MAJOR ARTICLE

Antifolate Resistance in *Plasmodium falciparum*: Multiple Origins and Identification of Novel *dhfr* Alleles

Andrea M. McCollum,[1,2,3] Amanda C. Poe,[2,3] Mary Hamel,[2,5] Curtis Huber,[2] Zhiyong Zhou,[2,3] Ya Ping Shi,[2,5] Peter Ouma,[5] John Vulule,[5] Peter Bloland,[2] Laurence Slutsker,[5] John W. Barnwell,[2] Venkatachalam Udhayakumar,[2] and Ananias A. Escalante[2,4]

[1]Emory University, Program in Population Biology, Ecology, and Evolution, [2]Centers for Disease Control and Prevention, Division of Parasitic Diseases, Malaria Branch, and [3]Atlanta Research and Education Foundation, Atlanta, Georgia; [4]Arizona State University, School of Life Sciences, Tempe; [5]Kenya Medical Research Institute, Centre for Vector Biology and Control Research, Kisumu, Kenya

Background. Sulfadoxine-pyrimethamine has been widely used as first-line therapy for uncomplicated malaria throughout sub-Saharan Africa. Recent studies conducted in Asia and Africa suggest the triple-mutant *dhfr* genotype (51I/59R/108N) may have been generated as a single event in Southeast Asia, with subsequent spread of the single lineage to the African continent, but this hypothesis needs further validation.

Methods. Direct sequencing of polymerase chain reaction (PCR) products, pyrosequencing, and cloning of PCR products were utilized to identify mutations in *dhfr*. To investigate the evolutionary history of *dhfr* alleles, we assayed microsatellite loci flanking *dhfr* along chromosome 4.

Results. A total of 15 of 479 samples from western Kenya showed the presence of I164L, in 5 different genotypes. We document C50R in 2 of our samples. Using microsatellite markers, we show 2 haplotypes for both the 51I/108N/164L and 51I/59R/108N/164L genotypes. Our results also show multiple lineages for the triple-mutant *dhfr* genotype in Africa.

Conclusions. These findings highlight the importance of local characterization of alleles before molecular surveillance of drug-resistant alleles is considered in different endemic settings and populations.

Sulfadoxine-pyrimethamine (SP) has been widely used as first-line therapy for uncomplicated *Plasmodium falciparum* malaria throughout sub-Saharan Africa, because of its affordability, its ease of administration, and, until recently, its effectiveness. It is currently the only option for intermittent treatment of malaria during pregnancy. Recently, increasing resistance to SP in Africa has become a major public health concern. SP acts as an inhibitor of the *P. falciparum* folic acid pathway. Pyrimethamine inhibits the enzyme dihydrofolate reductase (DHFR), and sulfadoxine inhibits the enzyme dihydropteroate synthase (DHPS). Mutations in the genes *dhfr* and *dhps* are associated with the development of clinical resistance [1]. The *dhfr* genotypes bearing a single mutation at codon 108 (S108N/T) yield a low level of resistance to SP. Resistance increases with the presence of double mutations (N51I and S108N/T or C59R and S108N/T) and more so with triple-mutant genotypes (N51I, C59R, and S108N/T). An additional nonsynonymous mutation at codon 50 (C50R) confers an increased level of resistance to pyrimethamine, and this mutation has been described only at sites in South America [2]. Occurrence of a fourth mutation in the triple mutant at codon 164 (I164L) confers a higher level of resistance to SP [3]. So far, the quadruple-mutant genotype has been found in multiple sites in Southeast Asia and South America. However, the pres-

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Reprints or correspondence: Dr. Ananias A. Escalante, School of Life Sciences, Arizona State University, PO Box 874501, Tempe, AZ 85287-4501 (ananias.escalante@asu.edu).

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Table 1. Primers used to amplify *dhfr* for direct sequencing and pyrosequencing.

Primer	Sequence (5′→3′)	Purpose
primFds	TCCTTTTTATGATGGAACAAG	Primary PCR for direct sequencing of *dhfr*
primRds	AGTATATACATCGCTAACAGA	Primary PCR for direct sequencing of *dhfr*
secFds	TTTATGATGGAACAAGTCTGC	Nested PCR for direct sequencing of *dhfr*
secRds	ACTCATTTTCATTTATTTCTGG	Nested PCR for direct sequencing of *dhfr*
primFpy	ATGATGGAACAAGTCTGCGAC	Primary PCR for pyrosequencing of *dhfr* codons 50, 51, 59, 108, and 164
primRpy	ACATTTTATTATTCGTTTTCT	Primary PCR for pyrosequencing of *dhfr* codons 50, 51, 59, 108, and 164
sec1Fpy	GCGACGTTTTCGATATTTATGC	Nested PCR for pyrosequencing of *dhfr* codons 50, 51, and 59
sec1Rpy	**B**-GGCATATCATTTACATTATCCACAGTTT	Nested PCR for pyrosequencing of *dhfr* codons 50, 51, and 59
sec2Fpy	**B**-CTAATTCTAAAAAATTACAAAATGT	Nested PCR for pyrosequencing of *dhfr* codons 108 and 164
sec2Rpy	CACATTCATATGTACTATTT	Nested PCR for pyrosequencing of *dhfr* codons 108 and 164
50/51F	GGTCTAGGAAATAAAGGAGT	Pyrosequencing primer for *dhfr* codons 50/51
59F	CCCTAGATATGAAATATTTT	Pyrosequencing primer for *dhfr* codon 59
108R	TGGAATGCTTTCCCAG	Pyrosequencing primer for *dhfr* codon 108
164R	ATTCTTGATAAACAACGGAA	Pyrosequencing primer for *dhfr* codon 164

NOTE. Boldface B denotes biotin tag. PCR, polymerase chain reaction.

ence of the I164L mutation in Africa has been elusive and has only recently been observed [4–7]. Further confirmation of the I164L mutation in other parts of Africa will have implications for antifolate use on the continent.

It is not known how SP-resistant alleles originate and spread across populations. Recent studies of microsatellite loci surrounding *dhfr* from areas of low and moderate transmission of malaria found a common haplotype for triple-mutant genotypes in sites 4000 km apart in Africa and a single haplotype for highly resistant genotypes in Southeast Asia [8, 9]. Further analysis of these haplotypes suggested that they originated in Southeast Asia, subsequently spread to Africa, and resulted in the triple-mutant genotypes present in Africa today [10]. This hypothesis needs to be validated in other endemic areas. If it is true that a single origin for highly resistant alleles exists, the public health implications would be considerable. Malaria control programs could use molecular markers as part of their surveillance system to assess the dispersion of drug resistance [11]; these tools could provide important information for making policy decisions about the emergence of drug resistance.

In the present study, we investigate the origins of highly resistant *P. falciparum* genotypes in an area of intense malaria transmission in western Kenya by use of microsatellite markers. The results demonstrate that multiple haplotypes representing the triple-mutant *dhfr* genotype are present in this study site, which is inconsistent with previous reports [9, 10]. We directly identify the presence of I164L in 4 different microsatellite haplotypes in western Kenya. We also identify the presence C50R—for the first time outside of South America, to our knowledge—in 2 samples. Taken together, our results suggest multiple origins for pyrimethamine-resistant alleles due to independent accumulation of mutations.

CLIENTS AND METHODS

Study clients. We analyzed blood samples collected from HIV-infected and HIV-uninfected adults enrolled in a longitudinal cotrimoxazole prophylaxis study in the Kisumu region of western Kenya, an area of year-round intense malaria transmission. Clients enrolled in the study had blood samples collected at enrollment, at 6 subsequent scheduled visits, and at all sick visits, for a total of 6 months. Samples were collected at the scheduled visits regardless of whether symptoms of illness were present. Clients who presented with history of fever and any *P. falciparum* parasitemia and those who had no reported fever and a parasitemia of ⩾400 asexual parasites per microliter were treated with SP and asked to return 7 and 14 days later, or at any time if their condition did not improve, to measure parasitemia. Informed consent was obtained from clients. The study was approved by the Human Subjects Review Committees of the Centers for Disease Control and Prevention (CDC) and the Kenya Medical Research Institute.

DNA isolation. DNA was isolated from whole blood or isocode blood spots from all samples with *P. falciparum* present on blood smears and from 70 samples that were negative for asexual *P. falciparum* on blood smears. The presence of *P. fal-*

Table 2. Summary of samples that contained I164L and the method used to genotype *dhfr*.

Sample from client	Amino acid at codon					Genotyping method[a]	
	50	51	59	108	164	DS	PS
1[b]	C	I	R	N	L	1	
2[b]	C	I	R	N	L	1	
3[c]	C	I	C	N	L	2	3
3[c]	C	I	C	N	L	3[d]	1
4	C	I	C	N	L	1	3
5	C	I	R	N	I/L	1	1
6	C	I	R	N	L	1	3
7	C	I	R	N	L		1
8	C	I	R	N	L		1
9	C	I	C	N	L	1	1
10	C	I	R	N	L	C (19/20)	
11	C	I	C	N	L	C (2/19)	
11	C	I	R	N	L	C (6/19)	
12	C	I	R	N	L	C (2/20)	
13	C	I	C	N	L	C (1/19)	
13	C	N	R	N	L	C (1/19)	
13	C	I	R	N	L	C (1/19)	
14	C	I	C	N	L	C (5/20)	
14	R	I	C	N	L	C (1/20)	
14	C	I	R	N	L	C (4/20)	

NOTE. DS, direct sequencing; PS, pyrosequencing.

[a] Nos. under DS or PS indicate no. of times each method was conducted on this sample. C indicates cloning of polymerase chain reaction products followed by DS for these samples; the nos. in parentheses represent the no. of clones with the given genotype/total no. of clones sequenced.

[b] These samples also had mutations at codons 30 and 57.

[c] Client had I164L mutations identified at 2 different time points 5 weeks apart (in November and December 2002) and possessed the same genotype (51I/108N/164L) and haplotype at each time point.

[d] The same *dhfr* sequence was detected in 3 aliquots of a single collection from client 3.

ciparum was confirmed in the smear-negative samples by species-specific polymerase chain reaction (PCR) [12]. DNA from whole blood was isolated using the QIAamp DNA Mini Kit (Qiagen). DNA was isolated from isocode stix (Schleicher & Schuell), as described by the manufacturer.

Genotyping methods. All selected samples were initially genotyped for mutations at codons 50, 51, 59, 108, and 164 in *dhfr* by use of restriction fragment–length polymorphism (RFLP) analysis, as described by Contreras et al. [13] (M.H., unpublished data). Those samples that were found to have an I164L mutation by RFLP analysis were then selected for further evaluation of mutations by use of direct sequencing and/or pyrosequencing. In addition, a subset of samples that did not have the I164L mutation was selected for genotyping by direct sequencing or pyrosequencing. It should be noted that one of the caveats of pyrosequencing, as with other commonly used methods such as RFLP analysis, is that the method identifies mutations independently and does not necessarily reconstruct the true genotype present in a sample.

PCR primers used to amplify *dhfr* for direct sequencing are shown in table 1. The primary PCR consisted of the following,

Table 3. Polymerase chain reaction primers used to amplify microsatellite loci.

dhfr locus, primer	Sequence (5′→3′)
−10 kb	
Forward[a]	CAGAAGGGTTATAATAAGAT
Forward (nested)[a]	CGACATACATACATTTATCA
Reverse[a]	TACAAATGAAGGTCGATTTT
−7.5 kb	
Forward	CATTCCTTTGTTAATTCATCA
Forward (nested)[b]	CCTTCTTATTTTAAAGGGTTA
Reverse[b]	TTTTATTACACACGTTTTTG
−5.3 kb	
Forward[a]	TACATAATTCATATGAACTTG
Forward (nested)[a]	CCTGCATTTGCAAGAAGTA
Reverse[a]	CACATATTATACAGGACGA
−4.5 kb	
Forward	TAGGACACAGAAAATGT
Forward (nested)[b]	TTCTACGAATTATTTTTCCA
Reverse[b]	ACAAGTTAAAAGACGAAACA
−4.4 kb	
Forward[a]	GTTGTCAATAATTTCTGATC
Forward (nested)[a]	TACCATAGCAGTCTTTGCA
Reverse[a]	CGATATATCTGATGGGTGA
−3.8 kb	
Forward	ATGCACGCTCATATTATAAAT
Forward (nested)[b]	ACAGTTATAAGATTTAATGCAA
Reverse[b]	ACTGATGAAATTGTAAATGA
−1.2 kb	
Forward	GAGTAAATATGAATGTGCATGCTTA
Forward (nested)[b]	ATTGAAACTAGCTCAACAAA
Reverse[b]	TTATAAGATTCCCATAGACA
−0.3 kb	
Forward[a]	ATTCCAACATTTTCAAGA
Reverse[a]	GGCATAAATATCGAAAAC
Reverse (nested)[a]	TCCATCATAAAAAGGAGA
0.2 kb	
Forward	TCAATGGATATGGCTGCT
Forward (nested)[b]	TATGAACAAATGATGACAAA
Reverse[b]	ATTTCATGTTCAGGTAAAAA
0.5 kb	
Forward	GACAGATGTATGTGTAACATAC
Forward (nested)[b]	TAAAGAAGGCATAATTTTCA
Reverse[b]	CATTGAGATAAATAAGTGTTCA
4.0 kb	
Forward	AACCTCTTGAAGTATCATT
Forward (nested)[b]	CACATATAAATAAATATATCTCGAA
Reverse[b]	TAAATAACCCAAAATCATGT

[a] Denotes primers designed and used by Roper et al. [9].

[b] Denotes primers designed and used by Nair et al. [8].

Table 4. *dhfr* genotypes identified by direct sequencing or pyrosequencing and their respective microsatellite haplotypes.

dhfr genotype, group, client	Allele at microsatellite locus[a]									
	−7.55 kb	−5.3 kb	−4.49 kb	−4.4 kb	−3.87 kb	−1.22 kb	−0.3 kb	0.2 kb	0.52 kb	4.05 kb
51I/108N										
15[b]	131	191	202	174	188	224	85	158	93	190
15[c]	131	191	202	174/180	188	224	85	158	93	190
16	131	191	202	179	188		85	158	93	189
17	131	199	202	178	188		85	158	93	189
18	133	191	202	176	188		85	158	93	188
19	131	191	202	176	188		85	158	93	190
20	131	191	202	176	188		85	158	93	189
21	115	191	202	164	188		85	158	95	
22	131	191	202	178	188		85	158	93	
23	131	191	202	171	188		85	158	93	188
24	133	191	198/202	174	188		105	158	93	189
25	131	191	202	178	188		110	158	100	
26		191	202	178	188		85	158	93	189
27	131		198	174	192		83/105	173	103	
28	131	191/202					85	158	99	
51I/59R/108N										
A										
29, 30, 31, 32	131	202	198	174	192	213	105	173	103	188
33, 34[d], 34[e], 35, 36, 37	131	202	198	174	192	213	105	173	103	189
38	131	202	198	174	192	213	105	158	103	188
34[f]	131	202	198	174	192	213	105	173	99	189
40	131		198	174	192		105	173	116	176
41	131	202	198	174	192		105	173	101	
B										
42, 43	131	202	198	174	192		102	173	103	188
44	131	202	198	174	192		102	173	87	188
45	131	202	198	174	192	213	102	173	110	189
C										
46	131	202	198	174	192	200/213	85	158	93	188
47	131	202	198	174	192		110/117	173	104	188
48	131	198/202	198	174	192	222/229	85	158	91	187
49	131	202	198	174	188	213	85	158	93	189
50	131	191	191/198	174	192	211	85	158	91/93	188
51	131	191/202	188/198	174	192		85	157	91/93	188
D										
52	131	191	202	188	186/198	213	85	172	91	196
53	131	191	202	176	188	224	85	158	93	190
54	131	191	202	172	188/192	213	85	158	93/103	189

(continued)

in a total volume of 20 μL: 2 μL of template, 20 μmol/L dNTPs, 2 ng/μL primFds (primer), 2 ng/μL primRds (primer), 1× Expand High Fidelity buffer with 15 mmol/L $MgCl_2$ (Roche), and 0.3 U of Expand High Fidelity Enzyme Mix (Roche). Thermal cycling conditions for the primary reaction were as follows: 94°C for 5 min; 35 cycles of 95°C for 30 s, 50°C for 30 s, and 68°C for 1 min; and 68°C for 5 min. The secondary PCR consisted of the same components as the primary, except that the template was 0.2 μL of the primary PCR and the primers used were secFds and secRds. Thermal cycling conditions for the secondary reaction were as follows: 94°C for 5 min; 30 cycles of 95°C for 30 s, 52°C for 30 s, and 68°C for 1 min; and 68°C for 5 min. Some samples were independently genotyped for *dhfr* by direct sequencing in an additional laboratory within the Malaria Branch, CDC.

Primers used to amplify products and to sequence mutations by pyrosequencing are shown in table 1. The primary and nested PCRs consisted of the following, in a total volume of

Table 4. ***(Continued.)***

dhfr genotype, group, client	Allele at microsatellite locus[a]									
	−7.55 kb	−5.3 kb	−4.49 kb	−4.4 kb	−3.87 kb	−1.22 kb	−0.3 kb	0.2 kb	0.52 kb	4.05 kb
51I/108N/164L										
E										
3[g], 3[h]	131	191	202	183	188	224	85	167	97	183
F										
4	131	191/202	202	174	184	200/213	85	158/166	110	187
51I/59R/108N/164L										
G										
5	131						92/120		108	
H										
6	135	191/212	202	180	184	200/219	85	166	97	187

NOTE. Haplotypes for the 51I/59R/108N genotype and genotypes containing 164L have been divided into groups on the basis of similar microsatellite alleles, to emphasize similarities

[a] The locations of the microsatellite loci with respect to *dhfr* are represented in kilobases; negative positions are 5′ to *dhfr*, and nonnegative positions are 3′ to *dhfr*. Some samples had 2 different alleles at a locus, in which case both are given. The alleles listed are the product sizes of polymerase chain reactions in base pairs.

[b] Sample collected January 2003.

[c] Sample collected March 2003.

[d] Sample collected November 2002.

[e] Sample collected 22 October 2002.

[f] Sample collected 8 October 2002.

[g] Sample collected November 2002.

[h] Sample collected December 2002.

100 μL: 1 μL of template, 16 μmol/L dNTPs, 0.5 μmol/L primFpy (primer), 0.5 μmol/L primRpy (primer), 1× PCR buffer with 15 mmol/L $MgCl_2$ (Applied Biosystems), 0.75 mmol/L $MgCl_2$, and 2.5 U of *Taq* DNA polymerase (Promega). Thermal cycling conditions for the primary reaction were as follows: 95°C for 3 min; 45 cycles of 92°C for 30 s, 45°C for 45 s, and 72°C for 45 s; and 72°C for 3 min. Thermal cycling conditions for the nested reaction for *dhfr* codons 50, 51, and 59 were as follows: 95°C for 5 min; 25 cycles of 92°C for 30 s, 45°C for 45 s, and 65°C for 45 s; and 72°C for 15 min. Thermal cycling conditions for the nested reaction for *dhfr* codons 108 and 164 were as follows: 95°C for 5 min; 25 cycles of 92°C for 30 s, 42°C for 30 s, and 65°C for 45 s; and 72°C for 15 min.

In 10 samples, the presence of I164L was confirmed by direct sequencing and/or pyrosequencing. In 5 samples, we cloned the entire *dhfr* gene in *Escherichia coli* and selected 20 colonies from each isolate for sequencing (table 2). The PCR primers were as follows: forward, 5′-ATGATGGAACAAGTCTGCGAC-3′; and reverse, 5′-ACATTTTATTATTCGTTTTC-3′. The PCRs consisted of the following, in a 100-μL reaction: 1 μL of template, 16 μmol/L dNTPs, 2 ng/μL each primer, 1× PCR buffer with 15 mmol/L $MgCl_2$ (Applied Biosystems), 0.75 mmol/L $MgCl_2$ *(dhfr)*, and 2.5 U of *Taq* DNA polymerase (Promega). Thermal cycling conditions were as follows: 95°C for 3 min; 45 cycles of 92°C for 30 s, 45°C for 45 s, and 72°C for 45 s; and 72°C for 3 min. Cloning was conducted using the pGEM-T Easy Vector System (Promega).

Microsatellite analysis. Microsatellite analysis was attempted on the samples that had I164L, as well as on a subset of samples that did not contain I164L. In several cases, we were unable to produce PCR products, and, therefore, there are no microsatellite results for these samples. Samples were assayed for 10 microsatellite loci that span 11.5 kb on chromosome 4 around *dhfr* [8–10]. Microsatellite PCR primers are detailed in table 3. A nested PCR was performed for each locus. PCR and thermal cycling conditions are described by Roper et al. [9]. PCR products were separated on an Applied Biosystems 3100 capillary sequencer and were scored using GeneMapper software (version 3.7; Applied Biosystems).

RESULTS

Sequencing of *dhfr* revealed several instances in which there was a nonsynonymous mutation at codon 164 (table 2) causing an amino acid change from isoleucine to leucine. A total of 15 samples from among those surveyed contained the I164L mutation. There were a total of 20 observations of the I164L mutation (table 2). The number of observations is greater than the number of samples because *dhfr* was cloned and sequenced from several clients; consequently, several clones were sequenced from each client, and those containing I164L are listed in table 2. Indeed, the I164L mutation was found in 5 different genotypes with mutations at other sites. Specifically, 7 samples contained the genotype 51I/108N/164L, 1 sample contained the genotype 59R/108N/164L, 1 sample contained the genotype 50R/51I/108N/164L, and 9 samples contained the genotype 51I/

59R/108N/164L. A sextuple *dhfr* mutant 30G/51I/57H/59R/108N/164L was also found in 2 samples via the sequencing survey. To our knowledge, this is the first description of this particular genotype. Mutations at codons 30 and 57 have not been described before. Only the presence of genotypes is reported, because the frequency of clones may reflect only the differential affinity of the primers and not the frequency of the allele in the infection. To rule out any potential artifacts, a subset of samples chosen for this analysis was tested in 2 different laboratories within the CDC, and the presence of I164L was confirmed in both laboratories.

The C50R mutation was detected in 2 samples in which the *dhfr* gene was PCR amplified and then cloned into *E. coli*. C50R was identified in client 13 in 1 of 19 clones that were sequenced and found to have the genotype 50R/51I/108N. The mutation was also identified in client 14, in 1 of 20 clones sequenced, with the genotype 50R/51I/108N/164L (table 2).

Microsatellite markers around *dhfr* were utilized to assess the evolutionary history of the genotypes containing I164L as well as double- and triple-mutant genotypes. Genotyping of *dhfr* was performed by sequencing for all samples utilized in the microsatellite analysis. For the microsatellite analysis, we chose samples that did not contain multiple infections detected by sequencing. Regardless of this provision, some of the samples contained multiple alleles at microsatellite loci, thus hampering our attempt to define exact haplotypes. Most of the haplotype groups we report here include samples in which there were single infections, and, in those cases, haplotype reconstruction was possible. We could not reconstruct the exact haplotypes for 3 of the 5 samples analyzed that contained I164L. Nevertheless, our data show that the I164L mutation is associated with at least 4 distinct groups of haplotypes, given that the samples did not share alleles at multiple loci (table 4). The genotype 51I/108N/164L was found in 2 distinct groups of microsatellite haplotypes, differing in 6 of 10 loci (groups E and F; table 4). Client 3 had I164L mutations identified at 2 different time points 5 weeks apart and possessed the same genotype (51I/108N/164L) and haplotype at each time point (group E; table 4). The haplotype found in client 3 is similar to what is seen for *dhfr* genotype 51I/108N in the chromosomal region 5′ to *dhfr* (table 4). The microsatellite alleles 3′ to the gene, however, are unique to this haplotype. Client 4 also harbored parasites with the 51I/108N/164L genotype (group F; table 4), but the haplotype group of client 4 is distinct from that of client 3. The quadruple-mutant genotype 51I/59R/108N/164L is represented by 2 groups of haplotypes (groups G and H; table 4). Although the genetic data are limited, group G is distinct from group H at every locus.

To validate the hypothesis of a single origin for the 51I/59R/108N *dhfr* genotype in Africa [9], we determined the microsatellite haplotype for 28 samples that were confirmed by sequencing to have the triple-mutant *dhfr* genotype. These haplotypes are presented in table 4. The haplotype previously described in Africa by Roper et al. [9] was present in 11 samples (group A). Three additional groups of haplotypes were also present (groups B, C, and D). Group D is quite distinct from the others and has a different set of alleles at the majority of the microsatellite loci surrounding *dhfr*. The loci at −0.3 kb and −5.3 kb are critical in determining different haplotype groups for the triple-mutant genotype. The data here show 5 different alleles for the −0.3 kb locus and 3 alleles for the −5.3 kb locus, 2 loci that have displayed no variation in previous studies [9, 10].

DISCUSSION

The *dhfr* genotype 51I/59R/108N/164L has been identified with clinically resistant parasites from Southeast Asia and South America, but its presence in Africa has been an elusive and a debatable issue until recently [6, 7, 14]. The concern has been that the rapid spread of this genotype in Africa could render SP widely ineffective for treatment of uncomplicated malaria or for the prevention of malaria during pregnancy. Mutations in *dhfr* that cause resistance to SP also confer resistance to other antifolates, including chlorproguanil, a component of the promising new antifolate antimalarial combination chlorproguanil-dapsone. Our findings corroborate those of other recent studies showing that the I164L mutation is beginning to appear in Africa [4–7]. Unlike these previous studies in Africa, our study included microsatellite markers as an additional tool to determine the haplotypes of isolates with I164L and to demonstrate that the I164L mutation is present in at least 4 distinct lineages, which indicates that, in this region, this mutation may have been independently selected for.

The progressive appearance of mutations in *dhfr* in natural populations has led to the suggestion that the mutations occur in an ordered, stepwise fashion [15]. In the present study, we observed the presence of the 51I/108N/164L genotype in at least 4 different haplotypes. If the *dhfr* genotypes arose by a progressive sequence, then the expectation would be that a mutation at codon 164 would occur only in a lineage that already had mutations at codons 108, 51, and 59. The presence of these genotypes demonstrates that mutations in *dhfr* do not always occur in a predictable, ordered, stepwise manner as observed previously [15, 16]. This finding supports the notion that convergent genotypes occur and could hamper approaches based on the tracking of specific alleles defined as groups of markers that are stable in time.

The results of our study suggest that local evolutionary history, as well as gene flow, is important to consider. Previous reports of the triple-mutant genotype implicate gene flow as the major force moving this genotype across continents [10, 11], but the situation in western Kenya does not appear to fit

Table 5. Clinical characteristics of the samples used in this study.

Client	HIV infected	Parasite density, parasites/mL	CTX[a]
1	Y	27	N
2	Y	0	Y
3[b]	Y	29,970	N
3[c]	Y	189	N
4	N	4779	N
5	N	135	N
6	Y	1512	N
7	Y	27	N
8	Y	0	Y
9	Y	0	N
10	Y	0	N
11	Y	0	N
12	Y	2025	N
13	Y	0	N
14	Y	0	N
15[d]	Y	1134	N
15[e]	Y	297	N
16	Y	5400	N
17	Y	5454	N
18	Y	19,305	N
19	N	2187	N
20	N	54	N
21	N	54	N
22	Y	81	N
23	Y	135	N
24	Y	54	N
25	Y	81	N
26	Y	162	N
27	Y	243	N
28	Y	702	N
29	Y	3267	N
30	Y	1053	N
31	Y	513	N
32	Y	2754	N
33	Y	8019	N
34[f]	Y	0	N
34[g]	Y	0	N
34[h]	Y	189	N
35	N	8343	N
36	Y	27,189	Y
37	Y	243	Y
38	Y	78,651	N
39	Y	27	Y
40	N	54	N
41	Y	378	N
42	Y	81	N
43	Y	81	Y
44	Y	621	N
45	Y	351	N
46	Y	108	N
47	Y	81	N
48	Y	5670	N
49	Y	405	N
50	Y	297	N
51	Y	486	N
52	Y	19,278	N
53	N	5211	N

(continued)

Table 5. ***(Continued.)***

[a] Denotes whether the client was receiving cotrimoxazole (CTX) at the time of sample collection.
[b] Sample collected November 2002.
[c] Sample collected December 2002.
[d] Sample collected January 2003.
[e] Sample collected March 2003.
[f] Sample collected November 2002.
[g] Sample collected 22 October 2002.
[h] Sample collected 8 October 2002.

that model. Group E, representing a 51I/108N/164L genotype, has similarities to a common haplotype for 51I/108N 5′ to the gene but is different 3′ to the gene. This suggests that recombination, rather than the accumulation of simple point mutations for haplotype E, may have played a role in the emergence of this novel genotype. The role of recombination is not surprising, given the high transmission intensity in western Kenya. The high transmission in this area results in a large number of multiple infections and, consequently, a greater amount of recombination among different alleles.

We cannot accurately assess the population prevalence of I164L in western Kenya on the basis of current data. Our study population was not representative of persons living in western Kenya; rather, it was a self-selected population of HIV-infected and HIV-uninfected persons who volunteered to participate in the study. Moreover, uniform selection criteria were not used for all specimens included in the analysis for the presence of the I164L mutation. The I164L mutation was found in both HIV-infected and HIV-uninfected clients (table 5 and M.H., unpublished data).

The samples used here were taken from a longitudinal cotrimoxazole prophylaxis study. Cross-resistance between trimethoprim, a component of cotrimoxazole, and pyrimethamine has been demonstrated in vitro [17, 18]. The presence of cotrimoxazole in the population may have influenced the presence of *dhfr* mutations in the samples. However, our results indicate that I164L and the other *dhfr* mutations do not exist in this population solely as the result of cotrimoxazole use: only 6 of 53 total samples were collected from clients who were using cotrimoxazole at the time of sample collection (table 5).

We also report the presence of C50R in 2 samples identified

from cloned PCR products. This nonsynonymous mutation at codon 50 has been described at several sites in South America but has not been detected in Africa. C50R does confer resistance to pyrimethamine, chlorcycloguanil, and cycloguanil, as demonstrated by an in vitro yeast study [2]; therefore, the presence of this mutation in Africa may be of clinical importance. If the mutation is present with other *dhfr* mutations and selectively spreads, there could be an increase in antifolate drug resistance on the continent. Many studies investigating *dhfr* mutations in Africa do not look for mutations at codon 50. Our results here suggest that researchers need to consider C50R in future studies.

In contrast to recent findings that *dhfr* triple-mutant genotypes from 2 different African countries and Southeast Asia shared a common microsatellite haplotype [10], we report that these mutant genotypes exist in multiple lineages. The previously documented [8–10] low level of microsatellite variation around *dhfr* genotypes was somewhat unexpected, because resistance to pyrimethamine spreads rapidly after introduction and has a simple genetic basis [15, 16]. Therefore, the expectation is that SP-resistant genotypes, even those with multiple mutations, would have several different lineages [19]. Nevertheless, the hypothesis of a single origin was appropriate in previous investigations, given the data [8–10]; however, our results demonstrate that SP-resistant *dhfr* genotypes may have multiple origins [19]. The question of why the present study documented a higher number of haplotypes in western Kenya than the previous study that used samples from Tanzania and South Africa then arises.

Our findings could be the combined result of high transmission intensity, the history of *P. falciparum* populations in western Kenya, and the selective pressure of drug use. This pattern of a large amount of genetic variation is consistent with previous studies of antigen-encoding genes: variation was greater in samples from western Kenya than in those from other parts of Africa or other regions of the world [20, 21]. High levels of transmission result in infections by multiple strains in a single individual. The result is a gene pool that maintains a higher effective population size—that is, a higher level of genetic diversity. There are more genetically distinct parasites mating and contributing to the next generation, reducing the fluctuation of population allele frequencies by chance and, therefore, maintaining a higher level of variation on which natural selection can act. However, it is worth emphasizing that the present study reports mutations observed in a specific population. When these mutations originate, whether they become fixed or lost, and how they could eventually be dispersed are matters to be addressed by further field and theoretical studies that are beyond the scope of this investigation [14, 19].

Microsatellite markers are a powerful tool to track drug-resistant parasite populations. The data presented here show that the use of multiple microsatellite markers is needed to track *dhfr* genotypes and that these markers may be different in different regions of the world. We also note that recombination can generate new drug-resistant alleles in parasite populations.

In conclusion, we have established the presence of multiple lineages of triple-mutant and quadruple-mutant *dhfr* genotypes in an area of high malaria transmission in western Kenya. We have also demonstrated the presence of novel and elusive mutations that have not been well documented in a holoendemic area of Africa. These findings highlight the importance of molecular surveillance [22] in different endemic settings and populations [23] for understanding how SP resistance in particular and drug resistance in general may originate and spread to populations.

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