RESEARCH NOTE

PREVALENCE OF POLYMORPHISMS IN *DHFR*, *DHPS*, *PFMDR*1 AND *PFCRT* GENES OF *PLASMODIUM FALCIPARUM* ISOLATES IN QUANG TRI PROVINCE, VIETNAM

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Abstract. In 2002 an antimalarial drug resistance survey was carried out in a seasonally endemic area of Vietnam. Sulfadoxine/pyrimethamine (S/P) was the standard treatment recommended for uncomplicated *Plasmodium falciparum* malaria in that area at the time. Early or late treatment failure as defined by WHO was observed in 14.9% (7/47) of patients. Molecular analysis of treatment failure isolates identified that 5/6 carried two or more *dhfr* and *dhps* polymorphisms associated with S/P resistance. Chloroquine resistance-associated polymorphisms occurred in 38.5% (15/39) of the isolates. These results support the move to artemisinin-based combination therapy for malaria in Vietnam.

INTRODUCTION

With the development and spread of antimalarial drug resistance in *Plasmodium falciparum* endemic regions of the world, use of many of the affordable antimalarial drugs is now not recommended (WHO, 2006). In Vietnam, *P. falciparum* resistance to chloroquine in the 1960s and later to sulfadoxine/pyrimethamine (S/P) became widespread (Masimirembwa *et al*, 1999). This led to the use of alternative antimalarial drugs including artemisinin derivatives. Since 1991, Vietnam has continued to revise standard treatment guidelines in response to the threat of drug resistant malaria. These guidelines have, until recently, supported the use of S/P as first-line treatment for *P. falciparum* in areas where treatment failure had not been recorded. Chloroquine continues to be used to treat cases of *P. vivax*. In 2002 a survey to assess *in vivo* responses to S/P treatment and to analyse molecular polymorphisms related to S/P and chloroquine resistance was undertaken in an area of Central Vietnam where S/P was still in use.

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MATERIALS AND METHODS

A 28-day drug efficacy survey (WHO, 2002) was carried out in Thanh commune, Quang Tri Province, located in the central highlands of Vietnam and adjacent to Lao PDR. Malaria transmission in the area is seasonal and intense. Forty-seven patients were enrolled. Participant inclusion and exclusion criteria

Table 1
Mutation specific oligonucleotides designed for PCR analysis of *pfmdr*1 Asn86Tyr and Tyr184Phe alleles.

Oligonucleotide name	Sequence	Target
rgright2	5′ TGCAACAGTTCTTATTCCCA 3′	Common
86wc	3′ GGTGTAATATTAAAGAACATCA 5′	86Asn
86mc	3′ GGTGTAATATTAAAGAACATCT 5′	86Tyr
184wc	3′ TGCCAGTTCCTTTTTAGGTTTACA 5′	184Tyr
184mc	3′ TGCCAGTTCCTTTTTAGGTTTACT 5′	184Phe

PCR cycling conditions were: 94ºC for 3 minutes, 25 cycles of 94ºC for 30 seconds, 56ºC for 45 seconds, 72ºC for 1 minute, and 72ºC for 5 minutes.

were according to WHO guidelines (2002). Excluded patients were treated independently by health center staff.

Patients with confirmed *P. faciparum* infection were treated with 1.25 mg/kg pyrimethamine and 25 mg/kg sulfadoxine (SR2, Central Pharmaceutical Factory No 1, Hanoi, Vietnam). Prior to treatment a blood sample was taken by finger prick, spotted onto Whatmann 3MM filter paper and stored individually in a sealed bag. Follow-up and treatment outcome were as defined by WHO guidelines (WHO, 2002).

Parasite DNA was extracted using the QIA-Kit (QIAGEN Sciences, MA) as per the kit protocol. PCR/RFLP was performed using standard methodology (Duraisingh *et al,* 1997). Amplification of *pfdhps* and *pfdhfr* fragments were performed as previously described (Masimirembwa *et al*, 1999) except for *pfdhps* nested PCR amplification conditions which used 60ºC annealing temperature over 30 cycles. Mutation specific PCR was used to analyse the *pfmdr*1 gene polymorphisms Asn86Tyr and Tyr184Phe (Table 1 for oligonucleotide and PCR cycle details). Analysis of *pfcrt* polymorphisms was conducted as described by Dorsey *et al* (2001).

RESULTS

The median age of patients was 12.5 years (range: 2-50 years) and the median pretreatment parasite density was 5,240 asexual parasites/µl (range: 400-85,000). Clinical response showed that 85.1% (40/47) of the patients had an adequate clinical and parasitological response (ACPR) and 14.9% (7/47) experienced early (n = 5) or late (n = 2) treatment failure. There was no difference in mean pretreatment parasite density between the ACPR and treatment failure groups.

Amplification and analysis of *dhfr* and *dhps* fragments was successful with 40 isolates and the results are summarized in Table 2. Of these, 77.5% (31/40) and 35% (14/40) of isolates carried at least one polymorphism for *dhfr* and *dhps* respectively. Comparison of isolate polymorphisms with *in vivo* responses to S/P treatment is also shown in Table 2. Double and triple mutation frequencies in *dhfr* were similar in the ACPR patients compared to treatment failures [73.5% (25/34) and 83.3% (5/6) respectively]. Multiple polymorphisms in *dhps* were observed in 29.4% (10/34) of ACPR patients, but 66.7% (4/6) of isolates from treatment failure patients carried multiple polymorphisms. Isolates from 75% (3/4) of early treatment failure (ETF) patients carried multiple polymorphisms in both *dhfr* and *dhps*.

Amplification and analysis of *pfcrt* and *pfmdr*1 was successfully performed with 39 isolates. Resistance-associated polymor-

Table 2
Molecular analysis of alleles in *dhfr* and *dhps* and their association with early (ETF) or late (LTF) treatment failure (Bold denotes resistance-associated polymorphisms).

Number of isolates *n* = 40	*Pfdhfr* allele					ETF	LTF
	16	51	59	108	164		
9	Ala	Asn	Cys	Ser	Ile		1
1	Ala	Asn	Cys	Asn	Ile		
15	Ala	Asn	Arg	Asn	Ile	2	
5	Ala	Asn	Arg	Asn	Leu	2	1
10[a]	Ala	Ile	Arg	Asn	Ile		
D10[b]	Ala	Asn	Cys	Ser	Ile		
W2mef[c]	Ala	Ile	Arg	Asn	Ile		
Total polymorphic isolates		10	30	31	5		
Number of isolates *n* = 40	*Pfdhps* allele						
	436	437	540	581	613	ETF	LTF
26	Ser	Ala	Lys	Ala	Ala	1	1
6	Ser	Gly	Lys	Ala	Ala		
1	Ser	Gly	Glu	Ala	Ala	3	
2	Ser	Gly	Lys	Gly	Ala		
2[a]	Phe	Gly	Lys	Ala	Ala		
2	Phe	Ala	Lys	Ala	Ser		
1	Ser	Gly	Glu	Gly	Ala		1
D10[b]	Ser	Ala	Lys	Ala	Ala		
W2mef[c]	Phe	Gly	Lys	Ala	Ser		
K1[c]	Ser	Gly	Lys	Gly	Ala		
Total polymorphic isolates	4	12	2	3	2		

ETF = Early Treatment Failure; LTF = Late Treatment Failure
[a] = Includes one wild type/polymorphic mixed infection.
[b]D10 = wildtype control parasite isolate,
[c]W2mef, K1 = mutant control parasite isolates.

phisms 76Thr and 75Glu in *pfcrt*, were observed in 28.2% (11/39) of isolates, and 25.6% (10/39) of isolates carried the 86Tyr and/or 184Phe polymorphisms in *pfmdr*1 (data not shown).

DISCUSSION

The results reported here indicate that high levels of multiple *dhfr* polymorphisms associated with resistance to pyrimethamine are present in the *P. falciparum* parasite population in Quang Tri Province, Vietnam. Multiple resistance polymorphisms were also present in *dhps* and a high proportion of isolates from treatment failure patients carried multiple resistance polymorphisms in both of these genes. Of particular concern was the high frequency of 59Arg, 108Asp and 437Gly *dhfr* polymorphisms among treatment failure patients.

Chloroquine resistance polymorphisms were present in *pfcrt* and *pfmdr*1 even though chloroquine treatment for *P. falciparum* had been phased out in this area. A previous

study carried out in south central Vietnam also indicated that these polymorphisms remained in the *P. falciparum* population after chloroquine use was discontinued (Nguyen *et al*, 2003).

Vietnam is currently moving to artimisinin-based combination therapy (ACT) (Ministry of Health, 2007) for uncomplicated *P. falciparum*. The results from this study support this change. In addition, the continuing presence of *pfcrt* and *pfmdr*1 mutations and their accepted association with chloroquine resistance suggests that ACT should be standard first-line therapy for all diagnosed cases of malaria. This would necessitate the phasing out of chloroquine treatment for *P. vivax* malaria as health workers would no longer be provided with chloroquine.

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