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SHORT REPORT: DYNAMICS OF *PLASMODIUM FALCIPARUM* MALARIA AFTER SUB-OPTIMAL THERAPY IN UGANDA

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Abstract. We followed parasite genotypes of 75 patients for 42 days after treatment of uncomplicated malaria with chloroquine + sulfadoxine-pyrimethamine in Kampala, Uganda. Infections were complex (mean, 2.88 strains) and followed three patterns: 27% of patients eliminated all strains and remained parasite-free, 48% had a long aparasitemic interval followed by reappearance of original strains after 3–33 days (mean, 9.2 days), and 25% failed to clear original strains and required therapy after 3–35 days (mean, 17 days). These results highlight the complexity of malaria in Africa and have implications for efficacy trials, because missing late reappearances of strains could lead to misclassification of outcomes.

INTRODUCTION

In sub-Saharan Africa, malaria is highly endemic, infections are often polyclonal, and antimalarial regimens are limited by drug resistance. Therefore, treatment is commonly followed by either recrudescent or new infections. In this setting, it is of interest to rigorously evaluate the dynamics of malaria infections after therapy. A number of studies have analyzed parasite dynamics during asymptomatic plasmodial infections.[1,2] Some reports noted a 48-hour periodicity in the appearance of strains, suggesting synchronicity of polyclonal *Plasmodium falciparum* infections.[1–4] The complexity of infection generally remained stable over time, but specific genotypes varied.[2,3,5] Increasing complexity of asymptomatic infection, increasing parasitemia, and the appearance of novel strains were associated with an increasing risk of developing clinical symptoms.[6,7]

Few studies have analyzed the dynamics of infection after treatment of malaria. In patients with uncomplicated malaria evaluated in France, parasite genotypes varied within hours of treatment with quinine.[8] In contrast, in patients with uncomplicated malaria evaluated in Sweden, genotypes at 12-hour intervals during therapy remained remarkably constant.[9] These studies were comprised of patients returning to nonendemic countries; thus, the impact of new infections on complexity of infection could not be examined. We were interested in the dynamics of malaria in Africans living in an area of high transmission and treated with a common, albeit sub-optimal regimen. We therefore evaluated 75 patients diagnosed with uncomplicated malaria in Uganda and treated with chloroquine + sulfadoxine/pyrimethamine (CQ/SP), the standard treatment for uncomplicated malaria in Uganda.

MATERIALS AND METHODS

Study site and population. Patients presenting to the outpatient department of Mulago Hospital in Kampala, Uganda, between December 2003 and August 2004 were evaluated. Patients with a positive screening thick blood smear were enrolled if they met the following inclusion criteria: 1) age of at least 6 months; 2) history of fever in the last 24 hours or tympanic temperature of at least 38.0°C; 3) *P. falciparum* mixed or mono-infection with a parasite density of no more than 400,000/μL; 4) no history of serious side effects to study medications; 5) no evidence of a concomitant febrile illness; 6) provision of informed consent; 7) ability to participate in 42-day follow-up; 8) absence of pregnancy based on history of last menstrual period; and 9) no danger signs (prostration, inability to drink, recent convulsion, persistent vomiting) or evidence of severe malaria. Patients received directly observed therapy with CQ/SP (Cosmoquine, Cosmos, sequential 10, 10, and 5 mg/kg daily doses; Fansidar, Roche, 25 mg/kg sulfadoxine and 1.25 mg/kg pyrimethamine as a single dose) and were followed for 42 days. Treatment outcomes were classified according to 2003 World Health Organization guidelines as early treatment failure (ETF)—danger signs, complicated malaria, or failure to adequately respond days 0–3—late clinical failure (LCF)—danger signs, complicated malaria, or fever and parasitemia on days 4–42 without meeting criteria for ETF—late parasitological failure (LPF)—asymptomatic parasitemia on day 42 without meeting criteria for ETF or LCF—and adequate clinical and parasitological response (ACPR)—absence of parasitemia on day 42 without meeting criteria for ETF, LCF, or LPF.[10] Patients classified as ETF, LTF, or LPF were treated with quinine 10 mg/kg three times a day for 7 days. The Institutional Review Boards of the University of California, San Francisco and Makerere University approved this study. Blood was collected on filter paper (Whatman no. 3) daily for the first week and thrice weekly for 5 subsequent weeks. After the first week a medical officer who recorded the patient's temperature and obtained a fingerprick blood sample performed home visits at 2- to 3-day intervals during the week. Also, patients were asked to return to clinic once a week, where interim history, a physical exam, and fingerprick blood samples were collected.

Molecular genotyping of parasite DNA. Parasite DNA was extracted with chelex.[11] The block 3 region of merozoite surface protein-2 (*msp-2*) was amplified by nested polymerase chain reaction (PCR) with primers corresponding to conserved sequences flanking this region[5] followed by primers to amplify the *IC3D7* and *FC27* allelic families.[12] 3D7 and HB3 genomic DNA was used for positive controls. PCR products were analyzed by electrophoresis using 2.0% Tris-Borate EDTA agarose gels. All samples from a single patient were

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run on the same gel. If there was no amplification for any allelic family on any day with a corresponding positive blood smear, PCR was repeated with 2.5 times the quantity of DNA for all samples from that patient. If no amplification was detected after this second attempt, genotyping was classified as unsuccessful. Of the patients included in this analysis, one patient had a single sampling day with a positive microscopy result but a negative PCR result. Gel images were digitized, and molecular weights were assigned to bands using GelCompar II software (Applied Maths, Austin, TX). Each band assigned a molecular weight was considered an individual strain. When comparing days, strains were considered the same if molecular weights were within 15 bp. Treatment outcomes were adjusted by comparing genotypes on the day of failure with those at presentation (day 0 or day 1). Recrudescences were defined as LCFs and LPFs when all strains detected on the day of failure were also seen at presentation, new infections as LCFs and LPFs when no strains present on the day of failure were seen on presentation, and mixed outcomes as LCFs and LPFs when the day of failure sample contained both original and new strains. For sequencing, PCR products were treated with ExoSAP-IT (US Biochemicals, Cleveland, OH) and sequenced by standard methods. ClustalW analysis was performed using Lasergene software (DNAStar, Madison, WI). For analysis of polymorphisms, PCR amplification of portions of the *P. falciparum* dihydrofolate reductase (*dhfr*) and dihydropteroate synthetase (*dhps*) genes and identification of genotypes by restriction endonuclease digestion were performed as previously described.[13,14]

Statistical analysis. Statistical tests were conducted using Stata version 8.0 (Stata Corp., College Station, TX). Comparisons of patient age and pre-treatment complexity of infection across the three genotyping patterns were made using a non-parametric test of trend (extension of Wilcoxon rank-sum test). Comparisons of pre-treatment geometric mean parasite density across the genotyping patterns were made using analysis of variance (ANOVA).

RESULTS AND DISCUSSION

Among the 75 patients who completed the study, the mean age was 7.8 years (range, 9 months to 37 years), and 45% of patients were women. On day 0, the geometric mean parasite density was 46,410 parasites/μL (range, 440–281,600 parasites/μL), and the mean complexity of infection was 2.88 (range, 1–10). The anti-malarial efficacy of CQ/SP was quite poor. Early treatment failures were uncommon (8%), but only 33% of patients had an adequate clinical and parasitological response after 42 days of follow-up. Forty-nine percent of patients were late clinical failures, and an additional 9% were late parasitological failures. Clinical failures occurred within 14 days of the onset of therapy in 30%, within 28 days in 87%, and by day 35 in the remaining subjects. Genotyping showed that, among late clinical and parasitological failures, 55% were caused by mixed or new infections and 45% by recrudescence.

Frequent sampling provided numerous data points by which to follow parasite dynamics over 42 days after treatment. Gel patterns generated from *msp-2* genotyping varied among patients. We identified three distinct patterns of parasite clearance. In pattern 1 (20 patients, 27%), parasites present on day 0 or day 1 cleared after therapy and did not recur through the course of follow-up (Figure 1A). All of these patients cleared original infecting strains within 3 days after treatment. New strains appeared in 65% of these patients but were usually cleared (77%).

In pattern 2 (36 patients, 48%), genotyping revealed a long aparasitemic interval (defined as at least 3 days between positive PCR results; mean, 9.2 days; range: 3–35 days) followed by reappearance of original infecting strains (Figure 1, B and C). Among these patients, 22 also had new strains appear during follow-up. In all cases, during intervals deemed aparasitemic based on PCR, parasite smears were also negative. No periodicity in the appearance of strains was observed. To confirm that strains that appeared after a long interval were recrudescent, as suggested by *msp-2* banding patterns, we sequenced *msp-2* alleles in nine patients with monoclonal infections. All studied patients had the same (> 98% identity) sequences, indicating that parasites commonly circulated at levels below microscopy and PCR limits of detection for many days before recrudescences. Previous studies of asymptomatic infection have shown that introduction of new strains is associated with increased risk of developing clinical symptoms.[6,7] We examined the timing of appearance of new strains in relation to time of clinical failure and found that, for the 24 patients requiring rescue therapy in this pattern, 5 (21%) had a new strain appear one sampling frame before or on the day of clinical failure.

In pattern 3 (19 patients, 25%), original strains were not cleared after treatment (parasites absent at only a single time-point, flanked by microscopy-positive time-points, were not considered to have been cleared); the majority of patients ($N = 16$) required rescue therapy 3–35 days after CQ/SP treatment (Figure 1D). Parasite density increased 1–2 days before presentation, with recurrent fever in 11 patients (58%). Complexity of infection increased before presentation with fever in only five patients (26%). Of the 16 patients who required rescue therapy in this pattern, 4 (25%) had new strains appear one sampling frame before or on the day of clinical failure. Therefore, the majority of failures could be attributed to original infecting strains.

Considering all patterns over the course of follow-up, most identified strains were those observed at the onset of the study (Table 1). For 44 (59%) patients, baseline strains persisted through follow-up. For 21 of these patients, only baseline strains were detected. A total of 42 (56%) patients had new strains appear during follow-up. Among these individuals, new strains persisted in the absence of original strains in 5 patients (12%), original and new strains persisted in 14 (33%), original strains persisted after clearance of new strains in 9 (21%), and all strains were cleared in 14 (33%). In contrast to results from studies of asymptomatic infection,[6,7] onset of recurrent malaria was mostly preceded by the presence of original infecting strains, rather than new strains.

A comparison of baseline data between the three genotyping patterns revealed some significant differences between the patient populations. As clearance of parasite strains diminished, there was a significant decrease in median age (pattern 1, 10 years; pattern 2, 7 years, pattern 3, 4 years; $P = 0.04$), a significant increase in pre-treatment geometric mean parasite density (pattern 1, 8,840/μL; pattern 2, 27,000/μL; pattern 3, 34,560/μL; $P < 0.001$), and a trend toward a higher mean complexity of infection (pattern 1, 2.3; patterns 2 and 3, 3.1; $P = 0.18$). Thus, age and parasite burden were significant

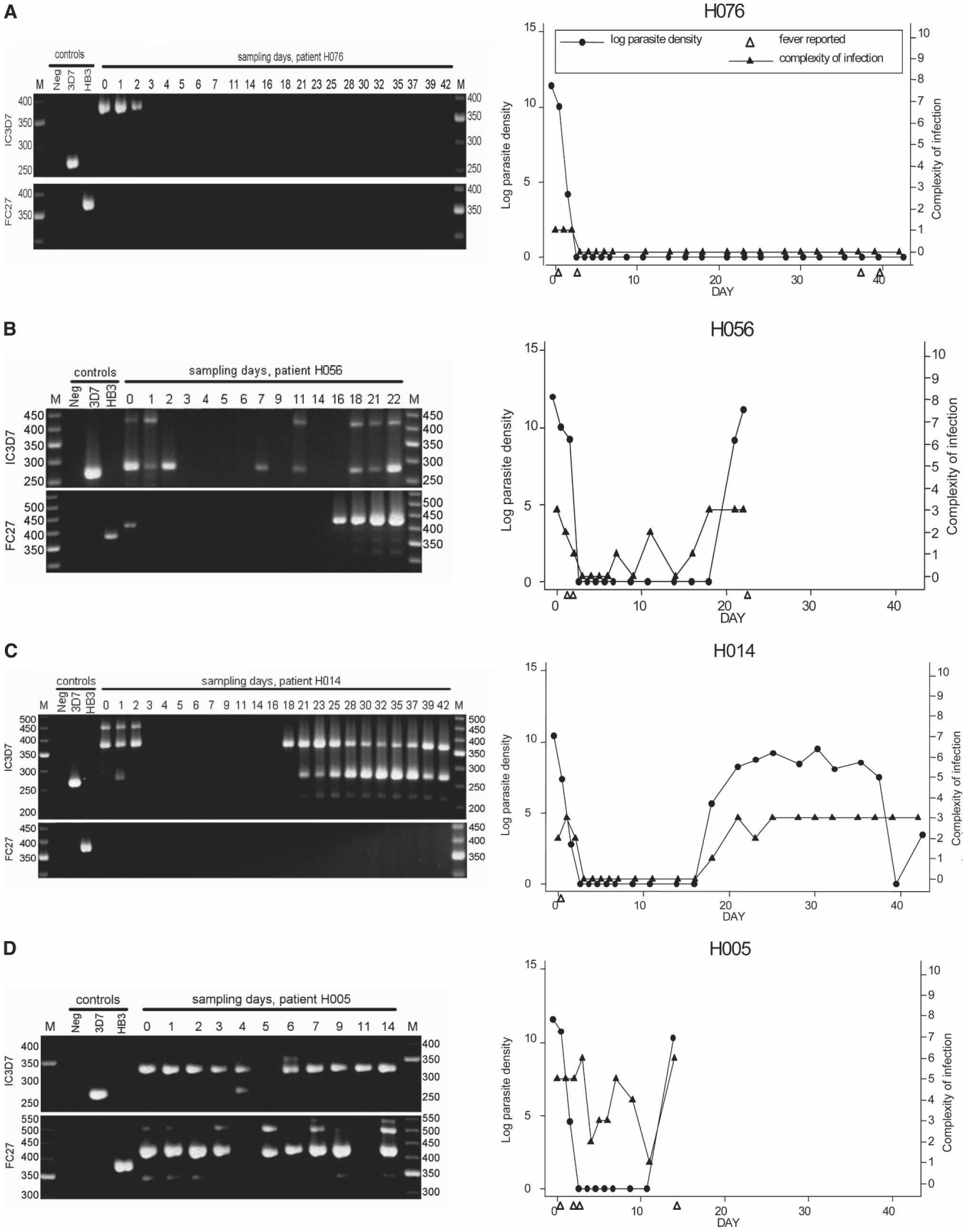

FIGURE 1. Genotyping patterns after chloroquine/sulfadoxine-pyrimethamine therapy. At the indicated time-points, the *IC3D7* and *FC27* alleles of *msp-2* were amplified, and products were resolved by electrophoresis. Representative results are shown for (**A**) a patient who cleared all strains (pattern 1), (**B** and **C**) two patients who experienced a long aparasitemic interval followed by recrudescence of original strains (pattern 2), and (**D**) a patient who did not clear infection, leading to treatment failure (pattern 3). Molecular weight markers (M), a negative control (Neg), and positive controls for the two alleles (3D7 for *IC3D7* and HB3 for *FC27*) are indicated on each gel. Accompanying graphs show the dynamics of infection in the same patients.

TABLE 1
Appearance of original and new strains through follow-up

Level of strain clearance	*N* (%)
All strains cleared	14 (18.7)
All strains persist	14 (18.7)
Original strains cleared, no new strains appear	12 (16.0)
Original strains cleared, new strains persist	5 (6.7)
Original strains persist, no new strains appear	21 (28.0)
Original strains persist, new strains cleared	9 (12.0)

factors in determining how well a patient cleared parasites after treatment.

These results indicate that exposure of parasites to drug pressure, with potential selection for resistance-mediating mutations, is common after treatment with CQ/SP. To assess the selection of resistant parasites, we evaluated key markers of resistance to SP (*dhfr* 59, *dhps* 540, and *dhps* 437) in 21 patients who had long aparasitemic intervals followed by reappearance of original isolates. Of evaluated patients, four had changes in molecular markers during the aparasitemic interval. Two patients showed shifts from mixed to mutant genotypes of *dhfr* 59, one from wild-type to mutant for *dhps* 437 and *dhps* 540 and mutant to mixed for *dhfr* 59, and one from mixed to mutant for *dhps* 437 and *dhps* 540. Although numbers for this analysis were small, the results suggest selection for resistance by CQ/SP.

In summary, after therapy with a standard, albeit suboptimal, regimen for uncomplicated malaria, the dynamics of infection and interplay between original and new strains were complex. Perhaps of greatest interest, a surprisingly large number of patients (48%) had long breaks without parasitemia detectable by microscopy or PCR followed by the reappearance of original infecting strains. These patients had lower mean age and higher pre-treatment parasite density than patients who were able to clear all original parasites. This observation indicates that, in malaria-immune individuals, parasites can persist in low numbers for extended periods of time, followed by multiplication to levels that cause recurrent illness. This result has important implications for drug efficacy studies. Follow-up that is not sufficiently long or that does not assess multiple time-points might miss late recrudescent infections, leading to misclassification of outcomes. It is likely that the use of CQ/SP, a drug that has a long half-life and intermediate antimalarial activity in Uganda,[15] contributed to the high frequency of aparasitemic intervals after therapy. It will be of interest to study parasite dynamics after treatment with regimens with different pharmacokinetic and drug resistance profiles.

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