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Drug Susceptibility and Genetic Evaluation of *Plasmodium falciparum* Isolates Obtained in Four Distinct Geographical Regions of Kenya

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The drug resistance profiles of *Plasmodium falciparum* isolated from four regions in Kenya were analyzed for drug resistance profiles. We observed variability in resistance to a broad range of antimalarial drugs across Kenya as determined from in vitro drug susceptibility screening and genotyping analysis.

Drug resistance is the greatest challenge in the fight against malaria in Africa. Resistance to chloroquine, the cheapest and most widely used antimalarial agent, has increased at an alarming rate. In 1998, chloroquine was officially replaced in Kenya as the first-line therapy with a combination of sulfadoxine and pyrimethamine (24), and resistance has subsequently developed to this treatment as well (1, 14, 15, 25, 31).

Resistance to several antimalarial drugs has been well characterized and shown to be associated with specific point mutations in *Pfdhfr*, *Pfdhps*, *Pfmdr1*, and *Pfcrt* genes. Mutations in *Pf*DHFR are routinely found as $^{Ala}16^{Val}$, $^{Cys}50^{Arg,Ile}$, $^{Asn}51^{Ile}$, $^{Cys}59^{Arg}$, $^{Ser}108^{Asn,Thr}$, $^{Val}140^{Leu}$, and $^{Ile}164^{Leu}$ substitutions and have been shown to confer resistance to pyrimethamine and chlorproguanil (3, 4, 27, 28). Resistance to sulfadoxine and dapsone develops through the acquisition of mutations in *Pf*DHPS at positions $^{Ser}436^{Phe,Ala}$, $^{Ala}437^{Gly}$, $^{Lys,Leu}540^{Glu}$, $^{Ala}581^{Gly}$, and $^{Ala}613^{Ser,Thr}$. Several studies have demonstrated that the differing degrees of antimalarial drug resistance are dependent upon the number and combination of mutations present (10, 12, 32).

The molecular mechanism of chloroquine resistance has been largely disputed. Originally the *Pfmdr1* gene was implicated in chloroquine resistance (2, 8, 23, 37). Five different mutations have been identified within *PfMDR1* that are associated with drug resistance: $^{Asp}86^{Tyr}$, $^{Phe}184^{Tyr}$, $^{Ser}1034^{Cys}$, $^{Asn}1042^{Asp}$, and $^{Asp}1246^{Tyr}$ (22, 38). In addition to chloroquine, evidence suggests that these mutations may also mediate drug resistance to quinine, halofantrine, and artemisinin derivatives (5, 22). Further analysis of a genetic association with chloroquine resistance identified the *Pfcrt* gene as having a central role in chloroquine resistance (6, 36). Despite discrepancies in the field about the gene responsible for chloroquine resistance, recent studies have verified that mutations in *Pfcrt* significantly correlate with chloroquine resistance (26). Specifically, mutations at codon 76 of *Pfcrt* most accurately and consistently correlate with chloroquine resistance (6, 21).

In this study, *Plasmodium falciparum* isolates collected from Kisumu, Kericho, Magadi, and Entosopia in Kenya between 1999 and 2000 were evaluated for genetic mutations and in vitro drug susceptibility as previously described (12, 16, 17, 35). Kisumu is a holoendemic area located in western Kenya near Lake Victoria. Kericho is located in the Rift Valley Highlands of western Kenya, 80 km from the holoendemic area of the Lake Victoria basin. Magadi lies in the southern part of the Rift Valley near Lake Magadi, while Entosopia is at the border of Kenya and Tanzania. Patients with a positive thick smear for *P. falciparum* at various clinics, who agreed to give informed consent, were eligible for the study. However, these individuals were excluded if antimalarial drugs were taken within 24 h. (This study was conducted under the approved Walter Reed Army Institute of Research and Kenya Medical Research Institute protocol no. 484; Epidemiology of malaria in Kenyan adults.)

Genotype analysis of *Pfdhfr* revealed a relatively consistent prevalence of mutations among the four areas of isolation. Most notable among the four areas is the predominance of the "triple mutant" genotype: mutations at codons 51, 59, and 108, which are generally associated with a high level of pyrimethamine resistance (16, 17, 19, 20) (Table 1). Consistent with other genotype studies of *Pfdhfr* from Kenyan isolates, we did not observe any mutations at codon 164 (16, 34) (Table 1). The only significant difference observed was the lower proportion of mutations at *Pfdhfr* codon 108 in Magadi isolates ($P = 0.007$). Serine 108 is often mutated to either asparagine or threonine. The asparagine substitution is associated with pyrimethamine resistance, while the threonine substitution correlates with cycloguanil resistance (7, 20). In this study, we limited our analysis to the serine-to-asparagine *Pf*DHFR mutation and found that the majority of parasites from Kenya contain this particular mutation. In concordance with the predominant triple-mutant genotype, we observed that all parasites in this study were resistant to pyrimethamine (Table 2).

Collectively, analysis of *Pfdhps* revealed a substantial pro-

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TABLE 1. Percentages of mutations at specific codons within malaria drug resistance genes

Location (n^a)	% of isolates with mutations at codon for gene group[b]:														
	DHFR				DHPS					MDR					CRT
	51	59	108	164	436	437	540	581	613	86	184	1034	1042	1246	76
Entosopia (31)	94	100	100	0	3	94	81	0	0	90	100	100	100	100	100
Magadi (22)	91	100	77	0	0	96	73	0	0	86	100	100	100	100	100
Kericho (38)	92	100	97	0	0	74	66	0	0	68	100	95	100	95	92
Kisumu (34)	97	100	91	0	27	41	65	0	0	35	100	100	97	35	82

[a] *n*, number of samples tested.
[b] DHFR, dihydrofolate reductase; DHPS, dihydropteroate synthase; MDR, multidrug resistance gene; CRT, chloroquine resistance transporter.

portion of mutations at codons 437 and 540, whereas no mutations were observed at codons 581 or 613 in isolates from these four areas (Table 1). Kisumu isolates had a higher prevalence of mutations at codon 436 ($P < 0.01$), and a significantly lower occurrence of mutations at codon 437 ($p <= 0.005$), than isolates from any other area (Table 1). While resistance to sulfadoxine was not observed among any of the isolates, there was a marked difference in the degree of sensitivity to sulfadoxine by region (Table 2). We observed at least a twofold difference in sulfadoxine 50% inhibitory concentrations (IC_{50}s) for Kisumu isolates compared to those for isolates from Entosopia or Kericho ($P = 0.001$; $P = 0.000$). It is interesting to speculate that the predominance of mutations at codon 436 may confer resistance to sulfadoxine, as was partially evidenced by the twofold increase in sulfadoxine IC_{50}s. However, more extensive surveillance studies would need to be performed to establish this correlation.

Mutations in *Pfmdr1* and *Pfcrt* have been implicated in conferring chloroquine resistance. In this regard, we observed extensive mutations (68 to 100%) at all codons evaluated, except codons 86 and 1246 of Kisumu isolates (Table 1). Mutations at these two codons are significantly less prevalent in Kisumu parasites than in those from the other three areas analyzed ($P <= 0.002$; $P = 0.000$). Studies place higher importance on mutations in the 86 and 1246 codons for conferring drug resistance than on mutations in the other three codons of *Pfmdr1*. Similar to *Pfmdr1*, we found a high frequency of mutations in *Pfcrt* from all isolates except those from Kisumu. Mutations at codon 76 were less prevalent in Kisumu isolates than in isolates from the other areas ($P = 0.021$), but this difference only reached statistical significance versus Entasopia isolates ($P = 0.014$) (Table 1). These genotype results suggest that Kisumu parasites are more sensitive to chloroquine than other parasites in this study.

With testing in the in vitro drug screen, we found that drug sensitivity to chloroquine falls into two groups that correspond with genetic mutations in *Pfmdr1* and *Pfcrt*. Isolates from Entosopia and Magadi, which have the greatest number of mutations in *Pfmdr1* and *Pfcrt*, had mean IC_{50}s of 30 and 45 ng/ml, respectively. Alternatively, isolates from Kerciho and Kisumu, which have lower prevalences of mutations, had mean IC_{50}s of 15 and 12 ng/mg, respectively. Based on the cutoff IC_{50} value of 45 ng/ml for chloroquine resistance, we observed significant chloroquine resistance in Entosopia and moderate resistance in Magadi. Studies conducted in western Kenya suggests that chloroquine resistance developed between 1982 and 1991 (18, 30). During these years, chloroquine was the recommended drug of choice for treatment of malaria. In 1998, extensive chloroquine resistance led to the recommendation of sulfadoxine-pyrimethamine (Fansidar) as the first-line drug of choice. Samples evaluated in this study were collected from 1999 to 2000, at a time in which chloroquine use in Kenya should have declined. Recent studies conducted in Malawi demonstrated that chloroquine sensitivity reemerged after the cessation of chloroquine use (11). Although the current national health policy discourages the use of chloroquine as the first-line treatment of malaria, many individuals continue to use chloroquine either in high doses or combined with other antimalarial drugs (9, 29, 33) (personal observations). This noncompliance with chloroquine treatment policy may contribute to the heterogeneity in chloroquine sensitivity across Kenya.

In an effort to obtain a more comprehensive understanding of the drug susceptibility profiles of *P. falciparum* from Kenya, we evaluated the malaria isolates against a panel of 11 addi-

TABLE 2. Susceptibility of *P. falciparum* isolated from Kenya to four common antimalarial drugs

Location (n^a)	IC_{50} (ng/ml)[b]			
	Chloroquine	Mefloquine	Sulphadoxine	Pyrimethamine
	Median (range)	Median (range)	Median (range)	Median (range)
Entosopia (7)	45 (33–75)	13 (3–27)	4,362 (1490–7412)	200 (99–375)
Magadi (7)	30 (11–71)	7.4 (0.8–15)	6,430 (5545–6966)	333 (41–742)
Kericho (5)	15 (2–31)	0.8 (0.6–1.0)	1,070 (705–1650)	295 (120–473)
Kisumu (4)	12 (1.0–33)	8.0 (5–9)	8,429 (7845–9438)	203 (109–240)

[a] *n*, number of samples tested.
[b] The following IC_{50}s are considered discriminative for resistance; chloroquine, >45.5 ng/ml; quinine, >275 ng/ml; mefloquine, >10 ng/ml; halofantrine, >2ng/ml; pyrimethamine, >15 ng/ml; cycloguanil, >15 ng/ml; sulfadoxine, >10,000 ng/ml; dapsone, >300 ng/ml (18, 24, 43, 47).

TABLE 3. Drug susceptibility testing of Kenyan *P. falciparum* against a panel of antimalarial compounds

Drug	IC_{50} (ng/ml) for isolates from[a]:			
	Entosopia (7)	Magadi (7)	Kericho (5)	Kisumu (4)
	Median (range)	Median (range)	Median (range)	Median (range)
Quinine	48 (27–79)	44 (11–96)	14 (10–17)	56 (20–122)
Artemisinin	1.0 (0.6–1.3)	2 (0.6–2)	1 (0.8–1)	2 (1–2)
Halofantrine	7 (.8–14)	7 (2–10)	0.2 (0.1–0.3)	8 (1–20)
Doxycycline	3,591 (1380–8527)	5,303 (3120–7686)	2,718 (1746–3670)	6,244 (5781–6333)
Atovoquone	2 (0.8–14)	2 (0.8–3)	NT[b]	0.8 (0.3–1)
Tafenoquine	848 (478–1528)	913 (813–1043)	NT	1,046 (812–1417)
Amodiaquine	10 (7–13)	13 (8–14)	3 (2–5)	10 (4–13)
Primaquine	656 (349–1255)	504 (270–1276)	416 (32–602)	464 (289–566)
Dapsone	28 (4–74)	1,926 (60–3522)	NT	2,804 (2345–3518)
Proguanil	54 (37–86)	905 (361–2475)	2275 (2172–2523)	556 (412–715)
Chlorcycloguanil	3 (2–5)	8 (1–17)	NT	5 (2–12)

[a] Number of samples tested is given in parentheses after name of region.
[b] NT, not tested.

tional antimalarial compounds. The mechanisms of action and drug targets of many of these compounds are unknown; however, in vitro drug susceptibility data will help characterize the larger picture of drug resistance in Kenya. While the IC_{50}s of many of the tested compounds were similar among the isolates from Entosopia, Magadi, Kericho, and Kisumu (Table 3), we did identify a few differences with regard to drug sensitivity. For example, we observed that the mean IC_{50}s for dapsone and proquanil (28 and 54 ng/ml) from Entosopia isolates were significantly lower than IC_{50}s of parasites from the other areas in this study ($P <= 0.015$ for dapsone and $P <= 0.016$, except for Kisumu, where the difference in proguanil IC_{50}s did not reach statistical significance). Although sensitivity values to dapsone were not uniform among the isolates in the study, there appears to be extensive resistance (Table 3). Dapsone resistance has been previously reported in Kenya and appears to be correlated with *Pfdhps* mutations and a high level of sulfadoxine resistance (15). The observation of multiple *Pfdhps* mutations and a low sensitivity to sulfadoxine supports the finding that there was a high degree of dapsone resistance in our study. The only exception to this trend is with Entosopia isolates, which were highly sensitive to dapsone in combination with multiple *Pfdhps* mutations. Recent studies demonstrate that the "quintuple mutant" (triple mutation in *Pf*DFHR [$^{Asn}51^{Ile}$, $^{Cys}59^{Arg}$, and $^{Ser}108^{Asn}$] and $^{Ala}437^{Gly}$ and $^{Lys,Leu}540^{Glu}$ mutations in *Pf*DHPS) is more strongly associated with sulfadoxine-pyrimethamine treatment failure than chlorproguanil-dapsone failure (12). Consistent with this finding, we observed that sensitivity to chlorcycloguanil, the active metabolite of chlorproguanil, is similar among the isolates from the four regions of Kenya. The predominance of the quintuple mutant, along with our in vitro drug screening results, suggests extensive sulfadoxine-pyrimethamine resistance in Kenya and thus favorable conditions for chlorproguanil-dapsone use (13). This observation is noteworthy, since the drug combination chlorproguanil-dapsone (Lapdap) is being introduced as an additional treatment option in Kenya (31).

The goal of this study was to establish a baseline for both genetic polymorphisms and drug sensitivity profiles of malaria parasites isolated from Kenya. This data will serve as a baseline for future studies to monitor the changes in antimalarial drug resistance throughout Kenya.

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