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Decline in Sulfadoxine-Pyrimethamine-Resistant Alleles after Change in Drug Policy in the Amazon Region of Peru▽†

Zhiyong Zhou,[1,2] Sean M. Griffing,[1,3] Alexandre Macedo de Oliveira,[1] Andrea M. McCollum,[1,2,3] Wilmer Marquino Quezada,[4] Nancy Arrospide,[4] Ananias A. Escalante,[5] and Venkatachalam Udhayakumar[1]*

Division of Parasitic Diseases, National Center for Zoonotic, Vector-Borne and Enteric Diseases, Coordinating Center for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia[1]; Atlanta Research and Education Foundation, Decatur, Georgia[2]; Program in Population Biology, Ecology, and Evolution, Emory University, Atlanta, Georgia[3]; National Institute of Health, Lima, Peru[4]; and School of Life Sciences, Arizona State University, Tempe, Arizona[5]

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The frequency of alleles with triple mutations conferring sulfadoxine-pyrimethamine (SP) resistance in the Peruvian Amazon Basin has declined (16.9% for *dhfr* and 0% for *dhps* compared to 47% for both alleles in 1997) 5 years after SP was replaced as the first-line treatment for *Plasmodium falciparum* malaria. Microsatellite analysis showed that the *dhfr* and *dhps* alleles are of common origin.

After sulfadoxine-pyrimethamine (SP) resistance became widely prevalent in the Peruvian Amazon Basin, artesunate plus mefloquine was chosen as the recommended first-line treatment for uncomplicated *Plasmodium falciparum* malaria in 2001 (1, 4, 8). In the north coastal region of Peru, SP is used in combination with artesunate, as it remains efficacious against *P. falciparum* parasites in this area (7).

Resistance to pyrimethamine is attributed to mutations in *dhfr* codons 50, 51, 59, 108, and 164; and resistance to sulfadoxine is attributed to mutations in *dhps* codons 436, 437, 540, 581, and 613 (for a review, see reference 2). In Peru, the triple mutant N51I/S108N/I164L *dhfr* allele and the triple mutant A437G/K540E/A581G *dhps* allele have been correlated with high levels of SP resistance (4). A recent study with microsatellite markers has shown that highly resistant *dhfr* and *dhps* alleles with triple mutations in isolates from Venezuela have a common ancestral origin (9). It is not known whether Peruvian *P. falciparum* populations, which have a distinct *dhfr* allele with triple mutations, have a single origin or multiple origins.

The prevalence of the CQ-resistant *pfcrt* mutation K76T declined in Malawi and China after chloroquine (CQ) was removed from governmental treatment recommendations (5, 10). This decline correlated with the return of the clinical efficacy of CQ for the treatment of falciparum malaria (6). In Cambodia, however, alleles conferring CQ and SP resistance still occur at a high frequency two decades after these drugs were replaced (3). In Venezuela, we recently showed the complete fixation of mutant *dhfr* and *dhps* alleles 8 years after the withdrawal of SP use (9). Further studies are required to understand the roles of various ecological factors that determine the fate of resistance-conferring alleles following a change in the drug use.

In the present study, we attempted to determine if there were any changes in the frequencies of *dhfr* and *dhps* mutations in *P. falciparum* isolates from Iquitos, Peruvian Amazon Basin, after SP was removed from the national treatment recommendation in 2001. We analyzed 208 blood samples on filter paper (all *P. falciparum* positive by microscopy) collected between April 2005 and March 2006 during an evaluation of the efficacy and effectiveness of mefloquine and artesunate combination therapy for the treatment of uncomplicated malaria at Santa Clara and Morona Cocha health facilities, Iquitos, Peru. The study was approved by the Institutional Review Board of the Centers for Disease Control and Prevention, Atlanta, GA, and the Ministry of Health, Peru.

DNA was extracted from the filter paper strips by using a QIAamp DNA mini kit (Qiagen, Valencia, CA). Pyrosequencing was used to genotype mutations in *dhfr* at positions 50, 51, 59, 108, and 164 and mutations in *dhps* at positions 436, 437, 540, 581, and 613, as described previously (11). Microsatellite analysis was conducted as reported previously (9). A heminested PCR was performed for each locus. For the *dhps* microsatellite reactions, a number of new primers were created in order to conduct the heminested reactions. Microsatellite PCR primer sequences are provided in the supplemental material. Haplotypes were grouped as being different if they contained more than one different allele across the loci.

All isolates (100%) were found to have mutations in *dhfr* codon 108; 17.9% and 20.1% of the isolates had the N51I and I164L mutations, respectively. At codons 50 and 59, all the isolates were wild type. Figure 1A shows that isolates with the allele with the N51I/S108N/I164L triple mutations had declined significantly to 16.9%, whereas the previously reported rate from the same region was 47% (4). Other mutant alleles did not decline in their frequencies. Mutations at codons 437 and 581 were found in 15.3% of the isolates. Mutations

* Corresponding author. Mailing address: Malaria Branch, Division of Parasitic Diseases, NCZVED, CCID, Centers for Disease Control and Prevention, Mail Stop F-12, 4770 Buford Highway, Atlanta, GA 30341. Phone: (770) 488-4047. Fax: (770) 488-4454. E-mail: vxu0@cdc.gov.

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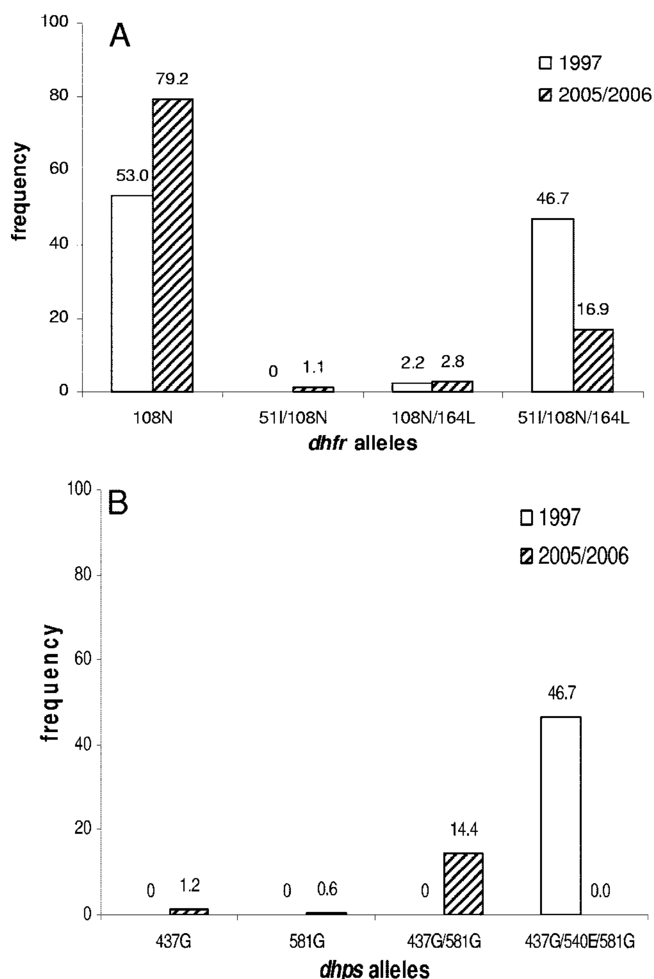

FIG. 1. Frequency of *dhfr* (A) and *dhps* (B) mutant alleles at two time points from the Iquitos region of Peru. The data for 2005 and 2006 came from this study; the data for 1997 were taken from the work of Kublin et al. (4). The difference in the frequencies of the *dhfr* and *dhps* alleles with triple mutations between the two points was highly significant ($P < 0.0001$), based on Fisher's exact test. The numbers of isolates were 141, 2, 5, and 30 for mutant *dhfr* alleles 108, 51/108, 108/164, and 51/108/164, respectively (a total of 178 samples were genotyped for *dhfr*). The numbers of isolates were 2, 1, 24, and 0 for mutant *dhps* alleles 437, 581, 437/581, and 437/540/581, respectively. The wild-type allele was present in 140 samples (a total of 167 samples were genotyped for *dhps*). One of the S108N/I164L allele isolates and all of the N51I/S108N/I164L allele isolates carried the Bolivia repeat.

at codons 436, 540, and 613 were absent. As shown in Fig. 1B, the *dhps* allele with the triple mutations A437G/K540E/A581G was completely absent in the samples from this study. Compared to the 47% prevalence in the previous data (4), this is a significant decline. The A437G/A581G allele was present in 14.4% of the isolates. Alleles with the A437G and A581G single mutations were each present in 1.2% and 0.6% of the isolates, respectively. A total of 83.8% isolates had wild-type *dhps* alleles.

Tables 1 and 2 show the *dhfr* and *dhps* haplotypes, respectively. Overall, the *dhfr* alleles showed five different haplotype groups. The *dhfr* alleles with triple mutations had at least three closely related haplotype groups, groups 1, 2, and 3. The allele with the N51I/S108N double mutation shared haplotype 3 and the S108N/I164L allele shared haplotype 1 with the allele with the triple mutations. The allele with the single mutation (S108N) was present in haplotype groups 3, 4, and 5 (Table 1). The *dhps* alleles also showed three haplotypes in this population (Table 2). The mutant alleles (A437G/A581G and A437G) had only one haplotype (group 1), while the wild-type alleles had three haplotype groups.

To our knowledge, this may be a first report on the reduction in the frequency of alleles with triple mutations that confer high-level SP resistance after drug policy changes. It appears that the *dhps* allele with triple mutations is being replaced faster than the *dhfr* allele with triple mutations as the drug pressure is removed from the population. We do not know whether this is because the *dhps* allele with triple mutations is less biologically fit than the *dhfr* allele with triple mutations or is due to other reasons.

It is unclear why, in Cambodia and Venezuela, SP resistance alleles have remained at a high frequency after the replacement of SP (3, 9). The key difference seems to be that in Peru alleles that confer high levels of resistance have not yet reached fixation. Thus, there is an opportunity for wild-type alleles to compete with drug resistance-conferring alleles, which are assumed to carry a high fitness cost to the parasite in the absence of drug pressure, and eventually replace the resistance-conferring alleles (9). If this hypothesis is true, our findings may have significant impact on drug policy development and implementation.

We have also shown, using microsatellite markers, that both

TABLE 1. *dhfr* microsatellite haplotypes by mutant alleles, Iquitos, Peru

Allele	Group	*dhfr* haplotype at the following microsatellite locus[a]:					No. of isolates
		−5.3	−3.87	−0.3	0.52	5.87	
50/108/164	1	204	215	101	97	122	9
50/108/164	1	224	215	101	97	122	7
50/108/164	1	204	194	101	97	122	6
50/108/164	1	204	202	101	97	122	1
50/108/164	2	224	202	101	97	122	3
50/108/164	2	224	194	101	97	122	2
50/108/164	3	204	194	101	97	108	5
50/108/164	3	204		101	97	108	1
51/108	3	204	194		97	108	3
108/164	1	204	215		97		1
108	3	204	194		103	108	11
108	3	204	194	101	103	108	5
108	3	204	194	92	97	108	1
108	3	204	194	95	97	108	1
108	3	204	194	90	97	108	1
108	4	204	209	97	103	108	8
108	4	204	194	97	103	108	5
108	4	204	209	97	101	108	1
108	5	224	194		97	108	5
108	5	224	194	92	97	108	4
108	5	224	194	78	97	108	3
108	5	224	194	74	97	108	2
108	5	224	194	97	97	108	1
108	5	224	194	100	97	108	1
108	5	224	194	103	97	108	1

[a] A total of 88 isolates were tested. The numbers for the microsatellite loci are the location with respect to *dhfr* (in kb), where negative positions are 5′ to *dhfr* and nonnegative positions are 3′ to *dhfr*. Empty cells represent loci that did not amplify.

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TABLE 2. *dhps* microsatellite haplotypes by mutant alleles, Iquitos, Peru

Allele	Group	*dhps* haplotype at the following microsatellite locus[a]:										No. of isolates
		−7.4	−2.74	−1.64	−0.8	0.006	0.14	1.59	6.19	9.79	10.1	
437/581	1	310	267	140	124	134	172	197	178	232	101	9
437/581	1	310		140	124	134	172	197	178	232	101	8
437/581	1	310		140	124	134	172	197	178	232		1
437/581	1	310	267	140	124		172	197	178	232	101	1
437/581	1			140	124		172	197	178	232		1
437/581	1			140	124	134	172	197	178		101	1
437/581	1			140	124	134	172		178	232		1
437	1	310	267	140	124	134	172	197	178	232	101	1
437	1	310		140	124	134	172	197	178	232	101	1
WT[b]	1	310	267	140	124	134	172	197	180	232	101	12
WT	1	310		140	124	134	172	197	180	232	101	8
WT	1		267	140	124	134	172	197	180	232		1
WT	1			140	124		172	197	180	232	101	1
WT	1			140	124	134	172	197		232		1
WT	2	285	246	140	130	134	190	189	180	221	122	1
WT	2	285	246	140	130	134		189	180	221	122	1
WT	2		246	140	130	134		189		221		1
WT	2			140	130	134		189	180		122	1
WT	2			140	132	134	172		180			1
WT	3	292	270	140	136	134	166	181	172	221	122	1

[a] A total of 53 isolates were tested. The numbers for the microsatellite loci are the location with respect to *dhps* (in kb), where negative positions are 5′ to *dhps* and nonnegative positions are 3′ to *dhps*. Empty cells represent loci that did not amplify.
[b] WT, wild type.

dhfr and *dhps* mutant alleles could have been derived from a common ancestor in this population. Although not all of the *dhfr* alleles with triple mutations had identical microsatellite haplotypes, they did appear to be closely related, and these additional haplotypes may have been generated by recombination and/or mutation. The *dhps* mutant and wild-type alleles all shared the same microsatellite haplotype, implying a common ancestor for these two mutant and wild-type alleles. In summary, molecular surveillance can be valuable in predicting the shifting trend in drug-resistant parasite populations following drug policy changes.

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