

## Mutations in *Plasmodium falciparum* Dihydrofolate Reductase and Dihydropteroate Synthase of Isolates from the Amazon Region of Brazil

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Since the late 1970s pyrimethamine-sulfadoxine (PS; Fansidar<sup>TM</sup> Hoffman-LaRoche, Basel) has been used as first line therapy for uncomplicated malaria in the Amazon basin. Unfortunately, resistance has developed over the last ten years in many regions of the Amazon and PS is no longer recommended for use in Brazil. *In vitro* resistance to pyrimethamine and cycloguanil (the active metabolite of proguanil) is caused by specific point mutations in *Plasmodium falciparum* dihydrofolate reductase (DHFR), and *in vitro* resistance to sulfadoxine has been associated with mutations in dihydropteroate synthase (DHPS). In association with a proguanil-sulfamethoxazole clinical trial in Brazil, we performed a nested mutation-specific polymerase chain reaction to measure the prevalence of DHFR mutations at codons 50, 51, 59, 108 and 164 and DHPS mutations at codons 436, 437, 540, 581 and 613 at three sites in the Brazilian Amazon. Samples from two isolated towns showed a high degree of homogeneity, with the DHFR Arg-50/Ile-51/Asn-108 and DHPS Gly-437/Glu-540/Gly-581 mutant genotype accounting for all infections in Peixoto de Azevedo ( $n = 15$ ) and 60% of infections in Apiacás ( $n = 10$ ), State of Mato Grosso. The remaining infections in Apiacás differed from this predominant genotype only by the addition of the Bolivia repeat at codon 30 and the Leu-164 mutation in DHFR. By contrast, 17 samples from Porto Velho, capital city of the State of Rondônia, with much in- and out-migration, showed a wide variety of DHFR and DHPS genotypes.

Key words: *Plasmodium falciparum* - malaria - dihydrofolate reductase - dihydropteroate synthase - drug resistance - Brazilian Amazon

Malaria is one of the major public health problems in Brazil, with 450,000 cases (about 77.2% *Plasmodium vivax* and 21.8% *P. falciparum*) and approximately 10,000 deaths reported annually (Marques 1995). The Amazon region, in which migrant populations, great distances, and poor access to diagnosis and treatment are the major obstacles to malaria control, accounts for more than 95% of the cases in Brazil (Marques 1993, 1995).

Compounding the existing problem is the emergence and spread of *P. falciparum* resistant to chloroquine and pyrimethamine-sulfadoxine (PS; Fansidar<sup>TM</sup> Hoffman-LaRoche, Basel), which were

the most commonly used antimalarials in Brazil. *In vitro* and *in vivo* drug response studies from the early 1980s documented 16-63% PS failure rates from various regions of the Amazon (Neifer & Kremsner 1991). After one decade with continued wide availability and use of the drug, 92% of the samples collected from the State of Acre in 1987 were found to be PS resistant. Proguanil was never introduced in Brazil and no current information is available on the *in vitro* efficacy against Brazilian isolates of cycloguanil, the active metabolite of proguanil (Kremsner et al. 1989, Neifer & Kremsner 1991).

Genetic analysis by polymerase chain reaction (PCR) has demonstrated the role of mutations in dihydrofolate reductase-thymidylate synthase (DHFR-TS) and dihydropteroate synthase (DHPS) genes that seem to be linked to *in vitro* resistance to the following drugs: pyrimethamine, cycloguanil and sulfadoxine (Cowman et al. 1988, Peterson et al. 1988, Foote et al. 1990).

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In the case of pyrimethamine and cycloguanil, resistance is conferred by point mutations in the parasite gene encoding DHFR, leading to amino acid changes in the active site pocket of the enzyme. A Ser at position 108 is linked to sensitivity to both pyrimethamine and cycloguanil, a mutation to Asn at position 108 confers resistance to pyrimethamine but only a moderate loss of response to cycloguanil, and a Thr at position 108 (paired with an Ala to Val change at position 16) is associated with resistance to cycloguanil with only a slight loss of response to pyrimethamine. An Asn to Ile change at position 51 and a Cys to Arg change at position 59 appear to modulate higher levels of pyrimethamine resistance when they occur with the Asn-108 mutation, and an Ile to Leu mutation at position 164 in combination with the Asn-108 plus Arg-59 and/or Ile-51 mutations has been found in *P. falciparum* lines that are highly resistant to both drugs (Cowman et al. 1988, Peterson et al. 1990, Foote & Cowman 1994). Recently, two new DHFR mutations were discovered in an area of widespread PS resistance in Bolivian samples: Cys→Arg-50 and a five amino acid repeat insertion between codons 30 and 31, termed the Bolivia repeat (Plowe et al. 1997). While both are associated with *in vivo* PS resistance, only the Arg-50 mutation has been shown to cause pyrimethamine resistance in transfection studies in yeast (Cortese & Plowe 1998).

Mutations at codons 436, 437, 540, 581 and 613 of the DHPS gene have been shown to cause resistance to sulfones and sulfonamides in *P. falciparum* (Wang et al. 1997b, Triglia et al. 1997). Mutations associated with resistance are Ala-436, Gly-437, Glu-540, Gly-581, and Phe-436 combined with Thr/Ser-613. The mutation Gly-437 is highly prevalent in areas of PS resistance, suggesting that this may be the first mutation in response to drug pressure (Wang et al. 1997b).

The present paper describes the use of nested mutation-specific PCR (Plowe et al. 1995, 1997), and restriction digestion methods to measure the prevalence of DHFR mutations at amino acid positions 50, 51, 59, 108 and 164 and DHPS mutations 436, 437, 540, 581 and 613 in *P. falciparum* isolates from two different gold-mining areas of the State of Mato Grosso, Peixoto de Azevedo and Apiacás, and Porto Velho, capital city of Rondônia.

## MATERIALS AND METHODS

**Study sites** - At present, clinical failures of chloroquine are reaching 100% in many areas of Brazil, and the drug is no longer recommended for treatment of uncomplicated *P. falciparum* malaria (Andrade et al. 1992). With the advent of chloroquine resistance, other antimalarials were intro-

duced, most commonly PS. Resistance to this drug emerged rapidly and different studies have demonstrated the widespread prevalence of the DHFR Asn-108 mutation which confers moderate resistance to pyrimethamine (Alecrim et al. 1982a, b, c, Peterson et al. 1991). As a result of evidence of reduced efficacy of chloroquine and PS, the standard treatment in Brazil for uncomplicated *P. falciparum* malaria has become quinine-tetracycline. Samples were collected from 1996 to 1998 in three malaria endemic areas in Brazil. Two places of the study are the gold-mining areas, Peixoto de Azevedo and Apiacás, State of Mato Grosso, which are localized in the southern Brazilian Amazon Forest. These two localities had the highest malaria transmission rates in Brazil, ~300 cases/week in 1996. The samples from Porto Velho, capital of the State of Rondônia, were collected in 1998.

**Sample collection and control parasites** - At the three sites of this study we performed the same sample collection procedure. Five milliliters of blood were collected from adult men (ages 18-65) with uncomplicated smear-confirmed falciparum malaria (1,000 - 100,000 ring forms/ $\mu$ l of blood) at the Fundação Nacional de Saúde (National Health Foundation) posts by venipuncture using vacutainers with EDTA (Beckton and Dickinson, NJ).

The samples were centrifuged and the pellet containing packed blood cells was mixed with equal volume of cryopreservation solution (NaCl 0.9%, sorbitol 4.2%, glycerol 28%) frozen and transported in liquid nitrogen containers to Rio de Janeiro. Nine samples from Peixoto de Azevedo and Apiacás were successfully grown *in vitro* by the modified method of Trager and Jensen (1997) and used for the *in vitro* drug test analysis. The W2 and HB3, PS resistant clones and D6, PS sensitive clone were used as controls (de Pecoulas et al. 1996).

**DNA extraction** - One ml of blood was centrifuged and suspended in NET Buffer (150mM NaCl, 10mM EDTA, 50mM Tris, pH 7.5) and treated with 1% sarcosyl and proteinase K (20 mg/ml, 2 h at 50°C). DNA was then extracted using phenol, phenol/chloroform and chloroform, precipitated with sodium acetate (0.3M) and ethanol, and resuspended in distilled water.

**Nested mutation-specific PCR** - PCR was done as previously described for analysis of the DHFR mutation sites (Plowe et al. 1995, 1997) (Fig. 1). The DHFR domain obtained from the first PCR amplification round, which used the oligonucleotide primer pair AMP1/AMP2, was used as a template in mutation-specific second amplification to assess all the DHFR mutation sites.

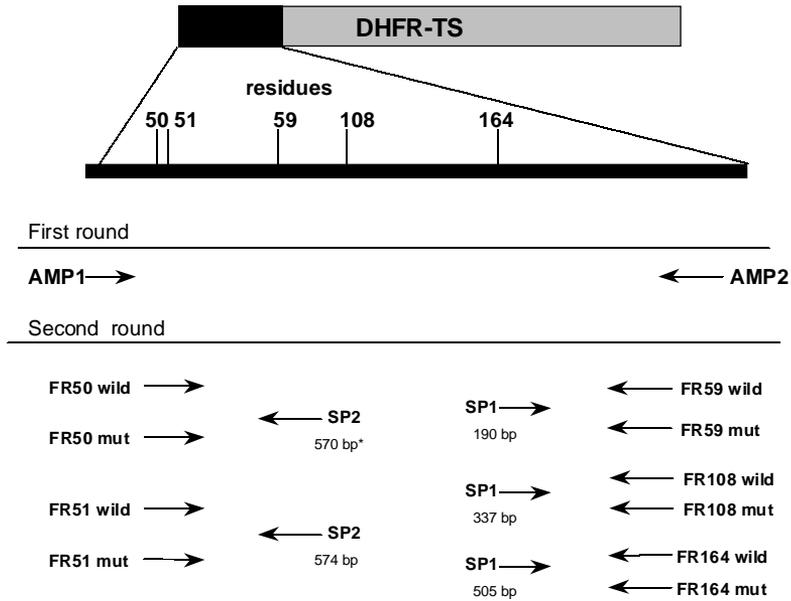


Fig. 1: strategy of the nested polymerase chain reaction (PCR) for dihydrofolate reductase-thymidylate synthase mutations analysis. \* PCR fragment size expected after the second round.

For codon 50, mutation-specific sense primers FR50w, which contains the codon for the wild type Cys and FR50m, which contains the mutant codon for Arg, were paired with the antisense primer SP2 for a PCR product of 570 bp. The mutation-specific sense primers FR51w, which specifically amplifies sequence containing the wild type AAT (Asn) codon, and FR51m, which is specific for the mutant codon ATT (Ile), were paired with the common antisense primer SP2, for a final product of 574 bp. The codon 59 was analyzed with the antisense primers FR59w, specific for the wild type Cys and FR59m specific for mutant codon Arg, were paired with the common sense primer SP1 with a product size of 190 bp. Detection of the DHFR Bolivia repeat was done by size determination of the amplified PCR product corresponding to the DHFR domain 5' to codon 59. For the analysis of the DHFR codon 108 the common sense SP1 were paired DIA-3 for the wild-type codon Ser, DIA-12 for the mutant codon Asn and DIA-9 for the mutant codon Thr. The final PCR product was 337 bp. For codon 164 the SP1 primer was paired with the antisense primers FR164w specific for the wild-type codon Ile and FR164m and gave a final product of 505 bp.

Detection for the known DHPS mutations at the codons 436, 437, 540, 581 and 613 was done as described (Wang et al. 1997a, b) (Fig. 2). Five microliters of the genomic *P. falciparum* DNA was amplified in a 50 ml reaction using mutation-

specific PCR and restriction digestion methods (Wang et al. 1995, Plowe et al. 1997).

*Determination of the antimalarial activity of antifolates* - The drugs used in this study were obtained from the Division of Experimental Therapeutics, The Walter Reed Army Institute of Research, Washington, D.C. Methods for the quantitative assessment of anti-malarial drugs on a semiautomated microdilution technique were described previously (Milhous et al. 1985). Determinations of the IC<sub>50</sub> (Inhibitory Concentration at 50%) were made for sulfadoxine, pyrimethamine, cycloguanil and proguanil in a folate-free and PABA-free RPMI-1640 medium (GIBCO BRL, Grand Island, NY) with 32 mM NaHCO<sub>3</sub> (GIBCO), 25 mM HEPES (GIBCO), dialyzed human plasma and A<sup>+</sup> erythrocytes.

Parasites were set up on the above deficient medium at initial 0.5% parasitemia, 2.5% haematocrit, 225 µl final volume, in 96-well microtiter plates. For each drug, two columns of seven wells was reserved. In the first seven rows (rows A-G) the drugs were added in a descending order of 3x concentration. The drug concentration range used to establish IC<sub>50</sub> values was 0-12 µg/ml for all the drugs. In the 8th row, the first six wells had only parasites with any drug (maximum radioactive uptake) and the last six wells had uninfected erythrocytes as controls for background incorporation of radioactive label. The microtiter plate was placed in a box, gassed with 5% CO<sub>2</sub>,

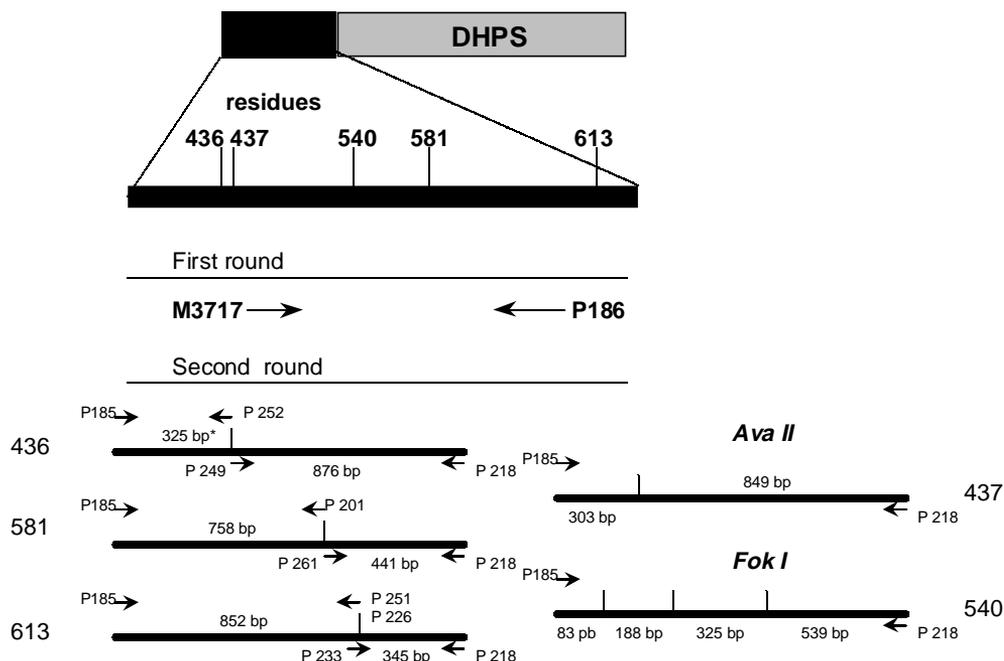


Fig. 2: strategy of the nested polymerase chain reaction (PCR) for dihydropteroate synthase mutation analysis. For residues analysis of the 437 and 540 amino acids, restriction digestion was performed after the second amplification. \* PCR fragment size expected after the second round.

5% O<sub>2</sub>, 90% N<sub>2</sub>, and sealed. After 48 h incubation with the drugs at 37°C, 0.5 µCi [<sup>3</sup>H]hypoxanthine in 25 µl of test medium was added to each well to monitor growth and samples were processed after a further 16 h. After harvesting the well contents, the filters were counted in a scintillation counter (Wallac) and the incorporation data were analyzed by non-linear regression software -NFIT (University of Texas, Medical Branch, Galveston) in order to generate IC<sub>50</sub>. IC<sub>50</sub> values were defined as the concentration of antifolate drugs at which parasite growth was reduced to half that of the untreated controls, as measured by tritium incorporation.

## RESULTS

**DHFR genotypes** - Seventeen samples from Peixoto de Azevedo, ten from Apicás and fifteen from Porto Velho were analyzed by PCR and restriction digestion. All fragments size corresponded to those described previously (Plowe et al. 1995, 1997, Wang et al. 1997b). DHFR genotypes are shown in Table I and DHPS genotypes in Table II. All samples collected in Peixoto showed only one genotype, with the following DHFR mutations: Arg-50, Ile-51, Cys-59, Asn-108 and Ile-164. Samples from Apicás, however, showed two distinct genotypes: six out of ten showed the same allelic variation observed in Peixoto, but four out of ten were found to have the genotype Cys-50, Ile-51, Cys-59,

Asn-108 and Leu-164 associated with high levels of resistance to both pyrimethamine and cycloguanil. These four samples were also found to contain the Bolivia repeat (BR) (Plowe et al. 1997).

The Thr-108 DHFR mutation, which confers resistance to cycloguanil, was not found in Peixoto or in Apicás, but was detected in eleven Porto Velho samples as a mixed population with the mutation Asn-108. The samples collected in Porto Velho showed considerably more allelic variation and fewer mutations than those from Peixoto and Apicás. Two samples from Porto Velho were mutated only at codon 108 with both Thr and Asn mutations in the same samples. Five Porto Velho samples had wild type Cys at codon 50 and seven had wild type Asn at codon 51, whereas all Peixoto and Apicás samples were mutated at these codons.

**DHPS genotypes** - All samples from Peixoto and Apicás had the wild-type Ser-436 and Ala-613 but sulfadoxine resistance-associated mutations Gly-437, Glu-540 and Gly-581 (Table II). Seven out of fifteen samples analyzed in Porto Velho had the Ala-436 DHPS mutation.

**In vitro drug test** - Four samples from Peixoto and five from Apicás were cultured and after two weeks *in vitro* tested against pyrimethamine, cycloguanil, proguanil and sulfadoxine (Table III). All the samples were found to be resistant to Pyr

TABLE I  
Frequency of dihydrofolate reductase genotypes of samples isolated from three different regions of the Brazilian Amazon

Site	BR	Amino acid position					Frequency % (n)
		50	51	59	108	164	
Peixoto de Azevedo n=17		<b>Arg</b>	<b>Ile</b>	Cys	<b>Asn</b>	Ile	100 (17)
Apiacás n= 10	—	<b>Arg</b>	<b>Ile</b>	Cys	<b>Asn</b>	Ile	60 (6)
	<b>BR</b>	<b>Arg</b>	<b>Ile</b>	Cys	<b>Asn</b>	<b>Leu</b>	40 (4)
Porto Velho n=15	—	<b>Arg</b>	<b>Ile</b>	Cys	<b>Asn+ Thr</b>	Ile	33.3 (5)
	—	Cys	<b>Ile</b>	Cys	<b>Asn</b>	Ile	13.3 (2)
	—	<b>Arg</b>	Asn	Cys	<b>Asn+ Thr</b>	Ile	26.7 (4)
	—	Cys	Asn	Cys	<b>Asn</b>	Ile	6.7 (1)
	—	Cys	Asn	Cys	<b>Asn+ Thr</b>	Ile	13.3 (2)
	—	<b>Arg</b>	<b>Ile</b>	Cys	<b>Asn</b>	Ile	6.7 (1)

BR: Bolivia repeat. Mutations are indicated in bold.

TABLE II  
Frequency of dihydropteroate synthase genotypes of samples isolated from three different regions of the Brazilian Amazon

Site	Amino acid position					Frequency % (n)
	436	437	540	581	613	
Peixoto de Azevedo n=17	Ser	<b>Gly</b>	<b>Glu</b>	<b>Gly</b>	Ala	100 (17)
Apiacás n=10	Ser	<b>Gly</b>	<b>Glu</b>	<b>Gly</b>	Ala	100 (10)
Porto Velho n=15	Ser	<b>Gly</b>	<b>Glu</b>	<b>Gly</b>	Ala	53.3 (8)
	<b>Ala</b>	<b>Gly</b>	<b>Glu</b>	<b>Gly</b>	Ala	46.7 (7)

Mutations are indicated in bold.

and sulfadoxine and showed to have reduced susceptibility to cycloguanil and proguanil when compared with the susceptible strain D6 (Sierra Leone). The inhibitory concentrations ( $IC_{50}$ ) values to pyrimethamine range from 48 to 98 ng/ml in the Peixoto and 50.8 to 87.4 ng/ml in Apiacás. For cycloguanil, the  $IC_{50}$  levels varied from 2.9 to 4.75 and 1.42 to 2.42 ng/ml in Peixoto and Apiacás, respectively. In Apiacás and Peixoto the  $IC_{50}$  for proguanil range from 331 to 1,125 ng/ml and 320 to 1,657 ng/ml, respectively.

Although proguanil is not the active metabolite, this drug showed an antimalarial activity in a nanomolar range, suggesting an independent mechanism of parasite inhibition by the proguanil. For sulfadoxine, the levels varied from 1,357 to 6,987 ng/ml in the Peixoto samples and from 1,043 to 1,686 ng/ml in Apiacás. In comparison to the sensitive strain D6, the Brazilian parasites were in average 700 fold more resistant to pyrimethamine, ten fold to cycloguanil, four fold to proguanil and eight fold more resistant to sulfadoxine.

## DISCUSSION

In 1972, resistance to PS was first reported in Brazil shortly thereafter in Colombia. In Brazil, *in vitro* and *in vivo* drug studies from the early 80s documented 16-63% PS failure rates in various regions of the Amazon (Alecrim et al. 1982a, b, c). After one decade of wide availability and use of the drug, 92% of samples collected from the State of Acre in 1987 were found to be PS resistant (Neifer et al. 1991). Today this drug combination is not recommended for use in any region of Brazil.

This study describes antifolate resistance mutations in three different communities of the Brazilian Amazon. In the two gold-mining areas Peixoto and Apiacás, only two distinct genotypes associated with pyrimethamine resistance were found, one with the DHFR mutations Ile-51 and Asn-108 and the second DHFR mutations at Cys-59, Asn-108 and Leu-164 associated with high level resistance to pyrimethamine and cycloguanil. The samples from Porto Velho were less highly

TABLE III  
Antifolate susceptibility of *Plasmodium falciparum* isolates with dihydrofolate reductase and dihydropteroate synthase amino acid position

Isolate	Site	IC50 (ng/ml±SD)					Amino acid position					DHFR			Amino acid position			DHPS		
		PYR	CYCG	SULF	50	51	59	108	164	BR	436	437	540	581	613					
ALR	Peixoto	97,73±6,8	4,75±0,86	1657±450	Arg	Ile	Cys	Asn	Ile	Asn	Ile	Ser	Gly	Ala						
ECP	Peixoto	48,13±2,9	3,72±0,47	320,15±98	Arg	Ile	Cys	Asn	Ile	Asn	Ile	Ser	Gly	Ala						
PAD	Peixoto	50,16±10	2,9±0,41	554±106	Arg	Ile	Cys	Asn	Ile	Asn	Ile	Ser	Gly	Ala						
RCS	Peixoto	78,50±7	4,34±0,67	1563±367	Arg	Ile	Cys	Asn	Ile	Asn	Ile	Ser	Gly	Ala						
A402	Apiacás	75,13±15,7	2,01±0,26	493,2±56	Arg	Ile	Cys	Asn	Ile	Leu	BR	Ser	Gly	Ala						
A403	Apiacás	79,81±8,6	2,42±0,76	468,61±94	Arg	Ile	Cys	Asn	Ile	Asn	Ile	Ser	Gly	Ala						
A408	Apiacás	50,75±13	1,23±0,36	1002,7±204	Arg	Ile	Cys	Asn	Ile	Asn	Ile	Ser	Gly	Ala						
A416	Apiacás	55,38±17	1,85±0,33	331,43±67	Arg	Ile	Cys	Asn	Ile	Asn	Ile	Ser	Gly	Ala						
A434	Apiacás	87,36±9,7	2,34±0,78	1125,55±300	Arg	Ile	Cys	Asn	Ile	Leu	BR	Ser	Gly	Ala						
D6	Africa	0,1	0,37±0,02	209,85±46	Arg	Ile	Cys	Asn	Ile	Asn	Ile	Ser	Gly	Ala						
W2	Indochina	277,66±32	8,79±0,45	896,2±100	Arg	Ile	Cys	Asn	Ile	Asn	Ile	Ser	Gly	Ala						

Mutations are indicated in bold; BR: Bolivia repeat; PYR: pyrimethamine; CYCG: cycloguanil; PROG: proguanil; SULF: sulfadoxine; DHFR: dihydrofolate reductase; DHPS: dihydropteroate synthase

mutated with more allelic variation than those from Peixoto and Apiacás. In Porto Velho, samples originated from several different river communities. In this region, there is intense internal migration from different parts of the Amazon as well as in- and out-migration from Bolivia and Peru. The higher degree of host mobility may explain the increased allelic variation in this area.

Interestingly, we found the relatively rare Thr-108 DHFR mutation in four samples from Porto Velho. These samples were analyzed three times to confirm the presence of this mutation. DNA sequencing studies are planned to verify these results and to rule out the presence of novel DHFR mutations.

In this study were found 40% of the analyzed samples from Apiacás the mutation Leu in position 164 of the *falciparum* DHFR, which is associated with *in vivo* and *in vitro* cycloguanil resistance. Although proguanil has never been reported as being used for the treatment of malaria in Brazil, the appearance of this mutation could be explained by the introduction in Brazil of a parasite population containing this mutation or by the previous pyrimethamine selection when the drug was used.

In addition, the Bolivia repeat previously reported by Plowe et al. (1997) was always in combination with this point mutation. This repeat which is localized in 5' domain of the DHFR, was previously found in all Bolivian samples with the mutant Leu-164. Antifolate resistance due to DHFR mutations expressed in yeast was not increased with the Bolivia repeat in addition to the Leu-164. Despite the Bolivian policy to stop the use of pyrimethamine-sulfadoxine, approximately 500,000 tables were consumed in 1995 (Kublin et al. 1998) in the country. With high in migration from Bolivia to Brazil, the Bolivia repeat in combination with the Arg-50 and Leu-164 was probably recently introduced by Bolivian migrants specifically in the Apiacás region and not in Peixoto. Apiacás is localized 200 km from Peixoto and has the same gold-miners migration pattern. This is the first evidence of these mutations in Brazil and we could trace this parasites studying other samples from different malaria endemic areas of Brazil.

The most common DHPS genotype at the study sites contained the mutations Gly-437, Glu-540 and Gly-581, which has also been observed in areas of high PS resistance in Peru and Bolivia (Kublin et al. 1998). The DHPS Ala-436, which was not found in Peru and Bolivia, was present only in Porto Velho, where overall mutation rates were lower. This is consistent with the observation that Ala-436, while associated with sulfa resistance *in vitro*, is inversely associated with *in vivo* PS resistance

(Kublin et al. 1998). The Gly-437, which is present in sulfa-resistant strains from different parts of the world, appears to be the first selected mutation in DHPS enzyme from resistant parasites.

The indiscriminate and unsupervised use of sulfonamides for bacterial infections in areas where malaria is prevalent is common in Brazil (Cor Jésus Fontes, pers. commun.) and despite the Brazilian policy to control the general use of sulfa drugs, they are widely available commercially in Brazil. Given that the role of DHPS mutations in clinical failure of PS remains controversial (Kublin et al. 1998), the use of sulfa drugs for bacterial infections must be considered as a possible explanation of the high prevalence of DHPS mutations at the study sites.

The DHFR and DHPS mutation patterns found in this study suggest that reintroduction of PS to these areas of Brazil cannot be recommended, based on a high prevalence of *in vivo* resistance in Peruvian samples containing similar mutation patterns. Our findings also have implications for the choice of other new antimalarial combinations for use in Brazil. For example, combinations of shorter-acting antifolates such as chlorproguanil/dapsone (Kublin et al. 1998) or proguanil/sulfamethoxazole have been considered as a strategy for deterring the emergence of antifolate-resistant parasites. Unfortunately, the presence of mutations such as DHFR Leu-164 that have been associated with resistance to these DHFR inhibitors may predict that these drugs would not perform well in the areas surveyed in this study. Additional studies of the relationship between DHFR and DHPS genotypes and therapeutic outcomes for these antifolate combinations are needed to validate the use of molecular assays for antifolate resistance as a basis for setting antimalarial drug treatment policy in different settings.

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