BioEdit sequence Alignment Editor version 7.0.5.3 (Ibis Therapeutics, Carlsbad, CA, USA). Nucleotide sequences of *kelch*, gene ID PF3D7_1343700 (for *P. falciparum*), PVX_083080 (for *P. vivax*), and AM910994.1 (for *P. knowlesi*) were used as reference sequences.

RESULTS

Parasite isolates

Archived clinical diagnostic samples from 2008 to 2017 were screened using microscopy for detection of plasmodium infection and confirmed at species level by nested-PCR assay. Samples (n = 185) with only *P. falciparum* infection were selected; these originated from Peninsular Malaysia (East, n = 38; West, n = 28; South, n = 60; and North, n = 26) and Malaysian Borneo (East Malaysia) (n = 33). All samples were subjected to amplification of *pfk13* fragment (849 bp) and samples successfully amplified (n = 125) were sequenced.

Distribution of *K13* mutant allele

Among the 125 *pfk13* propellar domain sequences from 17 samples, 16 different non-synonymous substitutions were obtained, 50% being novel (Table 1). Ten sequences harbored one substitution, while 7 contained two substitutions (Table 2). Two samples contained *k13* mutations associated with artemisinin resistance, while three carried a mutation classified as "*k13* validated and candidate/associated" conferred to artemisinin resistance (WHO, 2018b). The validated mutations conferring artemisinin resistance from the present study were P553L and C580Y, present in one sample each from East Coast and South Peninsular Malaysia respectively. In addition, mutations N537I and A675V, implicated as candidates or associated with resistance, was identified in two samples from East Coast Peninsular Malaysia and in one sample from South Peninsular Malaysia respectively (Table 1). The most frequent substitutions (5/17, 29%) among the samples was S600C, one sample with only this substitution and the remaining harboring another change (Table 2).

DISCUSSION

Prior studies on the distribution and frequency of pfk13 propellar mutations have been documented from Southeast Asian countries (Iwagami *et al*, 2018; Thuy-Nhien *et al*, 2017; Ménard *et al*, 2016; Imwong *et al*, 2015; Ashley *et al*, 2014; Thriemer *et al*, 2014; Kyaw *et al*, 2013; Tun *et al*, 2015; Amaratunga *et al*, 2012; Phyo *et al*, 2012; Hien *et al*, 2012), Africa (Boussaroque *et al*, 2016; Muwanguzi *et al*, 2016), South America (Chenet *et al*, 2016), Grande Comore (Huang *et al*, 2015a) and China (Huang *et al*, 2015b). With the growing list of *pfk13* mutant alleles

Table 1
Plasmodium falciparum kelch13 propeller domain variants detected in infected blood samples in Malaysia (2008 - 2017).

Substitution	Samples with propeller mutations (n = 125)(%)	Reference
G453A	1 (0.8)	This study
L462I	1 (0.8)	This study
D464E	1 (0.8)	This study
Y500N	1 (0.8)	This study
T593I	1 (0.8)	This study
S600C	5 (4.0)	This study
G674A	1 (0.8)	This study
A675L	1 (0.8)	This study
V494I	2 (1.6)	Escobar *et al* (2015) (Mozambique)
N537I[a]	1 (0.8)	Ariey *et al* (2014) (Cambodia); Feng *et al* (2015) (China-Myanmar border); Tun *et al* (2015) (Myanmar)
P553L[b]	1 (0.8)	Ariey *et al* (2014) (Cambodia)
R575K	1 (0.8)	Talundzic *et al* (2015b) (Thailand)
C580Y[b]	1 (0.8)	Chenet *et al* (2016) (Guyana); Ariey *et al* (2014) (Cambodia); Ashley *et a*l (2014); WHO (2018b) (mainly in Greater Mekong Subregion)
D584V	1 (0.8)	WHO (2018b)
L598G	1 (0.8)	WHO (2018b)
A675V[a]	1 (0.8)	Tun *et al* (2015) (Myanmar)

[a]Candidate of or associated with artemisinin resistance.
[b]Validated and confirmed conferring artemisinin drug resistance.

being reported (WHO, 2018a), none was reported from Malaysia to date. ACT was deployed in Malaysia as first line treatment for uncomplicated malaria since 2013 (Ministry of Health Malaysia, 2013). Drug Response Surveillance (DRS) Program was started as a pilot program in 2014 in Malaysia and become a national policy in 2015. Since then, the country has seen a substantial number of cases with delayed parasite clearance in ACT each year. In 2014, 26 microscopically examined cases were positive on day 3 (D3) after treatment with ACT, 22 cases in 2015, 38 cases in 2016, 204 cases in 2017 and 63 in 2018 (up to August). The increasing trend of delayed parasite clearance on D3 is worrying even though parasite are eventually cleared on D28 (Jelip, 2018). Although, all cases were considered as being artemisinin partial-resistant and do not necessarily lead to treatment failure as defined by WHO

Table 2
Plasmodium falciparum kelch13 propeller domain variants containing two substitutions from infected blood samples from various regions of Malaysia (2008 - 2017)

Sample origin	Sample ID	Substitution
North Peninsular Malaysia	F4	L598G, S600C
	F6	S600C, A675V[a]
	09/195	D584V
	10/45	R575K
	10/119	G453A
East Coast Peninsular Malaysia	F2	N537I[a], S600C
	F22	P553L[b]
	10/109	D464E
	11/352	L462I, G674A
South Peninsular Malaysia	F57	T593I
	F12	S600C, A675L
	16/651	C580Y[b]
	14/410	G674A
West Peninsular Malaysia	11/149	D452E, G674A
Malaysian Borneo	F17	S600C
	F50	V494I
	14/63	V494I, Y500N

[a]Candidate of or associated with artemisinin resistance.
[b]Validated and confirmed conferring artemisinin drug resistance.

(2018b), however this situation cannot be ignored, including the role of resistance to artemisinin partner drugs.

The study provides baseline data on the prevalence of *pfk13* variants in Malaysia. Although the frequency of validated and confirmed mutations detected were low compared to other countries, of the nine Asian *pfk13* mutations validated *in vitro* (F446I, N458Y, Y493H, R539T, I543T, P553L, R561H, C580Y and M476I (Ariey *et al*, 2014; Straimer *et al*, 2015; Tun *et al*, 2015; Ménard *et al*, 2016; Thuy-Nhien *et al*, 2017; Iwagami *et al*, 2018; WHO, 2018b), two validated and confirmed mutations (P553L and C580Y) were detected in the collection of isolates in Malaysia, including two mutations (N537I and A675V) classified as candidates for conferring artemisinin resistance.

There are a number of limitations to the present study. This study was using an archived diagnostic samples with no data on day of blood collection (*ie* before or on day-3 of drug treatment). Future studies will concentrate on collecting and analysing blood samples from the DRS program as well as new samples suspected to have partial artemisinin resistance together with demographic data and locality of the cases. Low numbers of samples successfully amplified for *pfk13* propellar domain and sequencing might be due poor DNA yields. Although there are no different between DNA

extracted from dried blood spot and whole blood, there are several factors that could contribute to poor DNA yield such as impact of storage condition, the time duration of the storage, and types of filter paper used (Chaisomchit *et al*, 2005; Halsall *et al*, 2008; Strom *et al*, 2014). Future studies will employ blood samples as it will also allow correlation of delayed parasite clearance with assay for status of parasite artemisinin resistance phenotype and *in vitro* transfection studies.

The Ministry of Health Malaysia (MOH) should take into consideration various factors to prevent spread of artemisinin resistance before reaching the country. The findings from such studies can help guide Malaysian MOH to prioritize strategies to prevent the spread of artemisinin (and analogs) resistance. Implementation of actions to delay emergence of artemisinin drug resistance, such as strengthening DRS programs, monitoring correct dosing of ACT, and improving lack of compliance to the full 3-day ACT treatment, poor follow up of the cases and identifying/destroying fake ACT compound, should be carried out. Tulloch *et al* (2013) have laid out a comprehensive plan of action addressing artemisinin resistance, especially in the Asia-Pacific region, such as assessing, containing, seeking suggestions and investments, creating integrated collaborations, and conducting research and development. Fairhurst and Dondorp (2016) more recently suggested other aspects worthy of investigation, such as understanding molecular mechanism of some *pfk13* propeller mutations that confer artemisinin resistance and gathering information on the fitness of artemisinin-resistant *P. falciparum* to be transmitted by native or non-native *Anopheles* spp.

In conclusion, although, artemisinin-resistant *P. falciparum* malaria has spread across the countries of Greater Mekong Subregion (Cambodia, Laos PDR, Myanmar, Thailand, Vietnam and China), Malaysia is, for now, free of artemisinin resistance, but episodes of delayed parasite clearance with ACT were observed in 2016. In order to eliminate the spread of artemisinin resistance within the country and to prevent import of such parasites as well as delaying development of resistance among native parasites, it is vital to come up with workable strategies. Findings from this study should provide background data towards achieving the goal of eliminating malaria in Malaysia by the year 2020.

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