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SHORT REPORT: DIFFERENCES IN DIHYDROFOLATE REDUCTASE BUT NOT DIHYDROPTEROATE SYNTHASE ALLELES IN *PLASMODIUM FALCIPARUM* ISOLATES FROM GEOGRAPHICALLY DISTINCT AREAS IN MALAYSIA

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Abstract. Dihydropteroate synthase (*dhps*) and dihydrofolate reductase (*dhfr*) alleles were typed in 67 Malaysian *Plasmodium falciparum* isolates. The isolates were collected from two geographically distinct locations: 51 from Sabah, Malaysian Borneo, where sulfadoxine/pyrimethamine (SDX/PYR) is used to treat uncomplicated malaria and 16 from Peninsular Malaysia where *in vivo* resistance to SDX/PYR has been reported. A total of seven *dhps* alleles were identified with no significant difference in allele frequency between the 2 populations. Two of the *dhps* alleles described here have not been previously reported. Four *dhfr* alleles were detected in 67 *P. falciparum* isolates. Eighty-seven percent of the isolates from the Peninsula, where clinical SDX/PYR failure has been reported, had *dhfr* alleles with triple point mutations while all of the isolates from Sabah had *dhfr* alleles with 2 or less point mutations. The difference in *dhfr* allele frequency between the two populations was highly significant. There was no correlation between *in vitro* PYR response and accumulation of *dhfr* point mutations.

Fansidar, sulfadoxine/pyrimethamine (SDX/PYR) antimalarial combination therapy, has been used to treat uncomplicated *Plasmodium falciparum* malaria in areas where there is chloroquine resistance. Sulfadoxine and pyrimethamine inhibit different enzymes in the folate biosynthetic pathway, dihydropteroate synthase (*dhps*) and dihydrofolate reductase (*dhfr*) respectively, and act synergistically when used in combination.[1] In endemic areas where SDX/PYR treatment has replaced chloroquine, clinical drug failure has been reported at various intervals following SDX/PYR introduction.[2] Clinical performance of antimalarial treatment may be measured by *in vivo* drug response assays and estimated by *in vitro* drug sensitivity assays. It is difficult to measure the response to the SDX/PYR combination *in vitro*[3,4] and the *in vitro* response to the PYR moiety alone has been used to indicate potential resistance to the SDX/PYR combination therapy.[5]

Non-silent point mutations on the *dhps* and *dhfr* genes have been reported following the introduction of SDX/PYR therapy.[6–8] The *dhfr* mutations have been shown to alter the PYR-*dhfr* association constant reducing the efficiency of the drug *in vitro*.[9] In addition there appears to be a sequential progression in the number of mutations on the *dhfr* gene that correlate with an increased incidence of clinical drug failure and reduced response *in vitro*. The point mutation at *dhfr* position 108 is detected first, followed by point mutations at positions 50 and/or 59 and finally position 164.[2,10,11] For *dhps*, mutations have also been detected following SDX/PYR therapy.[2,6,12] The evidence for sequential *dhps* mutational events is not as strong but the point mutation at position 437 is thought to be the first event and is associated with a decreased response to SDX.[6,7] SDX/PYR has been in use for more than 20 years in Malaysia. The epidemiology of malaria in Malaysia, shows a contrast between Peninsular Malaysia on mainland Asia and the states of Sabah and Sarawak on the island of Borneo. Malaria is largely under control in Peninsular Malaysia despite the fact that clinical resistance to SDX/PYR has been reported in various locations.[13] However, in Sabah (Malaysian Borneo), SDX/PYR remains the drug of choice for treating uncomplicated falciparum malaria. That the drug has been used for such a prolonged period in Sabah without the widespread emergence of clinical failure is unusual given that resistance to SDX/PYR has emerged rapidly in Southeast Asian (including Peninsular Malaysia), South American, and African countries.[2,14,15] In this study, point mutations on the *dhps* and *dhfr* genes were typed in *P. falciparum* isolates collected from Kelantan, Peninsular Malaysia where clinical resistance to SDX/PYR has been reported[13] and from Sabah where SDX/PYR remains the recommended drug of choice for uncomplicated malaria. *Dhps* and *dhfr* alleles were mapped and the potential of molecular typing as a marker for emergent drug resistance is discussed.

MATERIALS AND METHODS

The study was conducted on *P. falciparum* isolates collected from patients at Lahad Datu Hospital, Kunak Health Center, and Tawau General Hospital, in Southern Sabah, Malaysian Borneo during December 1996, May 1997, and December 1997. Patients with slide-positive *P. falciparum* single infections were recruited into the study following a full explanation of the study protocol and having obtained patient or parent/guardian consent.

Three ml venous blood were collected of which 0.5 ml was placed into heparin tubes for World Health Organization (WHO) *in vitro* drug assays and the remainder into EDTA blood collection tubes. The EDTA blood was used for making thick and thin blood films, 10 × 20 μl bloods spots on filter paper and the remainder cryopreserved. In addition, blood-spot samples from Peninsula Malaysia were included in the molecular typing study. These samples were collected between August 1994 and August 1995 from two district hospitals, namely Tanah Merah and Kuala Krai in the state of Kelantan situated on the Eastern Malaysian/Thai border. The study protocol was approved by the Ethics Committee of the Ministry of Health Malaysia.

For mutation detection, DNA was extracted from 20 μl of

TABLE 1

Dihydropteroate synthase (*dhps*) and dihydrofolate reductase (*dhfr*) alleles present in Malaysian Plasmodium falciparum isolates

a)

dhps amino acid position[a]					Prevalence (%)	
					Peninsula	Sabah
436	437	540	581	613	n = 16	n = 51
S	G	K	G	A	81	76
S	G	K	A	A	13	8
F[b]	G	K	A	A	0	6
S	G	E	A	A	0	6
F	G	K	A	S	6	0
S	G and A	K	A	A	0	2
S and F[b]	G	K	G	A	0	2

b)

dhfr amino acid position					Prevalence (%)	
16	51	59	108	164	Peninsula	Sabah
	2nd	2nd	1st	3rd	n = 16	n = 51
A	N	C	N	I	0	2
A	N	R	N	I	13	98
A	I	R	N	I	56	0
A	N	R	N	L	31	0

[a] Alleles are represented by the single letter amino acid code denoting wild type or as the result of a point mutation (underlined). For reference, the *dhps* wild type allele is SAKAA and for *dhfr* ANCSI.

[b] FGKAA and FGKGA are novel *dhps* alleles (Table 1a). Point mutations at *dhfr* codons occur in sequence noted as 1st through 3rd (Table 1b).

EDTA blood dried onto filter paper using the chelating resin Chelex™ as previously described.[16] A total of 67 *P. falciparum* isolates were examined, 51 from Sabah and 16 from Peninsular Malaysia. For nested PCR and mutation specific restriction enzyme digestion, the method described by Duraisingh and others[16] was used to identify *dhps* polymorphisms at codon positions as follows: 436 [Ser(S) to Phe(F) or Ala(A)], 437 [Ala(A) to Gly(G)], 540 [Lys(K) to Glu(E)], 581 [Ala(A) to Gly(G)], and 613 [Ala(A) to Ser(S) or Thr(T)] and at *dhfr* codon positions 16[Ala(A) to Val(V) or Ser(S)], 51[Asn(N) to Ile(I)], 59[Cys(C)to Arg(R)], 108 [Ser(S) to Asn(N) or Thr(T)] and 164[Ile(I) to Leu(L)]. The PCR primer pairs used in this method are specific to *P. falciparum* and did not amplify control *P. vivax* DNA. In addition to the in-built restriction enzyme digestion controls specific for the wild type and point mutation sequences incorporated in the method, *P. falciparum* clones 3D7, K1, and W2 were included in each assay. The clones collectively had mutations at all but 2 of the sites, namely *dhps* 540 and *dhfr* 164. These two mutations were found in some of our isolates, which were then included in subsequent assays to further control for digestion at these sites. Two μl of the Chelex™ supernatant was included in Nest 1 PCR amplifications and 2 μl of nest-1 product were included in nest-2 PCR amplifications (Roche, Molecular Biochemicals, Singapore). *Dhps* and *dhfr* allele frequencies were calculated for each population (Sabah and the Peninsula). The significance of differences in the allele distribution between the two populations was calculated using Fisher's exact test.

RESULTS AND DISCUSSION

Dhps and *dhfr* alleles were defined for a total of 67 *P. falciparum* isolates comprising 51 from Sabah, Borneo and 16 from Kelantan, Peninsular Malaysia. These are described as 5-codon alleles (Table 1), with positions which differ from the sensitive type underlined. In total, seven different *dhps* alleles were detected (Table 1a). The SGKGA allele was most prevalent in both Sabah (76%) and the Peninsula (81%). All of the isolates had the *dhps* 437 A to G mutation, which is common in areas of SDX/PYR use.[2,6] The 437 mutation is not thought to result in clinical drug failure but is rather a marker of SDX drug pressure and reduced SDX responsiveness *in vitro*.[18] One of the isolates had two different alleles at position 437 and another isolate had two alleles at position 436 indicating the presence of more than one clone within each of these two isolates. Three *dhps* alleles were recorded in the isolates from the Peninsula while 6 different alleles were recorded in the larger sample of isolates collected from Sabah, including 2 alleles (FGKAA and FGKGA) that had not been previously reported. (Table 1a). The *dhps* 540 K to E mutation, which has been described in areas where there are high levels of clinical resistance to SDX/PYR[2] had a prevalence of 6% in isolates from Sabah but was absent in isolates from the Peninsula. There were no significant differences in the frequencies of *dhps* alleles in the two locations ($P = 0.74$).

Four *dhfr* alleles were detected in the 67 *P. falciparum* isolates studied (Table 1b). All of the isolates had the 108 (S to N) mutation as expected in populations where SDX/PYR has been used extensively. This mutation while increasing the *in vitro* IC_{50} is not thought to be responsible for clinical PYR failure.[5] The most prevalent allele in Sabah was ANRNI (98%), but this was rare in the Peninsula (13%). Three different alleles were detected in isolates from the Peninsula and 87% of these had triple mutations, five of them involving the *dhfr* 164 I to L point mutation found in areas with high levels of clinical resistance to SDX/PYR.[2,9] The most common allele in isolates from Peninsula Malaysia was AIRNI (56%), which was not detected in Sabah (Table 1a). The presence of 3 *dhfr* point mutations appears to correlate with reduced clinical response to SDX/PYR in Peninsular Malaysia. In contrast to *dhps*, the difference in *dhfr* allelic frequency between Sabah and the Peninsula was highly significant ($P < 0.000001$). It is worth noting that SDX/PYR is still considered to be effective in treating falciparum malaria in Sabah where the isolates had ≤2 *dhfr* point mutations. This finding is consistent with *in vitro* responses of *P. falciparum* genetic crosses of wild type and mutant *dhps* and *dhfr* allele combinations to SDX/PYR[19] where the authors predicted that the presence of 3 *dhfr* point mutations would result in clinical resistance to SDX/PYR regardless of *dhps* alleles. In addition, they predicted that isolates with 2 or less *dhfr* point mutations would respond to SDX/PYR therapy.

In vitro PYR sensitivity was measured using the WHO *in vitro* micro-test kit (Mark II) supplied by the WHO regional office in the Philippines. The test kit comprised low folate and low *p*-aminobenzioc acid RPMI culture medium (LPLF RPMI) and microtiter plates coated with various concentrations of PYR. Parasites from 33 patients from Sabah who had not previously taken antimalarials and with between 1×10^3 and 8×10^4 asexual stage parasites per μl of blood were included in the *in vitro* study. For the assay, 300 μl of fresh heparinized blood was added to 2.7 ml LPLF RPMI. Diluted blood was incubated in the wells of microtiter plates pre-dosed with dilutions of PYR ($0–1 \times 10^4$ picomole

[*p*mol]) and parasites were harvested as thick films after 24 to 36 hours incubation depending on parasite development in control wells as per the WHO protocol. For consistency, schizont counts were performed by one experienced microscopist and the response reported as the PYR concentration resulting in 50% inhibition (IC_{50}) of schizont development to 8 nuclei or more compared to the control drug-free well. Valid tests were those with 10% of parasites developing to mature schizonts in control wells. IC_{50} values were converted from *p*mol (WHO scale) to nanomolar (*n*M) for comparison with other published studies.

Of the 33 isolates included in the in the *in vitro* response study only 12 (36%) isolates grew sufficiently for valid IC_{50} calculation. The IC_{50} values ranged from 15 *n*M to 4,000 *n*M (mean value 849 *n*M). Seven isolates had IC_{50} responses $<$ 400 *n*M (mean 104 *n*M, range 15–200 *n*M) and 5 isolates had IC_{50} responses $>$ 800 *n*M (mean 1,892 *n*M, range 960–4,000 *n*M). Two *dhfr* alleles were detected in the 12 isolates, with the majority of isolates (11 of 12) having ANRNI, and the remaining isolate being ANCNI which fell into the $<$ 400 *n*M response group. The wide range of *in vitro* responses to PYR among the isolates from Sabah was not reflected in the *dhfr* allele frequency or clinical value of the drug in Sabah. This study has shown that assessing the *in vitro* response of clinical isolates to PYR was not as useful an indicator of the clinical performance of SDX/PYR as was determining the accumulation of mutations on the *dhfr* gene. In the Malaysian populations studied, 3 point mutations correlate with areas of clinical resistance and 2 point mutations with areas where SDX/PYR remains the recommended drug of choice. It should be noted that with 2 *dhfr* point mutations already prevalent in Sabah that the efficacy of SDX/PYR treatment may need to be carefully monitored.

Each of the populations in this study were essentially under SDX/PYR pressure over a period of approximately 20 years, and the reason for the significant difference in alleles at the *dhfr* locus is not clear. The proximity of Thailand, a focus of multiple drug resistant *P. falciparum,* to the Peninsula study group may indicate that the *P. falciparum* isolates in this group originated in Thailand. Sabah, on the island of Borneo, is geographically distinct from Peninsular Malaysia, with *P. falciparum* gene pools and population migration more likely to be between Indonesian Borneo than mainland Asia. However, current knowledge of the *P. falciparum* genome is not sufficient for mapping the migration of particular *P. falciparum* populations. Alternatively, mathematical modeling demonstrates how high transmission in the presence of drug pressure may drive drug resistant mutants within populations, particularly where there is restricted parasite diversity, possibly leading to a higher rate of self-crossing during recombination events.[20] The host and parasite populations in the two distinct groups (Sabah and the Peninsula) would experience at least subtle, if not profound, differences in transmission rates, which alone may explain the population differences at the *dhfr* gene locus, but again this is very difficult to prove.

In the current situation where there is concern over the emergence of drug-resistant malaria parasites, we have demonstrated that prolonged drug pressure alone is not sufficient for the emergence of resistant *dhfr* mutant alleles. It is possible that by controlling the spread of mutant alleles we may prolong the useful life of antimalarial drugs. However, this strategy would depend on increased understanding of the emergence, maintenance and distribution of genetic mutations in malaria parasite populations.

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