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Effects of Mefloquine Usage on Genetic Polymorphism of *Plasmodium Falciparum* in Thai-Myanmarese Border

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We studied the polymorphism of *msp-1*, which encodes a major surface protein on the merozoite, isolated from blood samples from western Thailand in 1999. Our study area was a low-transmission area for malaria, where mefloquine has been used as an antimalarial drug since 1994. Forty-nine patients were confirmed to have contracted falciparum malaria twice within 24 weeks. The number of detected haplotypes in 49 patients was 89 at the first diagnosis and 68 at the second diagnosis. The mean number of haplotypes per patient significantly decreased from 1.82 to 1.39 but the frequency distributions of *msp-1* haplotypes did not change significantly with the use of mefloquine. Our study strongly suggests that the antigenic diversity of *Plasmodium falciparum* is retained during mefloquine therapy in low-transmission areas.

Falciparum malaria is an infectious disease caused by *Plasmodium falciparum*, and unless diagnosed and treated early, is often fatal. Approximately three hundred million clinical cases of malaria occur worldwide each year and over one million people die (22). The burden of this disease in disability-adjusted life years (DALYs) accounts for approximately 3% of all disease (23). Moreover, multidrug-resistant malaria has been a severe problem since about 1960 when chloroquine-resistant malaria was first reported in the Thai-Cambodian border regions, Colombia, and Venezuela (24).

Repetition of infection and a difficulty in acquiring immunity are characteristic of malaria. Antigenic diversity is also known to play a role in its characteristics and the immune response is believed to be strain-specific (1) (3). It is also reported that exposure to different antigens is more specifically important for acquiring immunity of malaria (2). Thus, it is important to examine the antigenic diversity of malaria parasites in malaria-endemic areas.

Currently, many kinds of antimalarial drugs are used for therapy, and the rate of expansion of multidrug-resistant malaria strains is known to differ between regions (4). It is believed that the use of antimalarial drugs decreases the degree of polymorphism and indirectly influences the immunological situation of a given area. However, little is known about the effects of drug therapy on antigenic diversity. It is reported that therapy with pyrimethamine/sulfadoxine and chlorproguanil/dapsone did not affect the number of clones of *Plasmodium falciparum* per individual (multiplicity) or the polymorphism of *Plasmodium*

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falciparum in Kenya, a high-transmission area (10). In low-transmission areas such as Thailand, however, these effects remain unclear.

We studied the polymorphism of *pfmsp-1* (*msp-1* of *Plasmodium falciparum*) using the *msp-1* haplotyping method (6) at Suan Phung in Rachaburi Province near the Myanmarese border in western Thailand in 1999. The present paper presents our findings regarding the effect of mefloquine therapy on the polymorphism of *Plasmodium falciparum* in this region.

MATERIALS AND METHODS

Study Area

Our study was carried out at the Rajanagarindra Tropical Disease International Centre (RTIC), which is operated by the Faculty of Tropical Medicine at Mahidol University in Suan Phung, Rachaburi Province in Thailand. Suan Phung is located near the Myanmarese border in western Thailand, about 170 km from Bangkok. The area is populated by numerous villages that are remote from each other and are only accessible by unpaved roads. The maximum temperature during 1999 was 34.2°C, the average minimum temperature was 22.5°C, and the annual rainfall was 1395.1 mm. The Karen hill tribes account for approximately 90% of the area population. The major vector of the malaria parasite in Suan Phung is *Anopheles minimus*, and other minor vectors include *An. maculates* and *An. dirus*.

Subjects

We took blood samples from those who visited the RTIC complaining of fever or general malaise between January and December 1999. Those patients that consulted the RTIC again for fever or general malaise at between 4 weeks and 24 weeks after the first diagnosis were asked for further blood samples. The last patient, who visited the RTIC on December 17, 1999, was followed until June 12, 2000.

Diagnosis of Falciparum Malaria

Patients infected with *Plasmodium falciparum* were detected by on-site microscopic examination of Giemsa-stained finger-pricked blood smears (thick and thin). When the patient was diagnosed as having falciparum malaria, 750 mg of mefloquine (15 mg per body weight for children) was immediately administered.

Blood Sampling

After obtaining informed consent, blood sampling was carried out. Parasite-positive finger-pricked blood (100 μl) was drawn quantitatively into a heparinized capillary tube and spotted onto a filter paper for chromatography (Whatman, UK). Each of the blood samples on filter papers was brought to Japan after being dried and placed into a plastic pouch.

DNA Extraction

DNA extraction was carried out according to the method described elsewhere (12). Briefly, (i) a half of the dried filter blot, equivalent to 50 μl of patient blood, was cut into 3 mm × 5 mm pieces. (ii) Hemolysis was performed in Hepes-buffer saline (HBS; 140 mM NaCl, 10 mM KCl, 10 mM Hepes buffer, pH 7.2) containing 0.5% (wt/wt) saponin (Merck, Germany). (iii) DNA was isolated using a QIAamp DNA Mini Kit (QIAGEN, Germany) and the eluted DNA was stored at -20°C.

Haplotyping

Pfmsp-1 consists of five conserved blocks, interspersed with seven variable and five semi-conserved blocks (Fig. 1) (19). In addition, block 4 is divided into block 4a and block 4b. The variation in *pfmsp-1* 5' is dimorphic, represented by the K1 and MAD20 allelic types, except for block 2, in which three allelic types, denoted K1, MAD20, and RO33, occur (5)

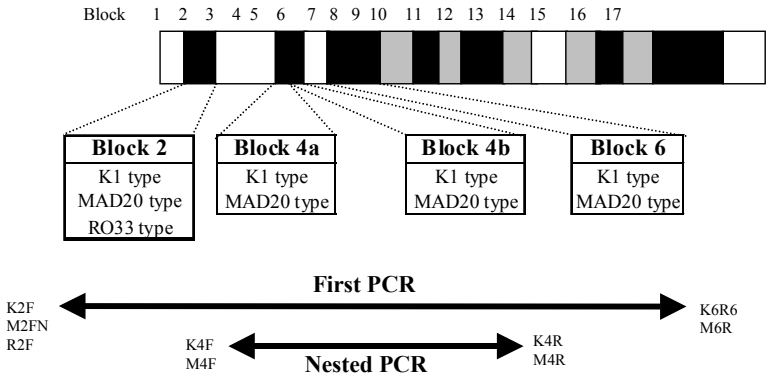

FIG. 1. Structure of *pfmsp-1* and outline of haplotyping. Upper bar represents the structure of *pfmsp-1* consisting of five conserved blocks (open bar), seven variable blocks (black bar), and five semi-conserved blocks (gray bar) (Tanabe *et al.*, 1987, Kaneko *et al.*, 1996). Block 2 can be divided into three types, K1, MAD20, and RO33. Block 4a, block 4b, and block 6 can be divided into two types, K1 and MAD20. Accordingly, *pfmsp-1* can be divided into 24 haplotypes using combinations of each type from block 2 to block 6. PCR was performed to amplify the region from block 2 to block 6 and nested PCR was then performed to amplify the region from block 4a to block 4b.

(8). The *pfmsp*-1 haplotype is defined in terms of the allelic types of the variable blocks: block 2, block 4a, block 4b, and block 6. Therefore, 24 kinds of haplotypes are detectable ($3 \times 2 \times 2 \times 2 = 24$).

PCR-based haplotyping of *pfmsp-1* was conducted using the following two steps (Kaneko *et al.*, 1997): (i) PCR to determine the allelic types of block 2 and block 6; and (ii) nested PCR to determine the allelic types of block 4a and block 4b using the first PCR products.

Each PCR was performed as follows. PCR was carried out in a 20 µl reaction mixture containing LA PCR Buffer II (Mg^{2+} free), 2.5 mM of $MgCl_2$, 0.4 mM of dNTP, 0.1 units per 20 micro-litter of Takara LA Taq (Takara Shuzo, Japan), 0.2 µM each of Primer F and Primer R (Table 1), H_2O, and 1 µl of extracted DNA from blood. Thirty-seven cycles of amplification (20 seconds at 93°C and 5 minutes at 62°C) were preceded by denaturation at 93°C for 1 minute and were followed by final elongation at 72°C for 10 minutes. Nested PCR was carried out in a 25 µl reaction mixture containing GeneAmp PCR Buffer (1.5 mM of Mg^{2+}), 0.2 mM dNTP, 0.5 units per 25 micro-litter of ABI AmpliTaq Gold (Applied Biosystems, USA), 0.5 µM each of Primer F and Primer R (Table 1), H_2O, and 1 µl of the

TABLE 1. Primer of PCR

		Type	Name	Sequence
First PCR	Primer F	K1	K2F	5'-TCTTAAATGAAGAAGAAATTACTACAAA-3'
		MAD20	M2FN	5'-GAACAAGTGGAACAGCTGTTACAACT-3'
		RO33	R2F	5'-TAAAGGATGGAGCAAATACTCAAGT-3'
	Primer R	K1	K6R6	5'-TCGTTATATGACAAAGGATAAGTAAC-3'
		MAD20	M6R	5'-ATTTGAACAGATTTCGTAGGATCTTG-3'
Nested PCR	Primer F	K1	K4F	5'-ATGAAATTAAAAATCCCCCACCGG-3'
		MAD20	M4F	5'-GAAGATATAGATAAAATTAAAACAGATG-3'
	Primer R	K1	K4R	5'-TCCTCGATTTTTTTGTTCTTATCAAG-3'
		MAD20	M4R	5'-TCGACTTCTTTTTTCTTATTCTCAG-3'

first PCR product diluted 100 fold. Twenty cycles of amplification (40 seconds at 95°C, 40 seconds at 50°C, and 40 seconds at 72°C) was preceded by initial denaturation at 95°C for 20 minutes and was followed by final elongation at 72°C for 5 minutes. Between one and three copies of target DNA were specifically detectable by the above protocol (14).

The PCR products were electrophoresed on 2% agarose gels and were visualized with ethidum bromide under UV illumination. Each haplotype is indicated as four letters with K, M, and R indicating the K1, MAD20, and RO33 allelic types, respectively (Table 2).

TABLE 2. *Pfmsp-1* haplotype

Haplotype #	Block 2/4a/4b/6
1	KKKK
2	MKKK
3	RKKK
4	KMKK
5	MMKK
6	RMKK
7	KKMK
8	MKMK
9	RKMK
10	KMMK
11	MMMK
12	RMMK
13	KKKM
14	MKKM
15	RKKM
16	KMKM
17	MMKM
18	RMKM
19	KKMM
20	MKMM
21	RKMM
22	KMMM
23	MMMM
24	RMMM

Each haplotype is expressed in terms of the allelic type of blocks 2, 4a, 4b, and 6. K, M, and R denote the K1, MAD20, and RO33 allelic types, respectively.

Statistical Analysis

Comparison of the mean number of haplotypes per person was assessed by Student's t-test. Frequency distributions of haplotypes at the first diagnosis and the second diagnosis were compared by a χ^2 test on a 2×6 contingency table. Minor haplotypes, in which the expected number was less than five, were grouped together as "others."

RESULTS

The number of patients who visited the RTIC with fever or general malaise more than twice within 24 weeks was 73. Of these, 49 patients were diagnosed with falciparum malaria twice (age range: 8 to 45 years; mean age: 22.9 years; median age: 20 years; 35 males, 14 females). For these 49 patients, the total number of parasites identified as being of the

pfmsp-1 haplotype was 89, consisting of 12 different haplotype-parasites at the first diagnosis. The number of *pfmsp-1* haplotypes per person was 1.82 ± 0.13 (mean ± standard error of the mean), ranging from one to four per person. The number of mixed infection that included more than two different haplotypes per patient was 27 (55.1%) of the 49 patients. The total number of parasites identified as being of the *pfmsp-1* haplotype was 68, consisting of eight different haplotype-parasites at the second diagnosis. The number of *pfmsp-1* haplotypes per person was 1.39 ± 0.11, ranging from one to four per person. The differences in the mean number of *pfmsp-1* haplotypes per person between cases at the first diagnosis and the second diagnosis were significant ($p = 0.01$).

In the detail of each haplotype identified at the first diagnosis and the second diagnosis, 48 parasites were identified as being of the *msp-1* haplotype at only the first diagnosis and not at the second diagnosis, 41 parasites were identified as being of the *msp-1* haplotype at both the first and second diagnosis, and 27 parasites were newly identified at the second diagnosis (Table 3).

TABLE 3. Details of each haplotype identified at the first diagnosis and the second diagnosis

Haplotype #*	1	2	13	14	16	17	18	19	20	21	22	23	Total
Only first diagnosis†	6	9	1	5	4	11	1	1	1	2	1	6	48
First and second diagnosis‡	0	4	0	2	0	12	0	0	0	4	1	18	41
Only second diagnosis§	4	3	0	3	2	3	0	0	0	3	1	8	27

*Haplotype number shown in Table 2. †Numbers of haplotypes observed at only the first diagnosis. ‡Numbers of same haplotypes observed twice. §Numbers of haplotypes observed at only the second diagnosis.

Twelve different haplotypes were detected at the first diagnosis, and MMMM (haplotype #23), MMKM (haplotype #17), and MKKK (haplotype #2) accounted for 67% of all types (Fig. 2a). Eight different haplotypes were detected at the second diagnosis, and MMMM and MMKM accounted for 60% of all types (Fig. 2b). Differences in the frequency distributions of the *pfmsp-1* haplotypes between at the first diagnosis and the second diagnosis were not significant ($p = 0.56$, Table 4).

TABLE 4. Comparison of frequency distribution of *pfmsp-1* haplotypes between cases at the first diagnosis and the second diagnosis

Haplotype #*	Observed		Expected	
	First diagnosis‡	Second diagnosis§	First diagnosis	Second diagnosis
2	13	7	11.3	8.7
14	7	5	6.8	5.2
17	23	15	21.5	16.5
21	6	7	7.4	5.6
23	24	26	28.3	21.7
Others†	16	8	13.6	10.4
Total	89	68	89.0	68.0

Frequency distributions of haplotypes at the first diagnosis and the second diagnosis were compared by a χ^2 test on a 2 × 6 contingency table.

*Haplotype number shown in Table 2.

†Minor haplotypes, for which the expected number was less than five, were grouped together as "others."

‡Numbers of haplotypes observed at the first diagnosis. §Numbers of haplotypes observed at the second diagnosis. $P = 0.56$.

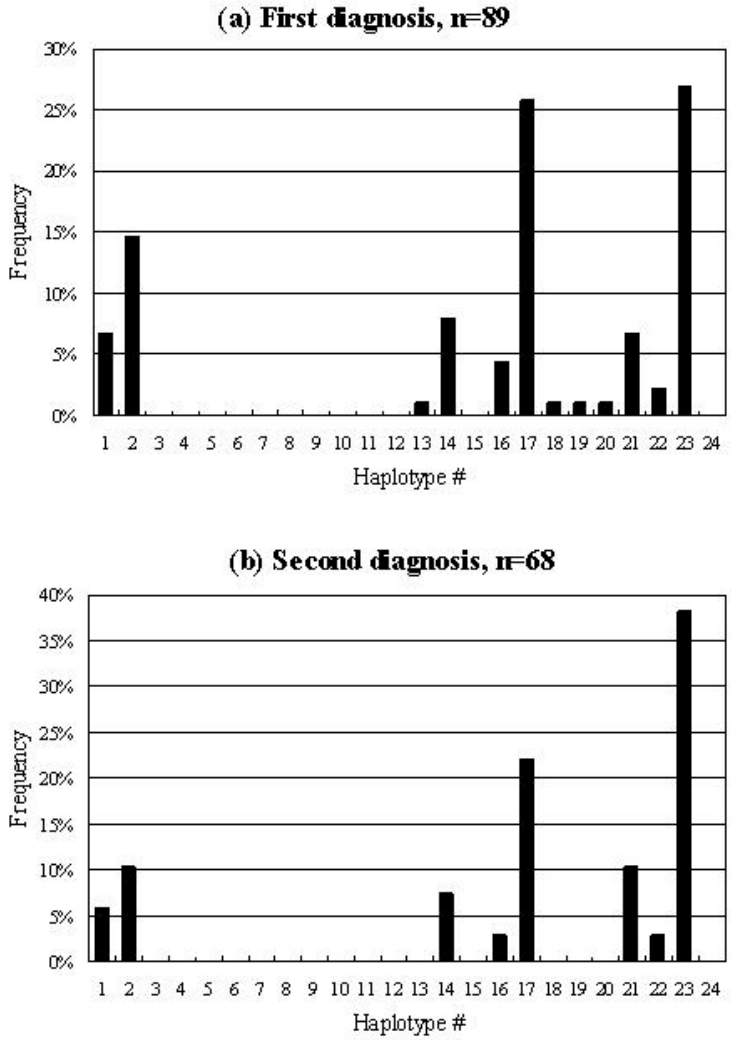

FIG. 2. Haplotype pattern at the first diagnosis and the second diagnosis. (a) The bar graph shows the ratio of each haplotype at the first diagnosis. The abscissa axis shows the haplotype number (see Table 2). (b) The bar graph shows the ratio of each haplotype at the second diagnosis.

DISCUSSION

The present study shows that the number of *pfmsp-1* haplotypes per individual (multiplicity) significantly decreased and that the pattern of *pfmsp-1* haplotype frequency did not change as a result of the mefloquine therapy. Our observation that the multiplicity decreased from 1.82 per person at the first diagnosis to 1.39 per person at the second diagnosis suggests that the mefloquine treatment introduced to the Suan Phung region in 1994 was still fairly effective as of 1999. However, mefloquine-resistant malaria has frequently been reported in Thailand, where mefloquine was first introduced in 1985. Resistant cases have been reported at Mae Sot (Tak Province, northwestern Thailand) (9) and have been demonstrated in *in vitro* studies (21). Moreover, the current prevalence of

mefloquine-resistant malaria is reported as 62% (7). Therefore, it is likely that mefloquine-resistant malaria has already occurred in the present study site, although we assume that a moderate number of mefloquine-sensitive malaria parasites existed in 1999 and that the multiplicity decreased due to the reduction of these mefloquine-sensitive malaria parasites by mefloquine therapy.

Most of the 41 haplotypes identified at both the first diagnosis and the second diagnosis are suspected to represent mefloquine-resistant malaria (Table 3). Most of the 27 haplotypes identified at only the second diagnosis are suspected to represent mefloquine-resistant malaria parasites which were not identified at the first diagnosis due to sequestration (Table 3). It is for this reason that the EIR (entomological inoculation rate: the number of infective mosquito bites received per person per unit time) (17) in Thailand is low, being one to two bites per person per year (18). Therefore, reinfection would be rare in a 24-week period under low EIR conditions, such as in Thailand. Forty-eight haplotypes identified at only the first diagnosis may also include parasites which were not identified at the second diagnosis due to sequestration (Table 3). Sequestration is defined that the ring forms and older trophozoites disappear from the peripheral circulation while being held in the capillaries of the internal organs (16). It was reported that a considerable number of parasites failed to be detected despite frank malaria infection, probably due to sequestration. Schleiermacher *et al.* compared placenta and peripheral blood *msp-1* genotype in 58 pregnant women. Their results indicated that in 52 women, there was partial or complete discrepancy in the genotype of the parasites in the placenta and those in the peripheral blood, and concluded considerable parasites have sequestered within the placenta (15).

It was reported that treatment with pyrimethamine/sulfadoxine and chlorproguanil/dapsone did not affect the antigenic diversity of *Plasmodium falciparum* in Kenya (10). In the present study, there were no significant differences in the pattern of *pfmsp-1* haplotype frequency between cases at the first diagnosis and the second diagnosis. This result shows that the antigenic diversity of *Plasmodium falciparum* is also retained by mefloquine therapy in a 24-week period. A further analysis of the mefloquine-resistant gene and a longer-term study are necessary to confirm whether the result means equilibrium (20).

The frequency distribution of *pfmsp-1* haplotypes has been shown to differ significantly between islands in the republic of Vanuatu in the southwest Pacific Ocean (13). The present results show that this phenomenon is also found on the continental mainland. The frequency distribution of *pfmsp-1* haplotypes at first diagnosis in the present study differs from that in Mae Sot, in the Tak Province of northwestern Thailand ($p < 0.01$, details not shown) (11). This clearly indicates the presence of interregional differences in the polymorphism of *Plasmodium falciparum* in Thailand. MSP-1 is known to be a major surface antigenic protein and an immune target (1), and further research may clarify whether haplotype-specific immune responses exist.

In conclusion, we observed significant decreases in the multiplicity and no changes in the patterns of *pfmsp-1* haplotype frequency as result of mefloquine therapy in Suan Phung in 1999. Therefore, it seems reasonable to conclude that the antigenic diversity of *Plasmodium falciparum* is retained during mefloquine therapy in low-transmission areas.

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