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SHORT REPORT: LIMITED ADVANTAGE OF MULTIPLE CONSECUTIVE SAMPLES FOR GENOTYPING *PLASMODIUM FALCIPARUM* POPULATIONS DURING THE FIRST DAYS OF TREATMENT

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Abstract. The informative value of genotyping *Plasmodium falciparum* populations in single blood samples was studied before and during treatment in 13 patients with *P. falciparum* malaria. Genotyping of the two merozoite surface proteins (*msp1* [block 2] and *msp2*) and the glutamate-rich protein showed multiple genotypes in seven patients, and single genotypes in the remaining six patients. The same genotype profiles were detected in consecutive samples obtained every 12 hours during treatment from the respective patients, although some genotypes were cleared earlier than others. These patterns are in contrast to the extensive daily dynamics previously described in asymptomatic infections. The genotypes detected in one pre-treatment sample thus appear to reflect the parasite subpopulations of the clinical malaria infection during the following days, and additional sampling does not provide any additional information.

Genotyping of *Plasmodium falciparum* infections by polymerase chain reaction (PCR)–based analysis of the highly polymorphic antigen genes, e.g. the merozoite surface proteins (*msp1* [block 2] and *msp2*) is a widely used methodology in molecular epidemiology studies of malaria. In clinical drug trials, the method is recommended to compare the genotypes in a pre-treatment sample to the profiles of recurrent parasites after treatment to distinguish between recrudescence and reinfections.[1–3] However, in view of the dynamics in asymptomatic individuals,[4–6] several consecutive samples may be required for an adequate determination of the parasite population within an individual at a certain time.

To assess the value of single samples for genotyping parasite populations during treatment, we analyzed *P. falciparum* infections on consecutive days in patients treated for malaria in Sweden, knowing that also travelers often are infected with multiple genotypes.[7,8] Venous blood samples were collected, after informed consent was obtained, in citrate-containing Vacutainer® tubes (Becton Dickinson, Franklin Lakes, NJ) before and every 12 hours for at least three days during treatment in 13 adult patients with microscopically confirmed *P. falciparum* infections. Eleven of the patients had been infected in different countries in Africa, one in Thailand, and one in Surinam. The study was conducted at the Departments of Infectious Diseases at Danderyd and Huddinge Hospital in Stockholm, Sweden, and was reviewed and approved by the Ethical Committee at the Karolinska Institutet (KI d NR 94: 230).

Genotyping of *P. falciparum* infections was performed on DNA purified from frozen whole blood by extraction with phenol-chloroform.[9] The amount of DNA analyzed in each reaction corresponded to 5 μL of whole blood. The PCR method included amplification of the three genetic regions *msp1* [block 2], *msp2*, and glutamate-rich protein [*glurp*][10] with allele family-specific oligonucleotide primers in a nested PCR, and a semi-nested reaction of the RII repeat region of *glurp*. The PCR products were analyzed by electrophoresis on MetaPhor™ agarose (FMC Bioproducts, Rockland, ME) gels and visualization by ultraviolet transillumination after staining with ethidium bromide.

Asexual parasitemias were detected by light microscopy (1,000 × magnification) of Giemsa-stained thin blood films for 2–5 days in 12 patients and for 8 days in 1 patient due to treatment failure. In six patients, there was an initial increase in parasite densities during the first 24 hours, otherwise densities (pre-treatment range = 0.1–23% infected erythrocytes, median = 1.2%) decreased steadily in all patients during the course of antimalarial therapy. Gametocytes were detected in four patients and appeared after two or three days of treatment. The PCR detected *P. falciparum* in all samples for all patients during the consecutive sampling period during admission, i.e., 3–9 days (median = 6 days). In four patients, the results of the PCR were positive for at least three days longer than microscopy. The total duration of PCR positivity after treatment could not be derived since repeated samples were not obtained after the patients had left the hospital.

Single genotype infections, i.e., one allele of each gene, were detected in six patients with the same profiles in all consecutive samples. Multiple genotypes, i.e., 2–5 alleles of *msp*1, *msp2*, and/or *glurp* were detected in seven patients. Three patients had the same profiles in all samples. In one patient with two alleles, one of the alleles was detected sporadically in three of nine samples, first on the second day and then in the two last samples of the study period. Three patients had some genotypes that disappeared after 2–3 days while the other could be detected 1 or 2 more days, suggesting a lower proportion of some genotypes (Figure 1). The profiles did not show any 48-hour periodicity in any patients. In the four patients with parasitemias more than 3% (i.e., 150,000 parasites/μL of blood), the PCR with first-day samples showed smears with unspecific bands interpreted as artifacts due to very high parasite loads. The PCR profiles were not different for the respective regimens of chemoprophylaxis or treatment (i.e., chloroquine, proguanil, mefloquine, quinine, and chloroquine, respectively). Thus, 12 of 13 patients had either identical genotypes throughout the pre-treatment and post-treatment periods or had only a subset of the original genotypes in the post-treatment period . Only one patient had a new genotype seen sporadically during the post-treatment period.

The genotyping profiles in these symptomatic patients during treatment were thus highly homogenous. This is in con-

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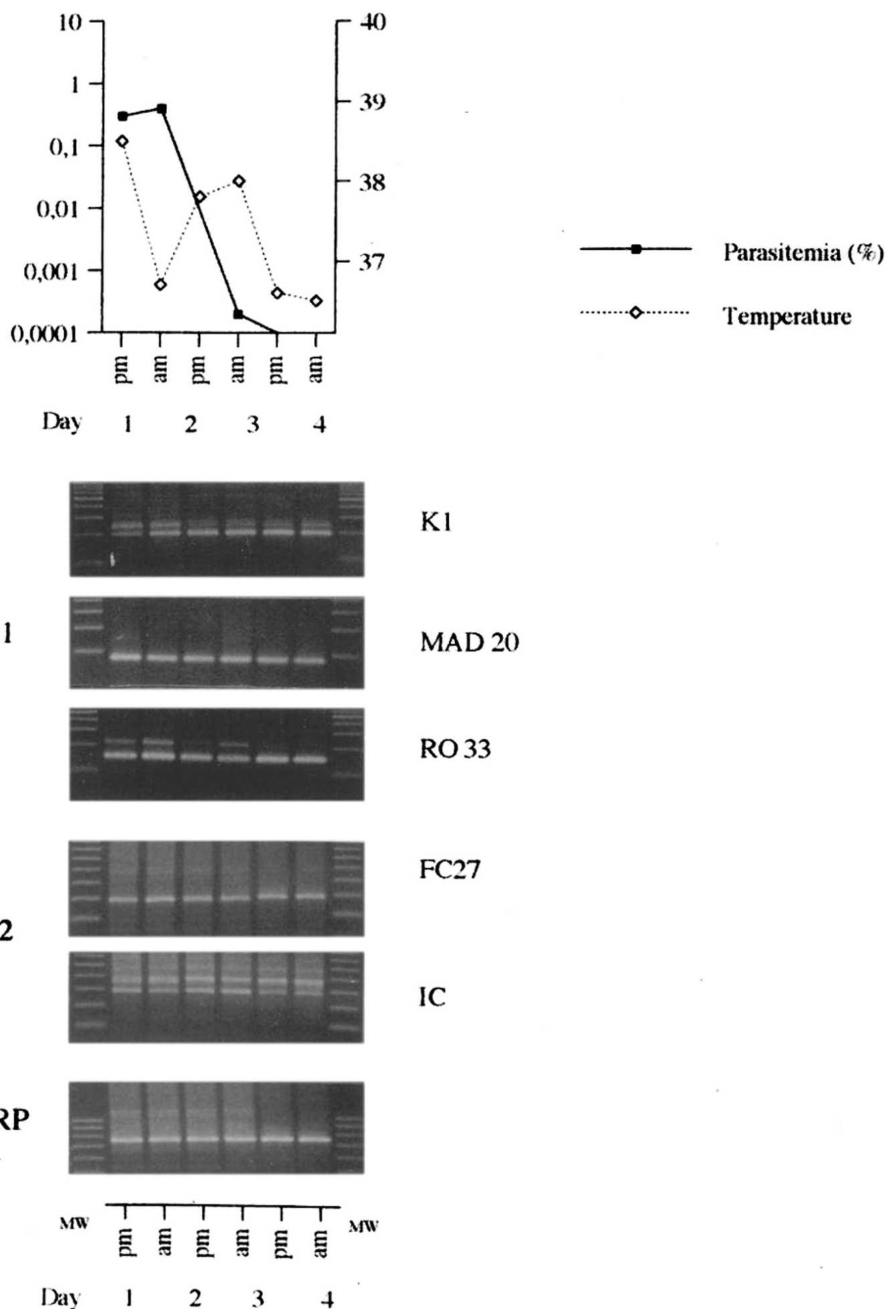

FIGURE 1. Genotyping profiles and parasite densities of *Plasmodium falciparum* in consecutive blood samples obtained from one patient during treatment. MSP = merozoite surface protein; GLURP = glutamate-rich protein. Lanes MW = molecular mass markers. Temperature is in °C.

trast to the extensive daily dynamics observed in asymptomatic individuals, where different genotyping profiles are detected on consecutive days with some genotypes appearing every 48 hours, suggesting inherent synchronous subpopulations.[5] The results are also in contrast to a recent study in travelers, in which different genotypes were found at different times using a new quantitative method.[11] Differences between these two studies may be explained both by differences in methodologies, as well as by the background of the patients. However, the present non-quantitative method for genotyping *P. falciparum* infections, which is commonly used in clinical and epidemiologic studies, did not show any varying patterns, but did show more homogenous profiles.

Detection of the same genotype profiles in consecutive samples may suggest that the parasite populations have an asynchronous growth during a clinical infection.[12,13] However, the findings may not fully reflect the natural population dynamics of symptomatic *P. falciparum* infections since these may be affected by the administration of antimalarial drugs. The finding of the same genotype profiles in the consecutive samples may also reflect amplification of parasite debris after breakdown of large parasite loads during treatment. The presence of gametocytes may also influence the results, but only four patients had gametocytes detected by microscopy.

Our findings further suggest that frequent sampling to obtain a representative sample of the parasite population may

not be as crucial in symptomatic infections as in asymptomatic infections. Although repeated sampling for determination of parasite densities is important when monitoring the effect of treatment in a patient with malaria, repeated sampling for characterization of the parasite populations did not show any additional genotypes during the first days of treatment. However, follow-up analysis in drug trials distinguishing recrudescent parasites from new infections may still be favored by analysis of additional samples, e.g., on two consecutive days, since an asymptomatic parasitemia may confer dynamics other than the infection in the acute phase and a single blood sample may then only partly reflect the infecting parasite population.

This study was conducted in patients residing in Sweden who contracted malaria after temporary visits to malaria-endemic areas. Symptomatic infections in partially immune individuals living in disease-endemic areas may confer different parasite population dynamics and genotyping profiles due to acquired immunity and accumulated chronic infections. Here, seven patients originated from malaria-endemic areas in Africa and six patients were Swedish tourists. The patients who originated from a disease-endemic area may have some partial immunity to malaria, but are less likely to have any chronic asymptomatic *P. falciparum* infections after several years in Sweden. Except for one patient with one sporadic genotype, these patients did not show dynamics different from those of Swedish non-immune travelers. Although repeated testing in symptomatic individuals residing in malaria-endemic areas needs further investigation, the parasite populations contributing to disease are still likely to dominate the genotyping profiles during the first days of treatment in semi-immune individuals.

In conclusion, the genetic profiles of *P. falciparum* populations were highly consistent in samples obtained before and during treatment, and do not substantially increase the possibility of detecting additional parasites below the threshold of detection. With regard to genotyping of asymptomatic infections, single samples may thus be sufficiently informative for determining the parasite population in an individual at the time of treatment.

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