MAJOR ARTICLE

Selective Sweeps and Genetic Lineages of *Plasmodium falciparum* Drug -Resistant Alleles in Ghana

Md Tauqeer Alam,[1,3] Dziedzom K. de Souza,[4] Sumiti Vinayak,[1,3] Sean M. Griffing,[1,3,2] Amanda C. Poe,[1,3] Nancy O. Duah,[5] Anita Ghansah,[4] Kwame Asamoa,[1] Laurence Slutsker,[1] Michael D. Wilson,[4] John W. Barnwell,[1] Venkatachalam Udhayakumar,[1] and Kwadwo A. Koram[5]

[1]Malaria Branch, Division of Parasitic Diseases and Malaria, Center for Global Health, Centers for Disease Control and Prevention; [2]Emory University, Atlanta; [3]Atlanta Research and Education Foundation, VA Medical Center, Georgia; and; [4]Parasitology Department and [5]Epidemiology Department, Noguchi Memorial Institute for Medical Research, University of Ghana, Legon, Ghana

Background. In 2005, Ghana adopted artemisinin-based combination therapy (ACT) for primary treatment of falciparum malaria. A comprehensive study of the drug-resistance–associated mutations and their genetic lineages will lead to a better understanding of the evolution of antimalarial drug resistance in this region.

Methods. The *pfcrt*, *pfmdr*1, *dhps*, and *dhfr* mutations associated with chloroquine (CQ) and sulfadoxine-pyrimethamine (SP) resistance and the microsatellite loci flanking these genes were genotyped in *Plasmodium falciparum* isolates from Ghana.

Results. The prevalence of mutations associated with both CQ and SP resistance was high in Ghana. However, we observed a decrease in prevalence of the *pfcrt* K76T mutation in northern Ghana after the change in drug policy from CQ to ACT. Analysis of genetic diversity and differentiation at microsatellite loci flanking all 4 genes indicated that they have been under strong selection, because of CQ and SP use. The triple-mutant *pfcrt* and *dhfr* alleles in Ghana were derived from Southeast Asia, whereas the double-mutant *dhfr*, *dhps*, and *pfmdr*1 alleles were of African lineage.

Conclusion. Because of the possible role of *pfmdr*1 in amodiaquine and mefloquine resistance, demonstrating selection on *pfmdr*1 and defining lineages of resistant alleles in an African population holds great importance.

Until 2004, chloroquine (CQ) and sulfadoxine-pyrimethamine (SP) were the official first- and second-line drugs against uncomplicated *Plasmodium falciparum* malaria in Ghana [1]. CQ-resistant parasites were first reported in Ghana in the mid-1980s [2]. Subsequent studies in different parts of the country indicated a significant drop in overall cure rate for CQ (to <50% in some areas) [1, 3–6], leading to a change in drug policy in 2005. Since then, artemisinin-based combination therapy (ACT) consisting of artesunate plus amodiaquine (AS+AQ) has been the first-line drug therapy against uncomplicated falciparum malaria [1]. Although SP's efficacy has always been higher than CQ's efficacy, resistance to SP has also been increasing in Ghana [1, 7, 8]. Since 2004, SP has been the Ghanaian drug of choice for intermittent preventive treatment in pregnant women [1]. Increases in resistance to CQ and SP were also supported by molecular studies, which registered a high prevalence of drug resistance–associated mutations in Ghanaian parasites [4, 9–13].

CQ resistance is determined by key mutations (C72S, M74I, N75E/D, and K76T) in the *P. falciparum* CQ-resistance transporter (*pfcrt*) gene, with K76T being the critical one [14]. There are several lines of evidence suggesting that mutations in the *P. falciparum* multidrug

Received 10 June 2010; accepted 24 August 2010.

Potential conflicts of interest: none reported.

Disclaimer: *The findings and conclusions in this article are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention.*

Reprints or correspondence: Dr Md Tauqeer Alam, Malaria Branch, Div of Parasitic Diseases and Malaria, Center for Global Health, Centers for Disease Control and Prevention, 1600 Clifton Rd NE, Mail Stop D-67, Atlanta, GA 30329 (hsf1@cdc.gov; tauqeer9@gmail.com).

The Journal of Infectious Diseases 2011;203:220–227

1537-6613/2011/2032-0001$15.00
DOI: 10.1093/infdis/jiq038

resistance gene 1 (*pfmdr1*) (N86Y, Y184F, S1034C, N1042D, and D1246Y) also confer or modulate CQ resistance [15]. Sulfadoxine and pyrimethamine resistance are conferred by mutations in the dihydropteroate synthase ([*dhps*]; S436A/F, A437G, K540E, A581G, and A613S/T) and dihydrofolate reductase [([*dhfr*]; A16V, C50R, N51I, C59R, S108N/T, and I164L) genes, respectively [16]. The critical mutation in *dhps* is A437G, and in *dhfr* it is S108N.

Characterization of the genetic backgrounds (microsatellite loci/markers) flanking these genes has helped to define the geographical origins and dissemination of CQ- and SP-resistant *P. falciparum*. Analyses of *pfcrt* and *dhfr* point mutations and flanking microsatellite loci showed that alleles conferring high levels of CQ and pyrimethamine resistance (3 or more mutations) originated only 4–5 times globally [17–20]. In contrast, *dhps* alleles conferring high levels of sulfadoxine resistance have multiple origins [21]. In Africa, the highly resistant *pfcrt* ($C_{72}V_{73}\underline{I}_{74}\underline{E}_{75}\underline{T}_{76}$, mutant amino acids are underlined) and *dhfr* ($C_{50}\underline{I}_{51}\underline{R}_{59}\underline{N}_{108}I_{164}$) alleles are originally derived from Southeast Asia [11, 12, 18–20, 22, 23]. The double-mutant *dhfr* alleles in Africa (CNRNI and CICNI) originated indigenously on multiple genetic backgrounds [11, 12, 22, 23]. However, indigenous evolution of triple-mutant *dhfr* CIRNI from these double mutants has also been observed in Ghana, Kenya, Senegal, and Republic of Congo [12, 22, 24]. Thus, in addition to predominant Southeast Asian triple-mutant lineage, there are 2–3 indigenous lineages of the triple-mutant *dhfr* alleles in Africa [12].

Regarding the origins and spread of resistant *dhps* alleles in Africa, studies have shown that the 2 predominant double-mutant *dhps* alleles in Africa, $\underline{A}_{436}\underline{G}_{437}K_{540}A_{581}A_{613}$ (mostly in West and Central Africa) and $S_{436}\underline{G}_{437}\underline{E}_{540}A_{581}A_{613}$ (mostly in East Africa) have emerged recently on multiple independent genetic backgrounds [13, 25]. Pearce et al observed at least 3 lineages for AGKAA and 2 lineages for SGEAA [13]. Moreover, we have recently shown that unlike highly resistant *pfcrt* and *dhfr*, the resistant *dhps* alleles in Africa do not share origins with Southeast Asian *dhps* alleles, which suggests sulfadoxine resistance evolved indigenously in Africa [21]. Surprisingly, selective sweep and genetic lineages of mutant *pfmdr*1 alleles have not been investigated in Africa, despite that the suggestion that mutation(s) in this gene are selected in response to CQ use, and more recently to lumefantrine and AQ (partner drugs in ACT) use, on this continent [26–28]. In addition, the *pfmdr*1 gene amplification plays an important role in resistance to mefloquine (MQ), another partner drug in ACT [29].

In this study, we systematically characterized *pfcrt*, *pfmdr*1, *dhps,* and *dhfr* mutations and 34 microsatellite loci flanking these genes in the Ghanaian parasites. Our aims were to investigate (1) the evidence of selection at these genes; (2) the genetic lineages of the alleles conferring CQ and SP resistance, and (3) any change in the prevalence of *pfcrt* 76T mutation 3 years after the change in drug policy from CQ to ACT.

MATERIALS AND METHODS

Study Sites and Samples

The *P. falciparum*–infected blood samples were collected on filter paper at 4 different sites (50 samples each from Navrongo, Sunyani, Bekwai, and Cape Coast, Ghana) during 2007–2008 (Figure 1). These areas are among the 10 sentinel sites set up for the monitoring of antimalarial drug efficacy in the country and belong to different ecological zones. Navrongo is located in the Sudan savanna zone, where malaria is hyperendemic and seasonal. Cape Coast is part of the coastal savanna area and Sunyani and Bekwai are part of the forest zone. Malaria in these 3 regions is hyperendemic and perennial. All samples were collected from children aged ⩽5 years with symptoms of malaria presenting at the local health clinic. Written informed consent was obtained from a parent or guardian of each patient before blood collection. The study was approved by the Institutional Review Board of the Noguchi Memorial Institute for Medical Research, University of Ghana (Legon, Ghana).

Sequencing of Codons Implicated in Drug Resistance

DNA samples from all 200 *P. falciparum*–positive filter paper blood spots were extracted using the Tris-EDTA method as described in a previous article [30]. They were genotyped for all key codons implicated in CQ (*pfcrt* codons 72–76; *pfmdr*1 codons 86, 184, 1034, 1042, and 1246), sulfadoxine (*dhps* codons 436, 437, 540, 581 and 613), and pyrimethamine (*dhfr* codons 50, 51, 59, 108 and 164) resistance. For amplification and sequencing of *pfcrt* [31], *pfmdr*1 [32] and *dhps* [21], we used previously described primers and methods. The *dhfr* gene

Figure 1. Map of Ghana showing the 4 sites (solid black circles) from which *Plasmodium falciparum* isolates were collected for this study.

fragment was amplified using a nested PCR approach. We performed the first round of polymerase chain reaction (PCR) using 5′-TCCTTTTTATGATGGAACAAG-3′(F1) and 5′-AGTATATACATCGCTAACAGA-3′(R1) primers with the following cycling parameters: initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 30 s, and extension at 68°C for 1 min. This was followed by a final extension at 68°C for 5 min. We performed secondary PCR using 5′-TTTATGATGGAACAAG-TCTGC-3′(F2) and 5′-ACTCATTTTCATTTATTTCTGG-3′(R2) primers. The cycling parameters for the secondary reaction were the same as for the primary, except that the annealing temperature was set at 52°C for 30 s and the number of cycles was reduced to 30. Sequencing of the *dhfr* fragment was done on both strands using F2/R2 primers following standard sequencing protocol on an ABI 3130xl Genetic Analyzer (Applied Biosystems) as described in previous studies [21, 32]. Samples with multiple peaks at any of the codons genotyped were not included in allele frequency calculations and were not carried forward for microsatellite typing.

Typing of Microsatellite Loci

We typed 34 microsatellite loci flanking the 4 drug-resistance genes. These comprised 8 loci each flanking *pfcrt* (at −45.1, −17.7, −4.8, −4.5, 1.5, 3.9, 18.8, and 45.3 kb), *dhfr* (at −7.5, −4.4, −3.8, −.06, .1, .45, 1.3, and 5.8 kb), *dhps* (at −7.5, −2.9, −1.5, −.13, .03, .5, 1.4, and 6.4 kb), and 10 loci flanking *pfmdr*1 (at −29.5, −9.3, −4.2, −3.3, 0, .16, .45, 3.6, 9.1, and 23.3 kb). Because microsatellite loci close to these genes would behave as nonneutral because of hitchhiking, we also analyzed 8 putatively neutral microsatellite loci. These included 4 loci on chromosome-2 spanning 78 kb (GenBank UniSTS ID/kb location: C2M27/380 kb, C2M29/319 kb, C2M34/313 kb, C2M33/302 kb) and 4 on chromosome-3 spanning 94 kb (C3M40/335 kb, C3M88/363 kb, C3M69/383 kb, and C3M39/429 kb). The primers and cycling parameters for *pfmdr*1, *dhps,* and neutral microsatellite loci were adapted from previous studies [21, 32–34]. Similarly, we also used previously published primers for *pfcrt* [19, 34] and *dhfr* [22,34,35] microsatellites with minor modifications as described in the supplementary table available online (Table S1). Allele scoring for all microsatellites was done using GeneMapper software, version 3.7 (Applied Biosystems). Samples presenting multiple alleles at any of the 42 loci were considered polyclonal infections and were not used for multilocus microsatellite haplotype construction and other analyses.

Estimating Expected Heterozygosity and Genetic Differentiation

There are several ways of detecting signature of selection on a gene/locus [33, 34, 36]. It has been suggested that loci directly under selection or linked to any other selected loci exhibit low expected heterozygosity (H_e) values [37]. This low H_e can be used as a signature to identify candidate loci that have been the target of selection [37]. Natural selection not only alters H_e but also affects genetic differentiation (F_{ST}). The F_{ST} values at selected loci are expected to be exceptionally low or high compared with loci that are not under the influence of any selection [38]. Thus, we estimated H_e and F_{ST} at microsatellite loci flanking *pfcrt*, *pfmdr*1, *dhfr,* and *dhps* in order to investigate evidence of selection occurring at these genes. Similar analyses were also done for all 8 neutral microsatellite loci. H_e at each microsatellite loci was estimated using the Excel Microsatellite Toolkit version 3.1.1 [39]. We used formulae $[n/(n-1)][1-\Sigma p_i^2]$ for H_e; and $2(n-1)/n^3\ \{2(n-2)\ [\Sigma(p_i^3-(\Sigma p_i^2)^2]\}$ for sampling variance, where n is the number of samples genotyped for any locus and p_i is the frequency of the ith allele. The Weir and Cockerham F_{ST} [40] was calculated by jackknifing per locus, using the software GENETIX version 4.05 [41]. 95% confidence intervals (CI) were obtained by bootstrapping over loci with 1000 permutations. Mean H_e between wild type and different mutant alleles (for all 4 genes) were compared using the Mann–Whitney test (Stata, version 8.1; StataCorp). Similarly, mean F_{ST} between drug-resistant (selected) and neutral (unselected) loci were compared using the Mann–Whitney test. Statistical significance was defined as $P \leq .05$. All microsatellite loci described in the preceding subsection were used for estimating H_e and F_{ST}.

Constructing Multilocus Microsatellite Haplotypes

We constructed multilocus microsatellite haplotypes for each gene in eBURST version 3 [42] to define the genetic lineages of the resistant alleles in Ghana. We compared the constructed haplotypes with the published data from Southeast Asia and other parts of Africa [12, 13, 18, 19, 21, 25, 32, 35]. For *pfcrt* (−4.8 to 3.9 kb) and *pfmdr*1 (−9.3 to 9.1 kb), only the loci closer to the genes were used for constructing multilocus haplotypes. For *dhfr* and *dhps,* all 8 loci were used for haplotype construction. Because the evolution of *pfmdr*1 and *dhps* mutations in Ghana is not well studied, the median-joining networks were constructed in NETWORK version 4.5.1.0 (http://www.fluxus-engineering.com/sharenet.htm) to visualize the relationships among different alleles of these genes.

RESULTS AND DISCUSSION

Prevalence of Drug-Resistant Alleles

Of 200 samples, 178 samples yielded the complete sequence with single genotype for *pfcrt*, 176 for *pfmdr*1, 167 for *dhfr*, and 171 for *dhps* (Table 1). The rest of the samples in each group had either multiple alleles (>1 peak in the sequencing electropherogram) or missing data at 1 or more codons, and thus were not included in calculating allelic frequencies. Overall, the mutations/alleles associated with CQ and SP resistance were high in Ghanaian parasites (Table 1). We observed the *pfcrt*-CVIET allele in 48% of the isolates, with greater prevalence ($P < .0001$) in southern Ghana (Bekwai, 65%; Cape Coast, 67%) than in

Table 1. Distribution of *pfcrt*, *pfmdr*1, *dhfr* and *dhps* alleles in Ghana.

Gene (Key Codons)	Alleles	Regions				Total No. (%)
		Navrongo No. (%)	Sunyani No. (%)	Bekwai No. (%)	Cape Coast No. (%)	
pfcrt (72–76)	CVMNK*	30 (73.2)	33 (67.3)	14 (35.0)	16 (33.3)	93 (52.2)
	CVIET	11 (26.8)	16 (32.6)	26 (65.0)	32 (66.7)	85 (47.8)
		Σ=41	Σ=49	Σ=40	Σ=48	Σ=178
*pfmdr*1 (86,184,1034, 1042, 1246)	NYSND*	3 (7.5)	9 (18.8)	13 (31.7)	8 (17.0)	33 (18.8)
	NFSND	25 (62.5)	18 (37.5)	13 (31.7)	18 (38.3)	74 (42.0)
	YYSND	1 (2.5)	2 (4.2)	3 (7.3)	2 (4.3)	8 (4.5)
	YFSND	10 (25.0)	14 (29.2)	12 (29.3)	16 (34.0)	52 (29.5)
	YYSNY	1 (2.5)	5 (10.4)	0	3 (6.4)	9 (5.1)
		Σ=40	Σ=48	Σ=41	Σ=47	Σ=176
dhfr (50, 51, 59, 108,164)	CNCSI*	18 (48.6)	13 (31.0)	2 (4.6)	5 (11.1)	38 (22.8)
	CNCNI	1 (2.7)	0	0	1 (2.2)	2 (1.2)
	CNRNI	5 (13.5)	6 (14.3)	4 (9.3)	10 (22.2)	25 (15.0)
	CICNI	1 (2.7)	0	2 (4.6)	1 (2.2)	4 (2.4)
	CIRNI	12 (32.4)	23 (54.7)	35 (81.0)	28 (62.0)	98 (58.7)
		Σ=37	Σ=42	Σ=43	Σ=45	Σ=167
dhps (436, 437, 540,581,613)	SAKAA*	2 (5.1)	1 (2.0)	0	0	3 (1.8)
	SGKAA	10 (25.6)	12 (24.5)	13 (37.1)	28 (58.3)	63 (36.8)
	AAKAA	14 (35.9)	2 (4.1)	3 (8.6)	2 (4.2)	21 (12.3)
	FAKAS	0	1 (2.0)	0	4 (8.3)	5 (3.0)
	YAKAS	2 (5.1)	1 (2.0)	1 (2.9)	0	4 (2.3)
	AGKAA	10 (25.6)	30 (61.2)	18 (51.4)	11 (23.0)	69 (40.3)
	SGEAA	0	1 (2.0)	0	2 (4.2)	3 (1.8)
	AGKAS	1 (2.6)	1 (2.0)	0	1 (2.1)	3 (1.8)
		Σ=39	Σ=49	Σ=35	Σ=48	Σ=171

NOTE. *Indicates wild-/ancestral-type alleles; mutated amino acids are underlined.

northern Ghana (Navrongo, 27%; Sunyani, 33%). Interestingly, we observed a decreased prevalence of *pfcrt* 76T mutation, especially in the northern Ghana, compared with frequencies reported among isolates before the change in drug policy to ACT from CQ [4, 10, 43]. Duah et al reported 46% and 61% prevalence of *pfcrt* 76T in Navrongo and Sunyani, respectively, in a sample set collected during 1998—2000 [4]. Our results are in agreement with several other studies wherein decline in drug-resistance-associated mutations or resistant parasites have been observed several years after the change in drug policy [44, 45].

The prevalence of mutant *pfmdr*1 alleles also remained high (81%) in these samples. The single-mutant NFSND (42%) *pfmdr*1 allele was predominant, followed by double-mutant YFSND (30%). Alleles YYSND and YYSNY were found in equal frequencies (5% each). When we analyzed the samples according to mutation at either codon 86 or 184, we observed that 39% of the isolates had 86Y, whereas 72% had 184F. We found no significant difference between northern and southern Ghanaian parasites in the prevalence of either 86Y (33/88 vs 36/88; $P = .38$) or 184F (67/88 vs 59/88; $P = .12$) mutations (Table 1). We could compare only the N86Y data from our study with earlier studies because this is the only codon that had been genotyped previously in Ghana. In all previous studies, the prevalence of 86Y mutations was high, ranging from 42%–78% [4, 46]. Previous studies have shown that the parasites harboring *pfmdr*1 haplotypes YYSNY and NFSND are selected in recrudescence and reinfection following AS+AQ and AL (artemether plus lumefantrine) treatment, respectively [26–28]. Lim et al found the *pfmdr*1 184F mutation to be associated with high half-maximal inhibitory concentration or resistance to MQ in Cambodia [47].

Approximately 77% of the isolates had mutations in *dhfr*, predominantly the triple-mutant CIRNI (59%), followed by double (CNRNI, 15%; CICNI, 2%) and single (CNCNI, 1%) mutants (Table 1). Similar to that of *pfcrt* 76T, prevalence of resistant *dhfr* alleles was also higher ($P < .0001$) in southern Ghana (Bekwai, 95%; Cape Coast, 89%) compared with northern Ghana (Navrongo, 51%; Sunyani, 69%). In *dhps*, 98% of the isolates had mutations, predominantly the AGKAA (40%) and SGKAA (37%), followed by AAKAA (12%), FAKAS (3%), YAKAS (2%), SGEAA (2%), and AGKAS (2%). As expected, we observed no decline in the frequency of *dhfr* and *dhps* mutations in the parasites after the change in drug policy [9–12, 48, 49].

Evidence of Selection at *pfcrt, pfmdr*1, *dhfr* and *dhps* Genes

We typed 42 microsatellite loci in samples that had complete sequencing data for all drug-resistance genes (Table 1). Samples presenting >1 allele at any of the 42 loci were excluded from further analyses. Though this approach led to the loss of a substantial number of samples, we still had a sufficient number of samples with unambiguous haplotype data ($n = 69$ for *pfcrt*; $n = 63$ for *pfmdr*1; $n = 65$ for *dhfr*; and $n = 66$ for *dhps*) and only these were used for constructing multilocus haplotypes and for H_e/F_{ST} analyses.

The mean H_e (standard deviation [SD]) at 8 microsatellite loci around mutant *pfcrt* allele CVIET ($H_e = .51$ [.07]; $n = 37$) was 22% lower ($P = .03$) than those around wild-type allele CVMNK ($H_e = .73$ [.06]; $n = 32$) (Figure 2A). Because there were 3 different alleles in *pfmdr*1, H_e was estimated around each of them separately. Interestingly, the reduction in H_e was more profound around double-mutant YFSND ($H_e = .50$ [.06]; $n = 21$) than around single-mutant NFSND ($H_e = .85$ [.01]; $n = 21$; $P = .0006$) and ancestral-type NYSND ($H_e = .88$ [.01]; $n = 14$; $P = .0004$). As shown in Figure 2B, there was no significant difference in mean H_e between NFSND and NYSND ($P = .10$). Another interesting observation was that the mean H_e around *pfcrt* CVIET and *pfmdr*1 YFSND alleles was almost equal ($P = .411$). This could be because in *pfcrt* we analyzed some loci farther from the gene (±45 kb) than in *pfmdr*1 (29.5 kb upstream and 23.3 kb downstream). The mean H_e [SD] around resistant *dhfr* alleles were significantly low (CIRNI triple mutants, .14 [.03]; $n = 41$; $P < .0001$; CNRNI double mutants, .58 [.07]; $n = 11$; $P = .025$) compared with the wild-type allele (CNCSI, .80 [.03]; $n = 11$) (Figure 2C).

For estimating H_e around *dhps*, we could include only AGKAA double-mutant and SGKAA single-mutant alleles (Figure 2D). Other alleles were limited in number. The mean H_e [SD] around AGKAA (.44 [.06]; $n = 19$) was 19% lower ($P = .017$) than that of SGKAA (.63[.05]; $n = 33$), confirming that an additional mutation at codon 436 (S436A) provided greater selective advantage than a single mutation at codon 437 (A437G). This is also in accordance with a kinetics study that showed that the enzyme with 436A + 437G mutations (AGKAA) exhibited an extremely high inhibitory constant for sulfadoxine (K_i [SD] = 19.9 [4] μM) compared with the enzyme with only the 437G (SGKAA) mutation (K_i [SD] = 1.39 [.23] μM) [50]. In contrast, in a recent study from Africa, only 3 loci (.8 kb, 4.3 kb, and 7.7 kb) flanking *dhps* were analyzed and no significant difference in the mean H_e around these 2 *dhps* alleles was observed [13].

Thus, we found a great reduction in H_e around mutant alleles as compared with that around their respective ancestral/wild-type alleles. Such a trend is consistent with a model of positive directional selection. The locus-by-locus F_{ST} analyses also supported our H_e results. The mean F_{ST} values at the microsatellite loci linked to drug-resistance genes (*pfcrt*, *pfmdr*1, *dhfr*, and *dhps*) were elevated and significantly greater compared with mean F_{ST} at 8 neutral loci ($P < .0001$ in all comparisons),

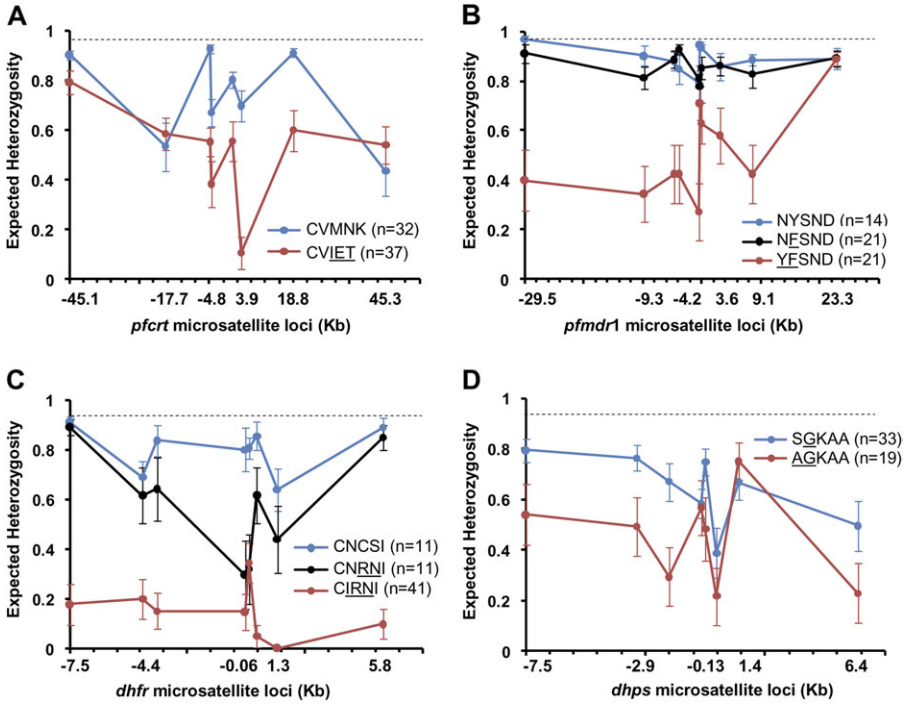

Figure 2. The expected heterozygosity (H_e) at microsatellite loci flanking (A) *pfcrt*, (B) *pfmdr*1, (C) *dhfr*, and (D) *dhps* alleles. The dashed line crossing the y-axis indicates the mean H_e at 8 neutral microsatellite loci on chromosomes 2 and 3. Mutant amino acids are underlined. Error bars indicate standard deviation (SD).

confirming strong selection at all 4 genes in Ghanaian parasites due to CQ and SP use (Figure S1). To the best of our knowledge, this is the first study in Africa demonstrating evidence of selection at *pfmdr*1. The selective sweeps and hitchhiking around this gene has not been reported so far in any African *P. falciparum* population. However, we and others have described "soft selective sweeps" of *pfmdr*1 alleles in Southeast Asian *P. falciparum* [32, 33].

Genetic Lineages of Drug-Resistant Alleles

Multilocus microsatellite haplotype profiles for all 4 genes are provided as supplementary information available online (Figure S2). The 4-loci (−4.8 to 3.9 kb) microsatellite haplotype profiles for *pfcrt* revealed that 27 of 32 isolates with CVMNK had unique haplotypes, whereas all 37 isolates with CVIET belonged to 13 closely related haplotypes (Figure S2A). This indicates that there was greater sharing of haplotypes among isolates with CVIET alleles. Most the CVIET alleles in Ghana had microsatellite haplotypes identical or nearly identical to Southeast Asian type, supporting earlier findings that CQ resistant alleles in Africa are originally derived from Southeast Asia [19]. However, we also observed few unique microsatellite haplotypes specific to Ghana, which could be the result of recombination between Southeast Asian lineage and Ghanaian parasites or possibly the indigenously evolved lineages of CVIET (Figure S2A).

For *pfmdr*1, we used the 8 closest microsatellite loci around the gene (−9.3 to 9.1 kb) to construct multilocus haplotypes (Figure S2B). All ancestral-type NYSND alleles had unique microsatellite haplotypes (Figures 3A and S2B). We found that the 184F mutation (NFSND) emerged on multiple genetic backgrounds. This is consistent with our previous study in Cambodia, where we observed multiple independent lineages of this *pfmdr*1 allele [32]. An increase in linkage disequilibrium was observed among loci around YFSND alleles, with 18 out of 21 isolates having identical or nearly identical microsatellite haplotypes, suggesting 1 major and few minor lineages of YFSND alleles in Ghana (Figures 3A and S2B).

In agreement with previous studies, we also observed that the triple-mutant *dhfr* alleles-CIRNI ($n = 41$) had a single origin in Ghana and were identical to Southeast Asian lineage (Figure S2C) [11, 12, 18]. The predominant double mutant *dhfr* alleles-CNRNI ($n = 11$) in Ghana was found to have originated indigenously, mostly on identical genetic background (Figure S2C). The minor frequency double mutant *dhfr* allele-CICNI ($n = 2$) had unique microsatellite haplotypes different from CIRNI and CNRNI. In contrast to a recent study, we did not find any indigenously evolved triple mutant *dhfr* alleles in our study [12]. Mita et al [12] analyzed 54 parasite isolates from the Winneba region of Ghana and observed 3 different genetic lineages of triple-mutant *dhfr*-CIRNI alleles: 1 Southeast Asian (70%) and 2 indigenous African lineages (27% Ghana-type and 3% Kenyan-type). Other researchers have observed the indigenous evolution of triple-mutant *dhfr* alleles in other African countries [22, 24].

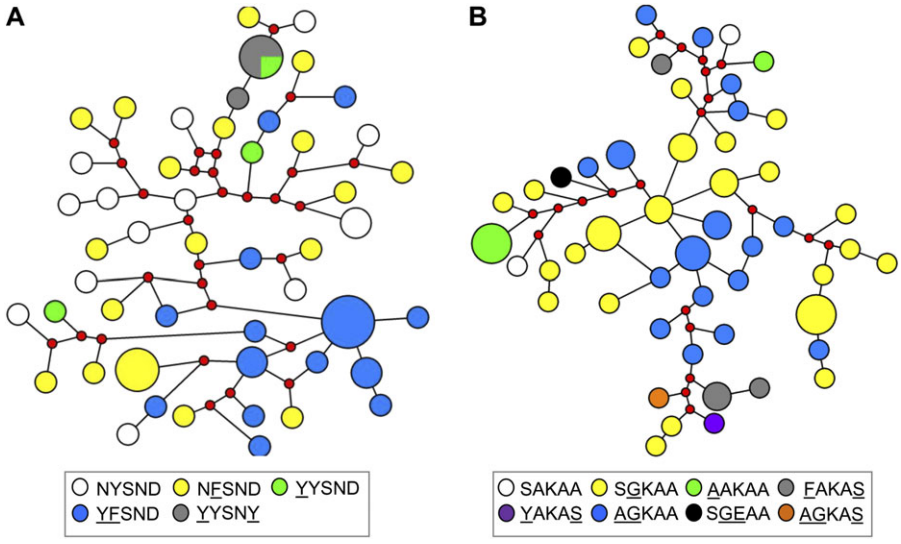

Figure 3. Median-joining network diagrams showing genetic lineages of (A) *pfmdr*1 and (B) *dhps* alleles in Ghana. The multilocus microsatellite haplotype profiles (using −9.3, −4.2, −3.3, 0, .16, .45, 3.6, and 9.1 kb loci flanking *pfmdr*1; and −7.5, −2.9, −1.5, −.13, .03, .5, 1.4, and 6.4 kb flanking *dhps*) were constructed for each gene to generate networks. The 63 *Plasmodium falciparum* isolates analyzed for *pfmdr*1 formed 50 unique 8-loci microsatellite haplotypes, whereas the 66 isolates analyzed for *dhps* formed 57 haplotypes. For allele sizes and other details please refer to Figures S2B and S2D, available online as supplementary material. Each circle in the network represents a unique microsatellite haplotype with the size of the circle being proportional to the number of isolates showing that particular haplotype, and color of the circle indicating the *pfmdr*1 or *dhps* allele. The red dots are hypothetical median vectors generated by the software to connect existing haplotypes within the network with maximum parsimony.

The 8-loci microsatellite haplotypes flanking *dhps* alleles showed that 11 of 19 isolates with double mutant-AGKAA had identical or nearly identical genetic backgrounds, whereas the remaining 8 isolates had different allele sizes at ⩾4 loci (Figure S2D). These findings suggest that the AGKAA alleles in Ghana have 1 major lineage with small numbers of other minor lineages (Figure 3B). Although SGKAA single mutant alleles ($n = 33$) had multiple independent microsatellite haplotypes, some of them were predominant and identical or nearly identical to the microsatellite haplotypes flanking AGKAA, suggesting that AGKAA have evolved on few SGKAA backgrounds (Figure 3B; Figure S2D). Comparison of the *dhps* microsatellite haplotypes of Ghana with Southeast Asian types revealed no similarity, suggesting that the *dhps* mutations in Ghana were not derived from Southeast Asia and likely have originated indigenously [21]. This suggestion is also supported by previous studies wherein evidence of multiple independent origins of resistant *dhps* alleles in Africa has been found [13, 25]. Pearce et al [13] had observed 2 lineages of resistant *dhps* in Ghana, the predominant being the AGKAA/SGKAA-2 with minor frequencies of AGKAA/SGKAA-1 and AGKAA/SGKAA-3–type lineages.

In conclusion, our study provides evidence of a decrease in the prevalence of the *pfcrt* 76T mutation in northern Ghana after CQ was removed from use. In addition to providing evidence of selection at *pfcrt*, *dhfr,* and *dhps*, we also demonstrated that the *pfmdr*1 gene has been under selection in Ghanaian parasites, possibly because of CQ use. There is evidence for 1 major and few minor genetic lineages of *pfmdr*1-YFSND alleles in Ghana. Defining genetic lineages of resistant *pfmdr*1 alleles in Africa and demonstrating selection on this gene is important because of its potential role in AQ and MQ resistance, the partner drugs in ACTs.

Supplementary Data

Supplementary data are available online.

Funding

This work was supported by the Atlanta Research and Education Foundation, VA Medical Center (IAA 09FED911106); CDC Antimicrobial Resistance Working Group (PID 1227); National Malaria Control Programme of Ghana with funds from the Global Fund to Fight AIDS, Tuberculosis and Malaria (GHN-405-G04-M) and International Atomic Energy Agency (IAEA) (RAF 6025); American Society for Microbiology and the Coordinating Center for Infectious Diseases postdoctoral fellowship (to M.T.A.). The funders had no role in the study design, data collection, analysis, decision to publish, or preparation of the manuscript.

Acknowledgments

We thank all the study participants and the field staff who helped in collecting samples. We also thank Ira Ford Goldman for proofreading this manuscript.

References

1. Koram KA, Abuaku B, Duah N, Quashie N. Comparative efficacy of antimalarial drugs including ACTs in the treatment of uncomplicated malaria among children under 5 years in Ghana. Acta Trop **2005**; 95:194–203.
2. Neequaye J. In vivo chloroquine-resistant falciparum in Western Africa. Lancet **1986**; 327:153–4.
3. Quashie NB, Duah NO, Abuaku B, Koram KA. The in vitro susceptibilities of Ghanaian *Plasmodium falciparum* to antimalarial drugs. Ann Trop Med Parasitol **2007**; 101:391–8.
4. Duah NO, Wilson MD, Ghansah A, et al. Mutations in *Plasmodium falciparum* chloroquine resistance transporter multidrug resistance genes, and treatment outcomes in Ghanaian children with uncomplicated malaria. J Trop Pediatr **2007**; 53:27–31.
5. Ehrhardt S, Mockenhaupt FP, Agana-Nsiire P, et al. Efficacy of chloroquine in the treatment of uncomplicated, *Plasmodium falciparum* malaria in northern Ghana. Ann Trop Med Parasitol **2002**; 96:239–47.
6. Afari EA, Akanmori BD, Nakano T, Ofori-Adjei D. *Plasmodium falciparum*: Sensitivity to chloroquine in vivo in three ecological zones in Ghana. Trans R Soc Trop Med Hyg **1992**; 86:231–2.
7. Landgraf B, Kollaritsch H, Wiedermann G, Wernsdorfer WH. *Plasmodium falciparum*: Susceptibility in vitro in vivo to chloroquine and sulfadoxine-pyrimethamine in Ghanaian schoolchildren. Trans R Soc Trop Med Hyg **1994**; 88:440–2.
8. Oduro AR, Anyorigiya T, Hodgson A, et al. A randomized comparative study of chloroquine, amodiaquine, sulphadoxine-pyrimethamine for the treatment of uncomplicated malaria in Ghana. Trop Med Int Health **2005**; 10:279–84.
9. Mockenhaupt FP, Bedu-Addo G, Eggelte TA, et al. Rapid increase in the prevalence of sulfadoxine-pyrimethamine resistance among *Plasmodium falciparum* isolated from pregnant women in Ghana. J Infect Dis **2008**; 198:1545–9.
10. Marks F, Evans J, Meyer CG, et al. High prevalence of markers for sulfadoxine pyrimethamine resistance in *Plasmodium falciparum* in the absence of drug pressure in the Ashanti region of Ghana. Antimicrob Agents Chemother **2005**; 49:1101–5.
11. Maïga O, Djimdé AA, Hubert V, et al. A shared Asian origin of the triple-mutant dhfr allele in *Plasmodium falciparum* from sites across Africa. J Infect Dis **2007**; 196:165–72.
12. Mita T, Tanabe K, Takahashi N, et al. Indigenous evolution of *Plasmodium falciparum* pyrimethamine resistance multiple times in Africa. J Antimicrob Chemother **2009**; 63:252–5.
13. Pearce RJ, Pota H, Evehe MS, et al. Multiple origins regional dispersal of resistant dhps in African *Plasmodium falciparum* malaria. PLoS Med **2009**; 6:e1000055.
14. Fidock DA, Nomura T, Talley AK, et al. Mutations in the *P. falciparum* digestive vacuole transmembrane protein PfCRT and evidence for their role in chloroquine resistance. Mol Cell **2000**; 6:861–71.
15. Duraisingh MT, Cowman AF. Contribution of the pfmdr1 gene to antimalarial drug-resistance. Acta Trop **2005**; 94:181–90.
16. Gregson A, Plowe CV. Mechanisms of resistance of malaria parasites to antifolates. Pharmacol Rev **2005**; 57:117–45.
17. Anderson TJ, Roper C. The origins spread of antimalarial drug resistance: Lessons for policy makers. Acta Trop **2005**; 94:269–80.
18. Roper C, Pearce R, Nair S, Sharp B, Nosten F, Anderson T. Intercontinental spread of pyrimethamine-resistant malaria. Science **2004**; 305:1124.
19. Wootton JC, Feng X, Ferdig MT, et al. Genetic diversity and chloroquine selective sweeps in *Plasmodium falciparum*. Nature **2002**; 418:320–3.
20. Mita T, Tanabe K, Takahashi N, et al. Independent evolution of pyrimethamine resistance in *Plasmodium falciparum* isolates in Melanesia. Antimicrob Agents Chemother **2007**; 51:1071–7.
21. Vinayak S, Alam MT, Mixson-Hayden T, et al. Origin and evolution of sulfadoxine resistant *Plasmodium falciparum*. PLoS Pathog **2010**; 6:e1000830.

22. McCollum AM, Poe AC, Hamel M, et al. Antifolate resistance in *Plasmodium falciparum*: Multiple origins identification of novel dhfr alleles. J Infect Dis **2006**; 194:189–97.
23. Certain LK, Briceño M, Kiara SM, Nzila AM, Watkins WM, Sibley CH. Characteristics of *Plasmodium falciparum* dhfr haplotypes that confer pyrimethamine resistance, Kilifi, Kenya, 1987–2006. J Infect Dis **2008**; 197:1743–51.
24. Ndiaye D, Daily JP, Sarr O, et al. Defining the origin of *Plasmodium falciparum* resistant dhfr isolates in Senegal. Acta Trop **2006**; 99: 106–11.
25. McCollum AM, Basco LK, Tahar R, Udhayakumar V, Escalante AA. Hitchhiking selective sweeps of *Plasmodium falciparum* sulfadoxine and pyrimethamine resistance alleles in a population from central Africa. Antimicrob Agents Chemother **2008**; 52:4089–97.
26. Holmgren G, Gil JP, Ferreira PM, Veiga MI, Obonyo CO, Bjorkman A. Amodiaquine resistant *Plasmodium falciparum* malaria in vivo is associated with selection of pfcrt 76T and pfmdr1 86Y. Infect Genet Evol **2006**; 6:309–14.
27. Humphreys GS, Merinopoulos I, Ahmed J, et al. Amodiaquine and artemether-lumefantrine select distinct alleles of the *Plasmodium falciparum* mdr1 gene in Tanzanian children treated for uncomplicated malaria. Antimicrob Agents Chemother **2007**; 51:991–7.
28. Dokomajilar C, Lankoande ZM, Dorsey G, Zongo I, Ouedraogo JB, Rosenthal PJ. Roles of specific *Plasmodium falciparum* mutations in resistance to amodiaquine and sulfadoxine-pyrimethamine in Burkina Faso. Am J Trop Med Hyg **2006**; 75:162–5.
29. Price RN, Uhlemann AC, Brockman A, et al. Mefloquine resistance in *Plasmodium falciparum* and increased pfmdr1 gene copy number. Lancet **2004**; 364:438–47.
30. Bereczky S, Mårtensson A, Gil JP, Färnert A. Short report: Rapid DNA extraction from archive blood spots on filter paper for genotyping of *Plasmodium falciparum*. Am J Trop Med Hyg **2005**; 72:249–51.
31. Griffing S, Syphard L, Sridaran S, et al. pfmdr1 amplification and fixation of pfcrt chloroquine resistance alleles in *Plasmodium falciparum* in Venezuela. Antimicrob Agents Chemother **2010**; 54:1572–9.
32. Vinayak S, Alam MT, Sem R, et al. Multiple genetic backgrounds of the amplified *Plasmodium falciparum* multidrug resistance (pfmdr1) gene selective sweep of 184F mutation in Cambodia. J Infect Dis **2010**; 201:1551–60.
33. Nair S, Nash D, Sudimack D, et al. Recurrent gene amplification and soft selective sweeps during evolution of multidrug resistance in malaria parasites. Mol Biol Evol **2007**; 24:562–73.
34. Nash D, Nair S, Mayxay M, et al. Selection strength and hitchhiking around two antimalarial resistance genes. Proc Biol Sci **2005**; 272:1153–61.
35. Nair S, Williams JT, Brockman A, et al. A selective sweep driven by pyrimethamine treatment in Southeast Asian malaria parasites. Mol Biol Evol **2003**; 20:1526–1536.
36. Harris EE, Meyer D. The molecular signature of selection underlying human adaptations. Am J Phys Anthropol **2006**; 43:89–130.
37. Schlötterer C. Hitchhiking mapping–functional genomics from the population genetics perspective. Trends Genet **2003**; 19:32–8.
38. Lewontin RC, Krakauer J. Distribution of gene frequency as a test of the theory of the selective neutrality of polymorphisms. Genetics **1973**; 74:175–95.
39. Park SDE. Trypanotolerance in West African cattle and the population genetic effects of selection. [Ph.D. thesis] University of Dublin **2001;** http://animalgenomics.ucd.ie/sdepark/ms-toolkit/.
40. Weir BS, Cockerham CC. Estimating F-statistics for the analysis of population structure. Evolution **1984**; 38:1358–70.
41. Belkhir K, Borsa P, Chikhi L, Raufaste N, Bonhomme F. GENETIX 4.05, logiciel sous Windows pour la génétique des populations. Laboratoire Génome, Populations, Interactions, CNRS UMR 5171. Montpellier, France: Université de Montpellier II Available at: http://www.genetix.univ-montp2.fr/genetix/intro.htm, 2004.
42. Feil EJ, Li BC, Aanensen DM, Hanage WP, Spratt BG. eBURST: Inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. J Bacteriol **2004**; 186:1518–30.
43. Ehrhardt S, Eggelte TA, Kaiser S, et al. Large-scale surveillance of *Plasmodium falciparum* crt(K76T) in northern Ghana. Antimicrob Agents Chemother **2007**; 51:3407–9.
44. Laufer MK, Thesing PC, Eddington ND, et al. Return of chloroquine antimalarial efficacy in Malawi. N Engl J Med **2006**; 355:1959–66.
45. Zhou Z, Griffing SM, de Oliveira AM, et al. Decline in sulfadoxine-pyrimethamine-resistant alleles after change in drug policy in the Amazon region of Peru. Antimicrob Agents Chemother **2008**; 52:739–41.
46. Mockenhaupt FP, Eggelte TA, Till H, Bienzle U. *Plasmodium falciparum* pfcrt and pfmdr1 polymorphisms are associated with the pfdhfr N108 pyrimethamine-resistance mutation in isolates from Ghana. Trop Med Int Health **2001**; 6:749–55.
47. Lim P, Wongsrichanalai C, Chim P, et al. Decreased in vitro susceptibility of *Plasmodium falciparum* isolates to artesunate, mefloquine, chloroquine, and quinine in Cambodia from 2001 to 2007. Antimicrob Agents Chemother **2010**; 54:2135–42.
48. Mockenhaupt FP, Eggelte TA, Böhme T, Thompson WN, Bienzle U. *Plasmodium falciparum* dihydrofolate reductase alleles and pyrimethamine use in pregnant Ghanaian women. Am J Trop Med Hyg **2001**; 65:21–6.
49. Mockenhaupt FP, Bousema JT, Eggelte TA, et al. Concurrence of *Plasmodium falciparum* dhfr and crt mutations in northern Ghana. Malar J **2005**; 4:42.
50. Triglia T, Menting JG, Wilson C, Cowman AF. Mutations in dihydropteroate synthase are responsible for sulfone and sulfonamide resistance in *Plasmodium falciparum*. Proc Natl Acad Sci U S A **1997**; 94:13944–9.