Parasitology International 67 (2018) 816–823

Contents lists available at ScienceDirect

Parasitology International

journal homepage: www.elsevier.com/locate/parint

Pfcrt genotypes and related microsatellite DNA polymorphisms on *Plasmodium falciparum* differed among populations in the Greater Mekong Subregion

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ABSTRACT

Malaria morbidity and mortality have decreased gradually in the Greater Mekong Subregion (GMS). Presently, WHO sets a goal to eliminate malaria by 2030 in the GMS. However, drug-resistant malaria has been reported from several endemic areas. To achieve the goal of elimination, the status of the emergence and spread of drug resistance should be monitored. In this study, the genotype of the *Plasmodium falciparum* chloroquine (CQ) resistance transporter gene (*pfcrt*) and 6 microsatellite DNA loci flanking the gene were examined. *P. falciparum* isolates (n = 136) was collected from malaria patients in Thailand (n = 50, 2002–2005), Vietnam (n = 39, 2004), Laos (n = 15, 2007) and Cambodia (n = 32, 2009). Amino acid sequences at codons 72–76 on the gene were determined. All of the isolates from Thailand were CQ-resistant (CVIET), as were all of the isolates from Cambodia (CVIET, CVIDT). Thirteen of the 15 isolates (87%) from Laos were CQ-resistant (CVIET, CVIDT), whereas the other 2 (13%) were CQ-susceptible (CVMNK). In contrast, 27 of the 39 isolates (69%) from Vietnam were CQ-susceptible (CVMNK), whereas the other 12 (31%) were CQ-resistant (CVIET, CVIDT, CVMDT) or mixed (CVMNK/CVIDT). The mean of expected heterozygosity of the microsatellite loci was 0.444 in the Thai population, 0.482 in the Cambodian population, and 0.734 in the Vietnamese population. Genetic diversity in the Thai population was significantly lower than that in the Vietnamese population. These results suggested that chloroquine selective pressure on *P. falciparum* populations is heterogeneous in the GMS. Therefore, further examination to understand the mechanisms behind the emergence and spread of drug-resistant malaria are needed.

Abbreviations: CQ, chloroquine; DNA, deoxyribonucleic acid; GMS, Greater Mekong Subregion; Lao PDR, The Lao People's Democratic Republic; PCR, polymerase chain reaction; *pfcrt*, *Plasmodium falciparum* chloroquine resistance transporter; WHO, World Health Organization

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https://doi.org/10.1016/j.parint.2018.08.008
Received 3 September 2017; Received in revised form 16 May 2018; Accepted 26 August 2018
Available online 28 August 2018
1383-5769/ © 2018 Published by Elsevier B.V.

1. Introduction

Malaria morbidity and mortality have decreased gradually in the Greater Mekong Subregion (GMS) due to the extensive efforts of each government in the Mekong countries along with the support of development partners such as the World Health Organization (WHO) and the Global Fund to Fight AIDS, Tuberculosis and Malaria [1]. Presently, WHO has set a goal to eliminate malaria by 2030 in the GMS. Even though the burden of malaria in the GMS has decreased, malaria is still prevalent, especially in remote, hard-to-access villages. Recently, molecular diagnostic methods such as polymerase chain reaction (PCR) revealed that there are a certain number of asymptomatic carriers (or parasite reservoirs, hidden malaria) in the malaria-endemic areas [2–4]. Moreover, drug-resistant malaria is reported from almost everywhere in the region [5–7]. Currently, artemisinin-based combination therapy is the first-line treatment for uncomplicated malaria in the region. However, the first case of artemisinin resistance and delayed parasite clearance was reported from Pailin, Cambodia near the border with Thailand in 2009 [8]. Thereafter, artemisinin resistance was reported from several endemic areas in Thailand, Vietnam, Myanmar, and Lao PDR [9–13].

To achieve malaria elimination in the region, the precise status of the emergence and spread of drug-resistant malaria should be fully understood for each antimalarial drug and each endemic area. Therefore, in the present study, the status of chloroquine (CQ)-resistant *Plasmodium falciparum* was examined in four countries, Thailand, Vietnam, Lao PDR, and Cambodia, because CQ was used for long time in the region.

A mutation in the *P. falciparum* CQ resistance transporter (*pfcrt*) gene at codon 76 (K76 T) is associated with CQ resistance and used to monitor the distribution and frequency of CQ-resistant falciparum malaria [7,14–16]. Microsatellite deoxyribonucleic acid (DNA) polymorphisms flanking the drug-resistant genes can be used to study the evolution of the genes [17]. In the present study, to understand the status and dissemination pattern of CQ-resistant malaria in the GMS, the frequency of mutations at codons 72–76 in the *pfcrt* gene and the microsatellite polymorphisms flanking the gene were examined using *P. falciparum* isolates collected from Thailand, Vietnam, Lao PDR, and Cambodia.

2. Materials and methods

2.1. Sample collection

A total of 136 *P. falciparum* isolates was collected from Thailand, Vietnam, Lao PDR, and Cambodia (Fig. 1). From a Thailand, 50 *P. falciparum* isolates were collected from malaria patients along the Thai-Myanmar border who had been admitted to the Bangkok Hospital for Tropical Diseases, Faculty of Tropical Medicine, Mahidol University during 2002 and 2005. About 2 ml of venous blood was taken from the malaria patients at the hospital and stored at -30 °C until DNA analyses. From Vietnam, 39 *P. falciparum* isolates were collected in villages near the Cambodian border in Binh Phuoc province in 2004. From Lao PDR, 15 *P. falciparum* isolates were collected from malaria patients in villages in Attapeu province, Lao PDR in 2007. From Cambodia, 32 *P. falciparum* isolates were collected in the eastern part of the country in 2009. The samples collected from the malaria patients in Vietnam, Lao PDR, and Cambodia were dried blood spot samples on filter paper, which were stored at 4 °C until DNA analyses.

2.2. DNA extraction and genotyping of the pfcrt gene

DNA was extracted from frozen blood or dried blood spots on filter papers with a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions with some modifications as described previously [18]. The extracted DNA was eluted with 50 μl of elution buffer in the kit and preserved at 4 °C until PCR was conducted. To amplify a partial *pfcrt* gene containing codon 76, PCR was conducted by using a primer set reported previous study [15]. The PCR products were purified by agarose gel extraction using the QIAquick gel extraction kit (Qiagen) in accordance with the manufacturer's instructions. Purified product was used with the ABI Big Dye Terminator Ready Reaction Kit Version 3.1 (Applied Biosystems, CA, USA) for the sequencing PCR. Templates were purified and sequenced on a Genetic Analyzer 310 (Applied Biosystems).

2.3. Genotyping of microsatellite DNA loci flanking the pfcrt gene

Six microsatellite DNA loci flanking the *pfcrt* gene were amplified by semi-nested PCR. The loci were B5M77, 2E10, 9B12, PE12A, 2H4, and PE14F. The location of the 6 microsatellite DNA loci and the *pfcrt* gene on chromosome 7 of *P. falciparum* is shown in Fig. 2. The PCR primer sets and amplification conditions followed the protocol of Wootton et al. [17] with some modifications. The original PCR protocol used by Wootton et al. was a single PCR method. However, to increase amplification efficacy of the PCR, a semi-nested PCR method was applied by designing one additional primer for each locus in the present study. The list of primer sets used is shown in Additional file 1. Sizes of fluorescence-labeled PCR products were measured on a Genetic Analyzer 310 (Applied Biosystems) using Gene Scan version 3.1.2 with a 500 ROX size standard (Applied Biosystems).

Different-sized PCR products amplified using the same primer set were considered to be individual alleles within a locus, as size variation among isolates is consistent with the repeat number in a microsatellite locus [19–23]. The electropherogram showed peak profiles for the microsatellite loci based on the fluorescence intensity of the labeled PCR products in this analysis. Multiple alleles per locus were scored if minor peaks were taller than at least one-third the height of the predominant allele for each locus.

2.4. Data analysis

Expected heterozygosity (H) was calculated for each locus based on the allele frequencies of the 6 examined microsatellite DNA loci. H values were calculated using the formula $H = [n/(n-1)][1 - \Sigma pi^2]$, where n corresponds to the number of isolates examined, and pi is the frequency of the *i*th allele.

3. Results

3.1. Genotype of the pfcrt gene codons 72-76

A partial DNA sequence of the *pfcrt* gene of the *P. falciparum* isolates from Thailand (50 isolates), Vietnam (39 isolates), Cambodia (32 isolates), and Lao PDR (15 isolates) was determined by PCR and DNA sequencing. When the amino acid sequence of the gene at codon 76 is threonine (T), the isolate acquires the CQ-resistant phenotype [14]. The amino acid sequences at codons 72-76 in the gene of the isolates are shown in Table 1. All of the Thai and Cambodian isolates possessed the CQ-resistant genotype, whereas the Vietnamese and the Lao isolates possessed both CQ-resistant and -susceptible genotypes.

All 50 of the Thai isolates possessed one CQ-resistant genotype, CVIET. The Cambodian isolates possessed two CQ-resistant genotypes, CVIET (30 isolates) and CVIDT (2 isolates). The Vietnamese isolates possessed one CQ-susceptible genotype, CVMNK (27 isolates); three CQ-resistant genotypes, CVIET (6 isolates), CVIDT (3 isolates), and CVMDT (1 isolate), and two isolates were multiple genotype infection with CQ-susceptible and -resistant genotypes, CVMNK/CVIDT. The Lao isolates possessed one CQ-susceptible genotype, CVMNK (2 isolates), and two CQ-resistant genotypes, CVIET (10 isolates) and CVIDT (3 isolates).

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