

Detection of mutated *Plasmodium vivax* Kelch Propeller Domain (PvK12) in Malaysian isolates

Shairah, D.¹, Fong, M.Y.¹, Amirah, A.¹, Ponnampalavanar, S.², Cheong, F.W.¹ and Lau, Y.L.^{1*}

¹Department of Parasitology, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia

²Department of Medicine, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia

*Corresponding author e-mail: lauyeeling@um.edu.my

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Abstract. Malaysia is located near the borders of countries where artemisinin resistant *Plasmodium falciparum* (mutations in the *P. falciparum* Kelch propeller domain [PfK13]) have been reported. *Plasmodium vivax* Kelch propeller domain, PvK12, the ortholog of PfK13, could assess resistance towards artemisinin in vivax malaria. Polymorphisms in PvK12 gene were determined by PCR and sequencing was done in 300 clinical isolates collected in recent years (2012–2017) from hospitals within the country. Among 48 *P. vivax* samples, all were Sal-1 wild type alleles except for two isolates, a synonymous and nonsynonymous mutation respectively. The nonsynonymous (V552I) isolate was collected from an immigrant. *P. vivax* with mutated PvK12 is speculated to be an imported case and is likely to be circulating at very low frequency in Malaysia. An in-depth drug resistance surveillance among immigrants needs to be investigated to provide information that may be crucial for drug policy changes.

INTRODUCTION

Artemisinin-combination therapies has been implemented as the first-line treatment in most malaria endemic countries, albeit, there has already been reports of artemisinin-resistant *P. falciparum* spreading in Greater Mekong Subregion (Ashley *et al.*, 2014; Hien *et al.*, 2012; Kyaw *et al.*, 2013; Noedl, 2008; Phyto, 2012; WHO, 2016). Molecular marker for artemisinin resistant *P. falciparum*, PfK13 is closely correlated with delayed rate of parasite clearance in patients treated with artemisinin (Ariey *et al.*, 2014 & Ashley *et al.*, 2014). Mutation in PvK12, the orthologue of PfK13, may mediate artemisinin resistance in *P. vivax* (Popovici *et al.*, 2015). The aim of this study was to investigate the polymorphisms of PvK12 gene and to determine the proportion of *P. vivax* parasites with mutant alleles in Malaysia.

METHODS

A total of 300 blood samples of malaria patients were collected from 10 states and district government hospitals in Peninsular and East Malaysia between the years 2012–2017. Diagnosis of *Plasmodium* spp. infection, was determined by microscopic examination and species-specific nested-PCR assays (Singh, 2004).

A single step PCR was conducted on 48 *P. vivax* positive samples for the amplification of *pvk12* (from codons 370 to 702) using a set of published primers, K12-F (5'-ACCACGTGACGAGGGATAAG-3') and K12-R (5'-AAAACGGAAATGTCCAAATCG-3') (Popovici *et al.*, 2015). The amplification was carried out under the following conditions: 95°C for 15 min, followed by 35 cycles at 95°C for 30 s, 62°C for 60 s, 72°C for 90 s, finally at 72°C for 10 min. PCR products were analyzed by electrophoresis using 2% agarose gel and sequenced.

Sequences were aligned and analyzed using BioEdit Version 7.2.5 Sequence Alignment Editor Software. Amino acid sequences were compared with PvK12 wild-type sequence (XM_001614165.1) where substitutions, insertions and deletions were verified manually.

RESULTS

Most isolates had Sal-1 wild-type alleles except two isolates which showed a synonymous and non-synonymous haplotype, respectively. The synonymous haplotype C1569T was seen in isolate UM0127 (Figure 1). Meanwhile, non-synonymous G1654A haplotype was seen in isolate PPNG 0002 resulting in a monomorphic amino acid change (V552I) (Figure 1 and Figure 2). UM0127 was isolated in 2015, from a male Chinese Malaysian who lived in Papua New Guinea for 10 years and had returned to Malaysia one week before he presented with symptoms of malaria. PPNG 0002 was isolated from a female Vietnamese patient in 2015.

DISCUSSION

The main reason for the failure of the Global Malaria Eradication Program is due mainly to drug resistant parasites (Nájera *et al.*, 2011; Sinha *et al.*, 2014; Smith *et al.*, 2014). Population movement from high to non-

endemic malaria areas can lead to imported infections, and continuing transmission (Martens, 2000). The relationship between migration and the spread of malaria is clearly seen where 98.8% of total reported malaria cases in Yunnan were due to imported cases from neighboring countries (International Organization for Migration, 2013; WHO, 2010). Malaysia is also at risk since it is a focal point for labor migration from neighboring countries. Illegal immigrants specifically, do not undergo health-screening programs for communicable diseases. Consequently, they may introduce or transmit drug-resistant malaria into the country. A study in Perak, found a high percentage of positive cases of malaria among workers from Indonesia (15.4%) and Myanmar (7.1%) and that vivax malaria prevalence was higher among foreign workers (Isa *et al.*, 2015). They have the possibility to spread the disease to local population thus causing an epidemic. More recently, a *P. vivax* outbreak occurred in Pos Kemar, Hulu Perak, with a total of 137 people infected (Perak State Health Department, 2017). The main risk factor identified from the outbreak was the entry of foreign workers into the village (Hospital Bahagia Ulu Kinta, 2016). It could be speculated that immigrants are responsible for the 84 imported *P. vivax* cases into Malaysia in 2015 (WHO, 2016).

In the current study, V552I mutation detected in isolate PPNG 0002, was also reported in Cambodia (Popovici *et al.*, 2015). This is in agreement with Popovici *et al.*

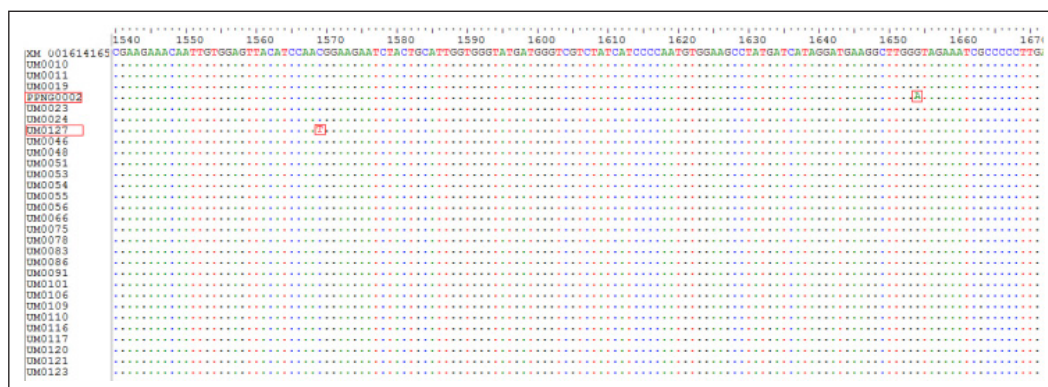


Figure 1. Nucleotide sequences polymorphism in *pvk12*. The mutations detected in PPNG 0002 (G1654A) and UM 0127 isolates (C1569T) are highlighted in red box.



Figure 2. Amino acid sequences polymorphism in PvK12. The V552I mutation detected in PPNG 0002 isolate is highlighted in red box.

(2015) where limited polymorphism of PvK12 was circulating in low frequency in Cambodia. Two isolates showed V552I, despite having high prevalence of Pfk13 mutation in the country (Popovici *et al.*, 2015). According to Wang *et al.* (2016), low levels of genetic diversity were also seen in the *pvk12* gene in South East Asia (Thailand, Myanmar, and Central China). One isolate showed nonsynonymous mutation (G581R), however the mutation has not been associated with artemisinin resistance (Wang *et al.*, 2016). Deng *et al.* (2016) also reported low prevalence of *pvk12* polymorphisms in the China-Myanmar border. Two isolates showed nonsynonymous mutation (M124I) in the PvK12 but since M124I is located outside of the propeller domain, it is unlikely to be involved in artemisinin resistance (Deng *et al.*, 2016).

CONCLUSIONS

Despite limited clinical and *in vitro* drug susceptibility data, this is the first report and

detection of PvK12 mutations in Malaysia. The genotyping results from this study may provide fundamental information, and perhaps, together with a future phenotypic study may be used to assess whether this V552I mutation is suitable for artemisinin-resistance marker in *P. vivax*. To date, there is no report of artemisinin-resistant *P. vivax*. Although PvK12 mutation may or may not confer resistance to artemisinin, it cannot be ruled out as a possible precursor (Popovici *et al.*, 2015). This warrants continuous in-depth surveillance to monitor this molecular marker, in concurrent with malaria elimination campaign to achieve a malaria free status.

AUTHOR'S CONTRIBUTIONS

SD, AA, MYF and YLL conceived the study and designed the study protocol; SD carried out the field study; SD, AA, MYF, FWC and YLL analyzed and interpreted data. SD drafted the manuscript; MYF, AA, SP, FWC and YLL critically revised the manuscript for intellectual content. All authors read and

approved the final manuscript. YLL and MYF are the guarantors of the paper.

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COMPETING INTEREST

The authors declare no conflicts of interest.

ETHICAL APPROVAL

Use of human samples in this study was approved by the University of Malaya Medical Centre Medical Ethics Committee (MEC Ref. No: 817.18) and Medical Research & Ethics Committee, Ministry of Health Malaysia (NMRR-15-67223975).

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