Complexity of *Plasmodium falciparum* Clinical Samples from Uganda during Short-Term Culture

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We cultured *Plasmodium falciparum* parasites from 98 Ugandan children with malaria and determined the complexity of infection (COI) on the basis of *msp-2* polymorphisms daily for 9 days. The mean COI decreased during culture from 1.73 to 1.56. New strains appeared after day 0 in 20 cultures. Strains disappeared after day 0 in 56% of 45 cultures that were initially mixed; persisting strains more commonly had wild-type *dhfr* (C59) and *dhps* (K540) sequences and mutant *pfmdr1* (86Y) sequences. Thus, initial genotypes offer an imperfect representation of clinical COI. Loss of strains in culture may be due to diminished fitness of some drug-resistant strains.

Techniques to culture erythrocytic stages of *Plasmodium falciparum* have been available for >30 years [1]. The study of culture-adapted strains is essential for much laboratory research on malaria; these strains have typically been cultured for months to years before analysis. Cultures of fresh parasites are widely used to study the in vitro drug sensitivity of infecting parasites and to search for associations between parasite features and clinical outcomes. However, *P. falciparum* infections are commonly polyclonal in many areas, and, because not all parasites successfully grow in culture, it is unclear how well in vitro culture represents the complexity of clinical infections.

In high-transmission areas, such as sub-Saharan Africa, the complexity of *P. falciparum* infections is typically high; for example, the complexity of infection (COI) was measured at 2.2–4.4 per subject by single time point assessments of individuals with uncomplicated malaria at 7 sites with varied transmission intensity in Uganda [2] and at 5.2–6.8 after intensive evaluation (at 8 time points) of asymptomatic parasitic children in Senegal [3]. COI is typically measured by assessment of the diversity of highly polymorphic *P. falciparum* genes, most commonly the gene encoding merozoite surface protein 2 (*msp-2*). To characterize changes in COI during short-term culture of *P. falciparum*, we cultured parasites from children with malaria in Kampala, Uganda, and characterized COI on the basis of *msp-2* polymorphisms in 98 samples for which culture was maintained for 9 days.

**Methods.** Samples were collected from a cohort of children in the Mulago III Parish of Kampala enrolled in a longitudinal drug efficacy comparison [4]. The clinical trial began in November 2004; samples for this study were collected between October 2005 and May 2006. The clinical trial and analysis of cultured parasites were approved by the Uganda National Council of Science and Technology, the Makerere University Research and Ethics Committee, and the University of California, San Francisco, Committee on Human Research.

At the time of new diagnosis of malaria, blood was spotted onto filter paper (Whatman 3MM), collected in an EDTA tube, and transported within 1 h to our laboratory. Giemsa-stained thin blood smears were examined, and if *P. falciparum* infection and the lack of other plasmodial species were confirmed, culture was initiated as follows: blood was centrifuged, plasma and buffy coat were removed, and the erythrocyte pellet was washed with RPMI 1640 medium supplemented with 25 mmol/L HEPES, 0.2% NaHCO3, 0.1 mmol/L hypoxanthine, and 0.25%–0.5% Albumax II serum substitute. Washed erythrocyte pellets (200 μL) were used to inoculate cultures in the same medium at 2% hematocrit in 10-mL sterile flasks. Parasites were then placed in a candle jar or in 5% CO2 at 37°C. Every 24 h, Giemsa-stained smears of cultures were examined, blood was spotted onto filter paper, and culture medium (prepared fresh at least every 2 weeks) was replaced. All cultures in which parasites remained for 9 days were considered in this analysis.

For analysis of COI, parasite DNA was extracted from filter paper using Chelex 100 resin, and a variant-specific nested polymerase chain reaction (PCR) protocol was used to amplify either the 3D7 or FC27 variants of the *msp-2* gene, as described elsewhere [5]. PCR products were resolved on agarose gels, and the sizes of products were compared with standards and between
days on densitometric digitized gel images analyzed by GelCompar II software (Applied Maths). Strains from different days were considered identical if estimated fragment lengths were within 15 bp.

Polymorphisms in individual loci of 3 genes, \(dhfr\) C59R, \(dhps\) K540E, and \(pfmdr1\) N86Y, were determined by nested PCR of relevant portions of the genes followed by sequence-specific restriction endonuclease digestion [6, 7], resolution of digestion products by agarose gel electrophoresis, and analysis of densitometric digitized gel images using GelCompar II software. For statistical analysis, we used a 1-sample test of proportions to test the null hypothesis that equal proportions of mutant and wild-type parasites would be selected in culture.

**Results and discussion.** We placed into culture 211 samples from children in Kampala (aged 1–0 years at the time of study enrollment) with acute malaria. Of the samples collected, 98 were successfully cultured for \(\geq 9\) days. Other samples were not maintained owing to failure of parasites to survive for 9 days (100 cultures) or bacterial contamination (13 cultures). Parasite density at diagnosis was greater in samples that successfully grew for 9 days (mean, 30,400 parasites/\(\mu\)L) than in those that did not (mean, 11,500 parasites/\(\mu\)L). COI was assessed daily on the basis of the analysis of polymorphic regions of \(msp-2\). Of the 98 samples, for 53 only a single genotype was detected at the time of the initiation of culture (day 0). The mean COI on day 0 was 1.73 (95% confidence interval [CI], 1.56–1.92); the COI was stable for the first 4 days of culture and then decreased gradually to 1.56 (95% CI, 1.40–1.73) on day 8 (figure 1A).

The measured COI was lower than that determined in a number of other areas of Africa [2, 3] and was also lower than the COI of 2.88 that we measured in children in Kampala in another study [8]. The relatively low COI reflects the fact that Kampala has relatively low transmission for sub-Saharan Africa. In addition, our genotyping was based on only a single polymorphic gene, likely underestimating the true diversity of cultured parasites. Finally, study children had benefited from prompt treatment of all malaria episodes for \(\geq 6\) months before evaluation for this study, likely decreasing parasite prevalence.
Fitness, considered here as the ability of a parasite to survive in
of the 45 cultures that were polyclonal on day 0, some strains
of circulating strains that were missed by genotyping at a single
time point on day 0. Strains not present on day 0 were first identified on days 1–5;
26 new strains were identified in 20 cultures (figure 1B). On the
basis of these results, the day 0 genotype understated the true
COI in 20% of samples. The appearance of new strains identifies
limitations in the sensitivity of a single msp-2 assessment. Prior
studies have shown changes in parasite genotypes within hours
after treatment in France [9], quite constant genotypes in Swe-
en studies from other areas showing that COI is underestimated by
20% of recurrent outcomes in a treatment efficacy trial were changed (from new
infection to recrudescence) by considering both day 0 and 1
rather than only day 0 genotypes as the baseline for comparison
with recurrent parasites. Our results similarly indicate a substan-
tial underrepresentation of the true complexity of a malarial
infection when only a single day 0 genotype is assessed. Because all
parasites that appear in culture after day 0 must necessarily have
been present initially, our results identify the minimum number of
circulating strains that were missed by genotyping at a single
time point on day 0.

We were also interested in the loss of strains after day 0. In 25
of the 45 cultures that were polyclonal on day 0, some strains
disappeared during 9 days of culture (figure 2); a total of 32
strains disappeared. We examined the possibility that parasite
fitness, considered here as the ability of a parasite to survive in
culture, played a role in the disappearance of certain strains.
Fitness might be diminished in drug-resistant parasites, as ap-
ppears to be the case with chloroquine-resistant strains, which
were replaced by wild-type parasites after drug pressure was re-
moved in China and Malawi [12]. We therefore evaluated 3
resistance-mediating polymorphisms in mixed cultures that
subsequently became monoclonal (within our limits of detec-
tion): the key mediators of antifolate resistance in East Africa,
dhfr C59R and dhps K540E [13], and a mediator of responses to
a number of antimalarials, pfmdr1 N86Y [14]. Of 12 cultures
that had mixed genotypes at dhfr 59 on day 0, 5 were subse-
sequently monoclonal on day 8 (4 C59 and 1 59R). Of 21 cultures
that were mixed at dhps 540 on day 0, 2 were subsequently
monoclonal on day 8, both pure wild type (K540). Thus, for the
mutations that mediate sulfadoxine-pyrimethamine resistance,
when cultures became monoclonal, wild-type parasites persisted
in 6 of 7 instances. With the small available sample size, results
did not differ significantly from a random distribution of alleles
(P = .18 and P = .16 for the selection of C59 and K540, re-
respectively).

Of 19 cultures that were mixed at pfmdr1 86 on day 0, 5 were
subsequently monoclonal on day 8, all with the 86Y genotype, a
significant selection for 86Y in culture (P = .03). Of note, the
pfmdr1 86Y genotype, which is typically classified as mutant,
actually leads to improved activity of a number of drugs, includ-
ing mefloquine, quinine, and artemisinins [15]. Thus, it is plau-
sible that all 3 studied polymorphisms may have come at some
cost to fitness, as suggested by our results showing parasites with
dhfr C59, dhps K540, and pfmdr1 86Y outcompeting those with
other sequences at these loci in culture. Of note, an important
factor not considered in our in vitro experiments is the impact of
circulating drugs on the removal of drug-sensitive parasites.

Our results offer, to our knowledge, the first assessment of
COI during culture of freshly isolated malarial parasites. They
have a number of ramifications. First, we confirm the findings of
studies from other areas showing that COI is underestimated by
a single characterization on day 0, presumably because of limits
in sensitivity for low-abundance strains. This observation is par-
ticularly relevant for drug efficacy trials. In this case, strains
missed on day 0 and seen after treatment will be misclassified as
new infections, thus understating levels of drug resistance. In
our study, 26 strains appeared only after day 0 (mostly on day 1),
indicating that genotyping using a combination of days 0 and 1
patterns as baseline, as has been suggested previously [11], might
make assessments of recrudescence rates more reliable, albeit at
the cost of added complexity in trial design. Second, we charac-
terize the dynamics of parasite complexity in fresh cultures.
Many samples did not survive in culture for 9 days, and surviving
cultures showed a gradual decrease in COI over time. Thus, par-
asites studied after culture adaptation represent only a subset of
those infecting a patient, and inferences regarding associations
between cultured parasites and clinical outcomes must consider

Figure 2. Genotype patterns over time for 2 representative cultures.
For each culture, DNA was extracted daily, the 3D7 and FC27 alleles of
msp-2 were amplified, and products were resolved by agarose gel
electrophoresis. Controls included no DNA (Neg) and DNA from labora-

tory strains known to express the 3D7 (3D7) or FC27 (HB3) allele. Sizes
were compared on the basis of 50-bp markers (M). The results shown
demonstrate a culture with an initial complexity of infection (COI) of 4,
with a loss of two 3D7 strains after day 2 (A), and a culture with an initial
COI of 1, with the appearance of an additional 3D7 strain on day 5 (B).

To gain insight into the sensitivity of single time point assess-
ment of COI, we followed the appearance of new strains after day
0. Strains not present on day 0 were first identified on days 1–5;
26 new strains were identified in 20 cultures (figure 1B). On the
basis of these results, the day 0 genotype understated the true
COI in 20% of samples. The appearance of new strains identifies
limitations in the sensitivity of a single msp-2 assessment. Prior
studies have shown changes in parasite genotypes within hours
after treatment in France [9], quite constant genotypes in Swe-
en [10], and a moderate level of change in genotype between
days 0 and 1 in Tanzania [11]. In Tanzania, ~20% of recurrent
outcomes in a treatment efficacy trial were changed (from new
infection to recrudescence) by considering both day 0 and 1
rather than only day 0 genotypes as the baseline for comparison
with recurrent parasites. Our results similarly indicate a substan-
tial underrepresentation of the true complexity of a malarial
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We were also interested in the loss of strains after day 0. In 25
of the 45 cultures that were polyclonal on day 0, some strains
disappeared during 9 days of culture (figure 2); a total of 32
strains disappeared. We examined the possibility that parasite
fitness, considered here as the ability of a parasite to survive in
of freshly prepared parasites. Thus, evaluation of parasites after culture adaptation may include a bias toward drug-sensitive parasites.

Our study had some limitations. First, it was limited to parasites that were successfully cultured for 9 days. For approximately half of our cultures, no strain survived for 9 days. Factors leading to rapid loss of these strains were not considered. Changes in techniques, including use of a defined gas mixture, increase in concentration of the serum substitute, or use of human serum, might have improved survival in culture. Second, we used only a single marker, *msp*-2, to distinguish parasite strains. The *msp*-2 marker has been shown to provide reasonable discrimination of *P. falciparum* strains in Kampala [5], although consideration of additional strains would likely increase estimates of COI. Third, we considered genotypes based on amplification of parasite DNA from dried blood spots. This technique matched that used in most clinical trials but might not be as sensitive as amplification of DNA purified from whole blood, again potentially underestimating COI. Fourth, for associations between parasite polymorphisms and survival in culture, our sample sizes were small, because relatively few cultures underwent changes in population structure, and our knowledge of the impact of polymorphisms on parasite fitness is incomplete. Despite these limitations, our results offer insight into the dynamics of freshly prepared *P. falciparum* cultures. In particular, our findings suggest potential limitations in both the use of a single time point assessment of COI as a baseline for drug efficacy determination and the reliability of associations between parasite genotypes and clinical outcomes that are based on assessment of stably cultured parasites.

**References**