RESEARCH NOTE

A MOLECULAR EPIDEMIOLOGIC STUDY OF POINT MUTATIONS FOR PYRIMETHAMINE-SULFADOXINE RESISTANCE OF *PLASMODIUM FALCIPARUM* ISOLATES FROM LAO PDR

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Abstract. To understand the current condition of pyrimethamine-sulfadoxine (PS) resistant falciparum malaria in Lao PDR, the frequency of point mutations in dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS) genes of *Plasmodium falciparum* were examined in 50 blood samples collected from the patients with *P. falciparum* infection in Southern Lao PDR. Point mutations in 5 codons of the DHFR gene, which is known to be related to pyrimethamine resistance, were detected in 15 out of the 50 samples (30%). Among the 15 samples, 10 samples showed a double mutation of codons 59 and 108 (Cys59Arg with Ser108Asn). In the remaining 5 samples, an additional mutation was observed in codon 51 (Asn51Ile), providing a triple mutation of codons 51, 59 and 108. On the other hand, point mutations in the 4 codons of DHPS gene related to sulfadoxine resistance were observed only in 2 samples (4.0%), namely in codon 437 (Ala437Gly). Only one sample showed mutations in both DHFR and DHPS genes. From the results, it should be considered that the frequency of PS resistant malaria is still low in Lao PDR. Continuous monitoring for the PS resistant malaria, however, is necessary because of the increasing use of PS in this country.

INTRODUCTION

Malaria is one of the most serious health problems facing populations in tropical and subtropical regions, because it infects approximately 300 million people in the world and kills 1.5 to 3 million people annually. One of the current problems for malaria control is the increase in drug resistant malaria parasites in endemic areas. The problem is also serious in Southeast Asia. Since 1961, *Plasmodium falciparum* strains resistant to chloroquine (CQ) have been detected in Thailand. In some areas, CQ is now almost ineffective for the treatment of malaria. Now, pyrimethamine-sulfadoxine (PS) is essential for the treatment of CQ resistant cases. Therefore, the emergence of PS drug resistant malaria parasites is posing another obstacle to control programs in these areas.

In Lao PDR, malaria is also a major public health problem as it causes high morbidity and mortality in children and severe losses in socio-economic development (Giboda *et al*, 1992; Pholsena, 1992; Toma *et al*, 2003). PS is being used as the recommended second-line antimalarial drug in this country, but there is only limited data available on the current status of PS resistant falciparum malaria. In the present study, we examined the frequency of point mutations in dihydrofolate reductase (DHFR) and dihydorpteroate synthase (DHPS) genes related to the pyrimethamine-sulfadoxine resistance of *P. falciparum* parasite.

MATERIALS AND METHODS

With approval from the Ministry of Health, a total of 1,635 inhabitants living in 9 villages in the southern province of Khammouane were examined in an active case detection survey with

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blood samples taken for microscopy in June, 2001. A total of 205 samples (12.5%) were found to be positive for the *P. falciparum* parasite, and 50 samples were selected randomly for analysis of mutations in DHFR and DHPS genes.

After extraction and purification of malaria DNA from blood samples, gene sequence analysis was done by dideoxynucleotide technology on amplified genes using appropriate PCR primers (Table 1). Briefly, for the DHFR gene, PCR primers AMP1 (Plowe *et al*, 1995) and ST6 were used to amplify the entire DHFR gene. Nested PCR products were further amplified for analysis of mutations at codons 16, 51, 59, 108, and 164 by using ST7 and ST8 primers. Similarly for the DHPS gene, the primary PCR was done by using two primers #3717 (Wang *et al*, 1995) and #186 (Wang *et al*, 1995), and the nested PCR was done by using other four primers to amplify regions containing codon sites 436, 437, 518, and 631 (Wang *et al*, 1995). Each of the PCR products was sequenced twice and to determine the mutations at each codon site.

RESULTS

Amino acid residues in the DHFR and DHPS domains of *P. falciparum* isolates in the present study are shown in Table 2. In the DHFR gene, point mutations in 5 codons were detected in 30 % (15/50) of the samples examined. Among the 15 isolates, the predominant DHFR mutant observed was in the double mutant form of Cys59Arg and Ser108Asn in 10 samples. The remaining 5 samples were found to be a triple mutant DHFR type with an additional mutation of Asn51Ile. For all samples, however, no paired mutation of Ala16Val and Ser108Thr was detected. On the other hand, point mutation in the DHPS gene was observed in only two samples. The mutation was Ala437Gly. There was only one sample with point mutations in both DHFR and DHPS genes in the present study.

DISCUSSION

From previous *in vivo* studies with CQ, an increasing emergence of resistant falciparum malaria has become evident, suggesting that the drug is ineffective in many cases (Pillai *et al*, 2001). Therefore, alternative treatment with PS is now receiving increased demand as the drug of choice for treatment of malaria patients in Lao PDR. The actual situation of drug resistant malaria in Lao PDR, however, is not known because of lack of sufficient data. Local observations suggest that PS resistant falciparum malaria is developing.

Pyrimethamine-sulfadoxine is used as a combination drug which blocks the synthesis of DNA in the folate pathway of the malaria parasite. Pyrimethamine inhibits DHFR and sulfadoxine DHPS. The acquisition of resistance to pyrimethamine and sulfadoxine is due by point mutations in the malaria parasiste gene encoding

Table 1
Oligonucleotide primers used in PCR.

Oligonucleotide	Code	S/A[a]
DHFR gene		
TTTATATTTTCTCCTTTTTA	AMP1	S
GTTACTAGTATATACATCGC	ST6	A
[b]TAATACGACTCACTATAGGGATTTATATTTTCTCCTTTTTA	ST7	S
GTATATACATCGCTAACAG	ST8	A
DHPS gene		
CCATTCCTCATGTGTATACAACAC	#3717	S
GTTTAATCACATGTTTGCACTTTC	#186	A
TGATACCCGAATATAAGCATAATG	#185	S
[c]ATTTAGGTGACACTATAGAAGGTTTCGCATCACATTTAAC	#185-A	A
[b]TAATACGACTCACTATAGGGCAATGGATAAACTAAC	#218-S	S
[c]ATTTAGGTGACACATTAGAATTTTCATTTTGTTGTTCATC	#218-A	A

[a]S indicates sense primer, A indicates antisense primer.
[b]The underlined segment is the added T7 sequence.
[c]The underlined segment is the added SP6 sequence.

Table 2
Amino acid residues in the DHFR and DHPS domains of *P. falciparum* isolates from Lao PDR.

DHFR					DHPS				No. of subjects	%
16	51	59	108	164	436	437	581	613		
Ala	Asn	Cys	Ser	*Ile*	Ser	Ala	Ala	Ala	34	68
Ala	Asn	Cys	Ser	*Ile*	Ser	*Gly*	Ala	Ala	1	34
Ala	Asn	*Arg*	*Asn*	*Ile*	Ser	Ala	Ala	Ala	9	18
Ala	Asn	*Arg*	*Asn*	*Ile*	Ser	*Gly*	Ala	Ala	1	2
Ala	*Ile*	*Arg*	*Asn*	*Ile*	Ser	Ala	Ala	Ala	5	10

The sequence of wild type is shown on the first line. Altered amino acid residues are indicated in italics. Three-letter code of amino acid: Ala, alanine; Asn, asparagine; Cys, cysteine; Ser, serine; Arg, arginine; Ile, isoleucine; Gly, glycine

DHFR and DHPS, respectively, leading to amino acid changes in the active sites of the enzymes (Triglia *et al*, 1997; Peterson *et al*, 1998).

In the case of DHFR mutation from Lao *P. falciparum* isolates, we detected 3 variants including the wild type with Asn 108 and Arg 59 being the most common mutation. Asn 108 is known to be as a key mutation site in resistance to pyrimethamine. For the DHPS gene, a single mutant form, Ala437Gly, was observed.

In Lao PDR, PS is not yet commonly used and the frequency of PS resistant malaria is still low in this country, as compared to those in the other countries in Southeast Asia. It is as expected, however, that the majority of falciparum malaria will acquire PS drug resistance because of increasing use of the drug in this country, as it was in neighboring Thailand where the high frequency of PS use was paralleled by the high emergence of mutations in both genes reflecting resistance to the drug (Biswas *et al*, 2000).

Unfortunately, the results in the present study on DHFR-DHPS mutations could not predict the *in vivo* efficacy of PS treatment because the patients were actually treated with CQ, not with PS. Hereafter, it is essential that we should investigate genotypes of malaria parasites from patients who have clinical resistance to PS. The information generated from such studies will be helpful in understanding the efficacy of PS treatments in falciparum malaria patients.

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