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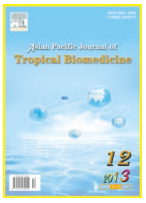

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Polymorphic patterns of *pfcrt* and *pfmdr1* in *Plasmodium falciparum* isolates along the Thai-Myanmar border

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PEER REVIEW

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Comments

This study is very interesting and applicable that established prevalence of *pfcrt* and *pfmdr1* polymorphims in provinces along Thai-Myanmar border after chloroquine withdrawal and estimately 15 years of artesunate and mefloquine usage. Application of well known drug resistance molecular markers; *pfcrt* and *pfmdr1* could be applied for surveillance of chloroquine, artesunate and mefloquine resistance progression among the four provinces. Furthermore, the degree of resistance estimated from this study is advantage to classify requirement of intensive monitoring in each location.
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ABSTRACT

Objective: To investigate the distribution and patterns of *pfcrt* and *pfmdr1* polymorphisms in *Plasmodium falciparum* (*P. falciparum*) isolates collected from the malaria endemic area of Thailand along Thai-Myanmar border.

Methods: Dried blood spot samples were collected from 172 falciparum malaria patients prior received treatment. The samples were extracted using chelex to obtain parasite DNA. PCR-RFLP was employed to detect *pfcrt* mutation at codons 76, 220, 271, 326, 356 and 371, and the *pfmdr1* mutation at codon 86. *Pfmdr1* gene copy number was determined by SYBR Green I real-time PCR.

Results: Mutant alleles of *pfcrt* and wild type allele of *pfmdr1* were found in almost all samples. *Pfmdr1* gene copy number in isolates collected from all areas ranged from 1.0 to 5.0 copies and proportion of isolates carrying>1 gene copies was 38.1%. The distribution and patterns of *pfcrt* and *pfmdr1* mutations were similar in *P. falciparum* isolates from all areas. However, significant differences in both number of *pfmdr1* copies and prevalence of isolates carrying>1 gene copies were observed among isolates collected from different areas. The median *pfmdr1* copy number in *P. falciparum* collected from Kanchanaburi and Mae Hongson were 2.5 and 2.0, respectively and more than half of the isolates carried>1 gene copies.

Conclusions: The observation of *pfmdr1* wild type and increasing of gene copy number may suggest declining of artesunate-mefloquine treatment efficacy in *P. falciparum* isolates in this border area.

KEYWORDS

Plasmodium falciparum, Multidrug resistance, *Pfcrt*, *Pfmdr1*, Gene mutation, Gene copy number

1. Introduction

Malaria is one of the major infectious diseases that causes a number of deaths in tropical and subtropical countries. In Thailand, the mortality rate had raised to 36 per 100 000 population in 1958, but continuously declined after the launch of malaria control program[1,2]. Most of the affected populations are those who reside in/near forests and hilly areas along the international borders. The highest incidence has been reported from areas bordering

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Thai-Myanmar, followed by Thai-Malaysia, Thai-Cambodia and Thai-Laos PDR. A serious problem that limits the effectiveness of malaria control program of the country is the emergence and spread of multidrug resistance *Plasmodium falciparum* (*P. falciparum*)[3,4]. To deal with the situation, the artemisinin-based combination therapy, a three-day artesunate-mefloquine combination is currently being used as first-line treatment of multidrug resistance *P. falciparum* according to recommendation of World Health Organization[4].

The gold standard for monitoring antimalarial drug efficacy mainly relies on *in vivo* investigation with supplemented information of *in vitro* parasite susceptibility. In recent years, attempt has been made to apply valid molecular markers of antimalarial drug resistance to predict treatment outcome following treatment with an antimalarial drug regimen[5]. The two candidate malarial parasite genes, *P. falciparum* chloroquine resistant transporter (*pfcrt*) and *P. falciparum* multidrug resistance 1 (*pfmdr1*) that express the transport proteins on the plasma membrane of the parasite's food vacuole *pfcrt* and *pfmdr1*, respectively, have been confirmed to link with resistance of the parasite to antimalarial drugs[6]. Mutation of *pfcrt* associated with chloroquine resistance in *P. falciparum* and distinct genotype polymorphisms depends on its origination. Most of *P. falciparum* isolates collected from Thailand carry *pfcrt* mutations at codons K76T, A220S, Q271E, N326S and R371I[7]. The mutation at codon 86 of *pfmdr1* (86Y) related with chlolroquine resistance, while *pfmdr1* wild type at the same codon (N86) including increased *pfmdr1* gene copy number linked to resistance of the parasite to mefloquine and artesunate[8-10]. The aim of the present study was to investigate the distribution and patterns of *pfcrt* and *pfmdr1* polymorphisms in *P. falciparum* isolates collected from the malaria endemic area of Thailand along Thai-Myanmar border.

2. Materials and methods

2.1. Sample collection and DNA extraction

A total of 172 *P. falciparum*-infected dried blood spot samples were collected prior to treatment, from patients with acute uncomplicated *P. falciparum* malaria during 2009-2010 from the four malaria endemic areas along Thai-Myanmar border of Thailand, *i.e.*, Mae Hongson (MH, 41 samples), Tak (TK, 82 samples), Kanchanaburi (KN, 6 samples) and Ranong (RN, 43 samples) provinces (Figure 1). Genomic DNA was extracted from each sample using chelex resin according to the previously described method[11].

Figure 1. Map of Thailand presenting the four malaria endemic areas along the Thai-Myanmar border.

2.2. Determination of pfcrt and pfmdr1 single nucleotide polymorphisms

PCR-RFLP was employed to detect *pfcrt* mutation at codons 76, 220, 271, 326, 356 and 371[12] and *pfmdr1* mutation at codon 86[13]. DNA of *P. falciparum* laboratory clones G112 and K1 served as control for chloroquine sensitive and chloroquine-resistant genotype, respectively.

2.3. Determination of pfmdr1 gene amplification

Pfmdr1 gene copy number in all samples was investigated by SYBR Green I real-time PCR[14]. DNA of 3D7 (1 *pfmdr1* copy number) and Dd2 (4 *pfmdr1* copy number) *P. falciparum* laboratory clones provided by Professor Dr. Steven A. Ward (School of Tropical Medicine, Liverpool, UK) were used as the internal control. The copy number was determined by relative quantification between *pfmdr1* (target gene) and *pf*β-*actin* (reference gene, an endogenous house-keeping gene which carries only a single copy) that was calculated using the comparative C_t method (also known as the $2^{-\Delta\Delta Ct}$ method).

2.4. Statistical analysis

Qualitative variables were summarized as proportions and

percentages, while quantitative variables were presented as median (95% *CI*). Differences among qualitative variables were determined using *Chi*-square test. Differences among quantitative variables were determined using Kruskal Wallis test. The statistical significance level was set at α=0.05 for all tests (SPSS version 17; SPSS, Chicago, Illinois, USA).

3. Results

3.1. Pfcrt and pfmdr1 single nucleotide polymorphisms

Mutations of the two candidate *P. falciparum* resistance genes, *i.e.*, seven codons of *pfcrt* (K76T, A220S, Q271E, A326S, I356T and R371I), one codon of *pfmdr1* (N86Y) were successful investigated in 172 *P. falciparum* isolates. The distribution and patterns of *pfcrt* and *pfmdr1* mutations were similar in *P. falciparum* isolates collected from the four different endemic areas (Figure 2). All isolates carried mutant allele of *pfcrt* at codons 76 and 371. The prevalence of mutations at codons 220, 271 and 326 was 99.4%, by only one isolate collected from Mae Hongson was wild type. The mutation at codon 356 was 98.8% of which wild type was observed in two isolates collected from Mae Hongson. In contrast, the *pfmdr1* mutation at codon 86 was identified in only two isolates (1.2%) which each from Mae Hongson and Ranong.

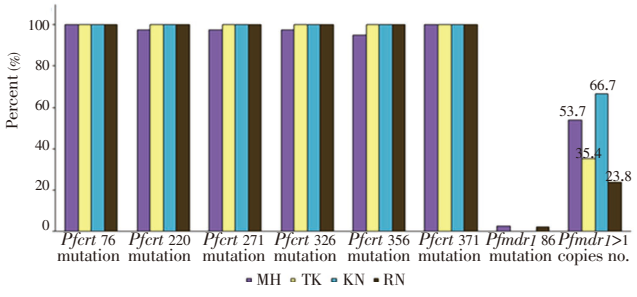

Figure 2. Proportions of *pfcrt* and *pfmdr1* polymorphisms in *P. falciparum* isolates from the four study areas.

3.2. Pfmdr1 gene amplification

Pfmdr1 copy number of 168 evaluable *P. falciparum* isolates collected from different areas has been summarized in Figure 2. The copy number ranged from 1.0 to 5.0 copies [median (95% *CI*)=1.0 (1.0-1.0) copies] and proportions of isolates carrying 1 and>1 gene copy number were 61.9% and 38.1%, respectively. Significant differences in both the number of gene copies (P=0.003) and prevalence of isolates carrying>1 gene copies (P=0.017) were observed among isolates collected from different areas. The median copy number and prevalence of isolates carrying more than one copy number were highest in samples from Kanchanaburi (median copy number=2.5, prevalence of isolates carrying >1 gene copies=66.7%), followed by Mae Hongson (median copy number=2.0, prevalence of isolates carrying>1 gene copies=53.7%), Tak (median copy number=1.0, prevalence of isolates carrying>1 gene copies=35.4%) and Ranong (median copy number 1.0, prevalence of isolates carrying>1 gene copies=23.8%).

4. Discussion

Information on distribution and patterns of antimalarial drug resistance is essential for implementation of effective malaria control program and disease surveillance in all malaria endemic areas of the world including Thailand. Early detection of occurrence and spreading of antimalarial drug resistance would be greatly enhanced by the application of valid antimalarial resistance molecular markers. Among the candidate genes investigated to date, *pfcrt* mutation is widely acceptable as a reliable marker of chloroquine resistance, while *pfmdr1* wild type including number of gene copies strong correlate to mefloquine and artesunate resistance in *P. falciparum*[15-17]. Despite the fact that chloroquine was withdrawn from treatment of falciparum malaria in Thailand for many decades, *pfcrt* mutation at least at the codon 76 was conserved in all *P. falciparum* isolates. This observation could be explained by a result of natural selection against chloroquine pressure which has been maintained in this area as chloroquine has been used as the first line treatment of *Plasmodium vivax* infection for more than six decades[4,18]. Furthermore the drug has always been prescribed in some case with *P. falciparum* infection due to misdiagnosis of *P. falciparum* with *Plasmodium vivax*, thus unintentionally exposing the *P. falciparum* parasite to subtherapeutic concentration of chloroquine[19,20].

Mefloquine was introduced for clinical use in Thailand since 1983, and since then, it has effectively select resistance genotype particularly the *pfmdr1* codon 86 wild type (N86) [21,22]. Extensive use of mefloquine in area along Thai-Myanmar border provided significant impact on the intensity and patterns of *P. falciparum* drug resistance genotypes, the *pfmdr1* mutation at codon 86 is gradually replaced by wild type (N86) genotype[21]. Prevalence of *pfmdr1* mutation at codon 86 in Tak and Kanchanaburi during 2001-2003 was 2.0% and 5.8%, respectively[23], and it was completely cleared from these areas in 2009-2010. Nevertheless, a number of isolates were found to carry mutant allele of *pfmdr1* codon 86, 1 isolate each from Mae Hongson and Ranong. This decreasing trend of *pfmdr1* mutation at codon 86 also observed in isolates collected from the Thai-Cambodia border by the prevalence of *pfmdr1* mutation at codon 86 was reduced from 11.4% during 1988-1993 to 4.8% in 2003[7].

A three-day combination regimen of artesunate-mefloquine has been adopted as first-line treatment of acute uncomplicated *P. falciparum* in Thailand since 2005[2]. The observation of good correlation between *pfmdr1* and declining of artesunate and mefloquine sensitivity supports results from the current study that both *pfmdr1* wild type at codon 86 and amplification of *pfmdr1* could apply as reliable tools for prediction of mefloquine and artesunate resistance[15,24].

With regards to *pfmdr1* copy number, the increase of copy number found in some *P. falciparum* isolates in this study correlates well with the observation of decline in sensitivity of *P. falciparum* to artesunate and mefloquine and therefore, the chance of recrudescence/treatment failure following

treatment with this artemisinin-based combination therapy regimen[25,26]. Application of the median copy number and prevalence of isolates carrying more than one gene copies can sort out the provinces with relatively high possibility of treatment failure and require close monitoring for drug resistance as Kanchanaburi, followed by Mae Hongson, Tak and Ranong, respectively.

As mefloquine resistance had occurred in these areas before the implementation of artesunate-mefloquine combination, emergence of *P. falciparum* resistance to artesunate is alarming and of greater concern since artesunate-mefloquine combination regimen is being used as first-line treatment for falciparum malaria in Thailand. In recent years, evidence of confirmed emerging resistance to artemisinins has been gathered from clinical studies in western Cambodia as well as the bordering areas between Thailand and Myanmar[27-30]. Furthermore, the observation of *pfmdr1* wild type and increasing of gene copy number may suggest declining of artesunate-mefloquine treatment efficacy in *P. falciparum* isolates in this border area. Continued monitoring of distribution and patterns of these two candidate molecular markers of antimalarial drug resistance is essential for adjusting the policy of malarial control program of the country.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

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Comments

Background

Malaria is usually found in area along Thai-international border. Several antimalarial drugs were used in malaria treatment due to spreading of multidrug resistance *P. falciparum*. The drug resistance *P. falciparum* developed by gene mutation and/or gene amplification. However, alteration of prevalence of polymorphisms always occurs after changed treatment policy.

Research frontiers

This work investigated prevalence of *pfcrt* and *pfmdr1* polymorphisms in *P. falciparum* collected from 4 provinces of malaria endemic area along Thai-Myanmar border during 2009-2010 using PCR-RFLP and real-time PCR. The prevalence of gene mutation and amplification including median copy number were compared among the sample collection areas.

Related reports

Food vacuole is a location for heam degradation. Drug accumulation in food vacuole correlates to treatment efficacy of chloquine, mefloquine and may also artesunate. Mutations of the drug transport proteins lead to resistance. Previous publications confirmed relationship between *pfcrt* mutations and chloroquine resistance while *pfmdr1* mutation at codon 86 and gene amplification contribute to declining of mefloquine and artesunate efficacy.

Innovations and breakthroughs

Using of *pfcrt* and *pfmdr1* polymorphims reveal *P. falciparum* resistance situation to chloroquine and artesunate-mefloquine in Kanchanaburi, Mae Hongson, Tak and Ranong during 2009-2010. Even the four provinces located at the same border, the prevalence of isolates carried more than one *pfmdr1* copy number including median copy number which linked to artesunate and mefloquine resistance, are significantly different among the areas that means to different degree of drug resistance.

Applications

Investigation of antimalarial resistance molecular markers of chloroquine including artesunate-mefloquine combination, *pfcrt* and *pfmdr1*, respectively can apply for monitoring of emerging of the antimalarial drug resistance and further adapt to estimate treatment efficacy of artesunate-mefloquine combination regimen indirectly instead of *in vivo* study. In addition, the information also useful in processes of treatment policy designs.

Peer review

This study is very interesting and applicable that established prevalence of *pfcrt* and *pfmdr1* polymorphims in provinces along Thai- Myanmar border after chloroquine withdrawal and estimately 15 years of artesunate and mefloquine usage. Application of well known drug resistance molecular markers; *pfcrt* and *pfmdr1* could be applied for surveillance of chloroquine, artesunate and mefloquine resistance progression among the four provinces. Furthermore, the degree of resistance estimated from this study is advantage to classify requirement of intensive monitoring in each location.

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