RESEARCH NOTE

THE OCCURENCE OF POINT MUTATIONS IN THE DIHYDROFOLATE REDUCTASE- THYMIDYLATE SYNTHASE (DHFR) GENE IN THAI ISOLATES OF *PLASMODIUM FALCIPARUM*

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The decreased susceptibility of malaria parasites to the drugs currently used for treatment of malaria is one of the main reasons for failure to control this disease in many endemic areas in Thailand. Several groups have reported that many of the *Plasmodium falciparum* isolates from Thailand have resisted antimalarials other than chloroquine which include cycloguanils, pyrimethamine and sulfadoxine (Edstein *et al,* 1996, 1997; Wilairatana *et al,* 1997). Resistance to chloroquine and pyrimethamine is widespread in Thailand and it has been reported that pyrimethamine resistance is still rising (Thaithong *et al,* 1990). Genetic analysis has demonstrated associations between point mutations in codon 108 of the DHFR gene and resistance to pyrimethamine and cycloguanil. A serine at position 108 is linked to sensitivity to both these drugs while a mutation to asparagine (Asn-108) or threonine (Thr-108) at this position confers resistance to pyrimethamine and cycloguanil respectively (Foote *et al,* 1990; Peterson *et al,* 1990). Polymerase chain reaction (PCR) using specific primers has been used successfully to detect these mutations in *P. falciparum* isolates from other geographical areas such as Africa (Plowe *et al,* 1995) and the Brazilian Amazon (Peterson *et al,* 1991). However, in a study with Malaysian *P. falciparum* isolates, there was no clear association found between the presence of Asn-108 and pyrimethamine resistance (Lim *et al,* 1998). In addition, there was considerable heterogeneity found in a large number of these isolates with both Ser-108 and Asn-108 found in the same individual. Therefore we would like to determine whether these mutations occur frequently in *P. falciparum* isolates from Thailand and whether any heterogeneity of these markers exists.

Thirty-eight filter paper blood specimens (20 µl) from malaria patients infected with *P. falciparum* from the Hospital for Tropical Diseases, Faculty of Tropical Medicine, Mahidol University were used in the study. The blood samples were collected from the patients prior to treatment with artemesinin derivatives such as artesunate and artemeter and the parasitemia determined by microscopy. Of the 38 samples collected, 23 patients responded well to treatment (sensitive) while 4 other showed R1 resistance. For 11 other cases, data regarding treatment response was either not available or the patients dropped out of the study. The DNA template for PCR was prepared according to the method described by Wang *et al* (1995). Individual filter paper blood samples were cut out and treated with 200 µl of 0.05% saponin in PBS, pH 7.2 for 30 minutes at 4°C. The filter paper was then washed once in 500 µl PBS and transferred to a PCR tube containing 100 µl of 1X Taq polymerase buffer (Redline). The PCR tubes were then placed in a thermocycler (Perkin Elmer 9600) and the mixture cycled at 95°C for 5 seconds followed by 50°C for 5 seconds over 5 cycles. Five µl of the suspension was used as template for the PCR experiments. Three types of PCR assays were performed to detect either Ser-108 or the mutant markers Asn-108 and Thr-108 according to the protocol described by Peterson *et al* (1991). The diagnostic primers were DIA-3′ 5′- GAATGCTTTCCAGC-3′ (specific for Ser-108), DIA-9 5′- GAATGCTTTCCCAGG-3′ (specific for Thr-108) and DIA-12 5′ - GGAATGCTTTCCCAGT-3′ (specific for Asn-108) which were used in conjunction with counterprimer SP1 5′ - ATGATGGAACAAGTCTGCGAC-3′). Forty-five cycles of amplification were performed, each consisting of denaturation at 94°C for 30 seconds, renaturation at 56°C for 45 seconds and extension at 74°C for 45 seconds. The PCR products were stained with ethidium bromide and photographed after electrophoresis in 1% agarose gel.

Table 1

Occurence of Ser-108, Thr-108 and Asn-108 in isolates of *Plasmodium falciparum* from Thailand.

Patient ref	Parasite count/μl	Drug response	Ser-108*	Thr-108	Asn-108	Ser-108+ Asn-108	Thr-108+ Asn-108	Ser-108*+ Asn-108	PCR Neg
PF-1	17,720	NA		+					
PF-2	4,850	S		+					
PF-3	12,240	S		+					
PF-4	NA	NA					+		
PF-5	NA	NA					+		
PF-6	NA	NA					+		
PF-7	NA	NA					+		
PF-8	18,400	S					+		
PF-9	142,820	S					+		
PF-10	23,250	S							
PF-11	1,102,460	S							+
PF-12	382	S				+	+		
PF-13	1,033,320	S				+			
PF-14	185,120	S			+				
PF-15	471,970	S			+				
PF-16	15,150	S			+				
PF-17	337,400	S	+						
PF-18	225,680	S			+				
PF-19	219,000	DO (D8)			+				
PF-20	352,560	R1 (D27)			+				
PF-21	202,304	S			+				
PF-22	84,360	R1 (D23)			+				
PF-23	225,570	DO (D22)					+		
PF-24	282,960	R1 (D12)					+		
PF-25	304,980	S			+				
PF-26	5,980	S			+				
PF-27	16,360	R1 (D15)					+		
PF-28	154,560	S			+				
PF-29	799	S					+		
PF-30	3,680	S					+		
PF-31	3,980	S				+			
PF-32	9,870	S						+	
PF-33	18,640	DO(D14)			+				
PF-34	759	DO(D15)		+					
PF-35	5,180	DO(D14)				+			
PF-36	4,700	S				+			
PF-37	8,920	DO(D12)				+			
PF-38	5,220	S				+			

Ser-108* = PCD product (130 bp), NA = Not available, S = Sensitive, R1 = R1 resistance, DO = Drop out

The results of the PCR for the 38 *P. falciparum* isolates are as shown in Table 1. The expected size of the PCR products for all the three experiments was 337 bp. No PCR products were obtained for 1 isolate (2.6%) while PCR was positive for the other 37 isolates. The negative PCR result may be due to

sequence variation in this particular isolate which could not be detected by the three sets of primers used in the study. The normal marker (Ser-108) did not appear to be commonly present in this group of isolates studied. It was observed that only 1 isolate (2.6%) had only the Ser-108 marker; however the PCR product obtained was much shorter (~130 bp) than the expected length which is 337 bp; this product was referred to as Ser-108*. This again may be due to some variation in the nucleotide sequence of the DHFR region amplified. Seven other isolates (18.4%) had both Ser-108 (337 bp in size) and Asn-108 while 1 other had a mixture of Ser-108* and Asn-108. Four isolates (10.5%) had only the Thr-108 mutation while 12 had only the Asn-108 mutation (31.6%) indicating that this mutation is quite prevalent in Thai isolates. Both Thr-108 and Asn-108 mutations were found together in 12 other isolates (31.6%). This finding is not surprising as multidrug resistance in *P. falciparum* isolates from Thailand has been reported (Looareesuwan *et al,* 1992; Wilairatana *et al*, 1997). Therefore the number of isolates with single mutations was 17/38 (45.5%) while 19/38 (50%) had mixed mutations indicating the heterogeneity of these isolates. Similar findings were observed in a study with Malaysian *P. falciparum* isolates whereby a large proportion of isolates were heterogeneous, having both Ser-108 and Asn-108 markers (Lim *et al,* 1998). In this study, four of the isolates showed R1 type of resistance to artemesinin derivatives. All four isolates had the Asn-108 mutation indicating resistance also to pyrimethamine. However, the mere presence of mutations or structural variations in the DHFR gene cannot be taken to be the only factor involved in drug susceptibility in malaria parasites as other genetic factors have been demonstrated. Clonal diversity with regards to isoenzyme patterns in a single isolate of *Plasmodium falciparum* has been reported (Thaithong *et al,* 1984) while Creasy *et al* (1990) reported extensive polymor- phism in many of the genetic characters of this parasite including geographical variations in the frequencies in which variant forms of certain enzyme and protein markers occur. From this study, it can be concluded that both the Asn-108 and Thr-108 mutations in the DHFR gene should not be used as the only markers to determine the parasite response to pyrimethamine and cycloguanil in Thai isolates as variant forms and heterogeneity of markers may exist thus complicating the diagnosis. Therefore, more information on the structural and genetic variants need to be looked into to identify suitable mutations as determinant markers for drug resistance in these parasites.

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