# Influence of Consecutive-Day Blood Sampling on Polymerase Chain Reaction–Adjusted Parasitological Cure Rates in an Antimalarial-Drug Trial Conducted in Tanzania

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**We assessed the influence that consecutive-day blood sampling, compared with single-day blood sampling, had on polymerase chain reaction (PCR)–adjusted parasitological cure after stepwise genotyping of merozoite surface proteins 2 *(msp2)* and 1 *(msp1)* in 106 children in Tanzania who had uncomplicated falciparum malaria treated with either sulfadoxine-pyrimethamine or artemether-lumefantrine; 78 of these children developed recurrent parasitemia during the 42-day follow-up period. Initial *msp2* genotyping identified 27 and 33 recrudescences by use of single- and consecutive-day sampling, respectively; in subsequent *msp1* genotyping, 17 and 21 of these episodes, respectively, were still classified as recrudescences; these results indicate a similar sensitivity of the standard single-day PCR protocol—that is, 82% (27/33) and 81% (17/21), in both genotyping steps. Interpretation of PCR-adjusted results will significantly depend on methodology.**

To ensure effective and life-saving treatment for sick children, accurate results from efficacy trials are essential for adequate policy decisions on the use of antimalarial drugs in Africa. The World Health Organization (WHO) recommends that a new antimalarial treatment should have an average cure rate of ⩾95%, as assessed in clinical trials, and that a review-/change-of-treatment policy should be initiated when the efficacy of a recommended treatment falls to <90% [1].

The recent introduction of genotyping of *Plasmodium falciparum* by polymerase chain reaction (PCR), as a tool to distinguish between treatment failure (recrudescence) and new infection (reinfection), has significantly improved the assessment of parasitological cure rates, particularly in antimalarial-drug trials conducted in high-transmission areas with extended follow-up beyond day 14. However, because of inherent limitations of the PCR technique and constraints imposed by the biology of the parasite [2], PCR-adjusted outcomes should be interpreted with caution.

PCR adjustment to differentiate between recrudescence and reinfection is presently achieved by analysis of one or a combination of highly polymorphic genetic markers—for example, merozoite surface proteins 1 *(msp1)* and 2 *(msp2)*—from paired blood samples collected at the day of enrollment and the day of recurrent parasitemia. However, the use of PCR analysis of such paired blood samples in antimalarial-drug trials will not reflect the daily dynamics of *P. falciparum* populations previously shown in asymptomatic children in high-transmission areas [3]. This observation may also have implications for symptomatic *P. falciparum* infections and needs to be considered in clinical-trial protocols, to establish PCR-adjusted parasitological cure. In previous studies of parasite population dynamics during the early phase of treatment of travelers returning with symptomatic malarial infection, Färnert et al. found a consistent genotype pattern during consecutive-day sampling [4], whereas Jafari et al., using a novel quantitative method, detected different genotypes at different time points [5]. In Gabonese children treated with quinine, the same genotype pattern was observed during the infection, although alleles disappeared and reappeared over time in some children [6]. However, none of these studies applied consecutive-day sampling during recurrent parasitemia.

We report here data from a clinical trial of uncomplicated childhood *P. falciparum* malaria, a trial that was conducted in a high-transmission area in Tanzania and that had the primary objective of assessing the influence that consecutive-day blood

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sampling had on PCR-adjusted parasitological cure rates (PCR-APCRs), compared with a standard protocol using paired blood samples only. This scientific question was recently identified, by WHO, as a research priority [7].

***Methods.*** The study was conducted during April–July 2004 in Fukayosi, Bagamoyo District, Tanzania, which is located in a rural area with holoendemic malaria transmission. The trial was conducted in accordance with the Declaration of Helsinki and was approved by the ethics committees at the Muhimbili University College of Health Sciences (Tanzania) and the Karolinska Institutet (Sweden). Informed consent was obtained from the parents/guardians of all children enrolled in the study. Inclusion criteria included a blood sample positive for *P. falciparum* at a density of 2000–200,000 asexual parasites/$\mu$L of blood, age $\geqslant$6 months, body weight $\geqslant$6 kg, and either axillary temperature $\geqslant$37.5°C or a history of fever during the preceding 24 h; exclusion criteria included symptoms/signs of severe malaria, hemoglobin level <50g/L, and any serious underlying disease or known allergy to study drugs.

Enrolled children were assigned randomly to receive a fixed combination of either (1) sulfadoxine-pyrimethamine (Fansidar; Roche) as a single dose under supervision or (2) artemether-lumefantrine (Coartem; Novartis), twice daily and on the basis of body weight, for 3 days. For logistic reasons, only the first of the 2 daily doses of artemether-lumefantrine were administered under supervision.

The duration of outpatient follow-up of the children was 42 days. Clinical and laboratory assessments were conducted on days 0, 1, 2, 3, 7, 14, 21, 28, 35, and 42 or on any day of recurrent illness.

Giemsa-stained thick-blood films were examined, quantified (in terms of parasites per microliter), and recorded at each clinic visit. Quality control was performed according to WHO recommendations [7].

PCR-APCRs were analyzed by use of stepwise genotyping, as described by Mugittu et al. [8]. Initially, all blood samples collected at enrollment and during recurrent parasitemia were analyzed by use of the *msp2* marker, for both assessment of parasite-population complexity and establishment of PCR-adjusted cure. In a second step, performed by use of another highly polymorphic genetic marker, all children with recurrent parasitemia underwent additional genotyping, using the *msp1* marker, to confirm the presence of recrudescences and to assess the distribution of PCR outcome.

All genotyping was performed from dried blood spots on filter paper (3MM; Whatman), on the basis of previously described nested-PCR protocols [9]. PCR results for paired blood samples collected on the day of enrollment and the day of recurrent parasitemia (standard protocol) were compared, as were those for paired blood samples collected on 4 consecutive days after enrollment and on 2 consecutive days of recurrent parasitemia (enhanced protocol). For each child, all samples were amplified in the same PCR run. The PCR products were loaded in parallel on the same gel and were separated by electrophoresis, for analysis of both the number of genotypes and size polymorphism.

For each step and protocol, recrudescence was defined as the presence of at least 1 matching allelic band, and reinfection was defined as the absence of any matching allelic band in samples collected at enrollment and during recurrent parasitemia. Children with PCR results that, on the basis of either the *msp2* or *msp1* genotyping, fulfilled the reinfection criteria were considered to be treatment successes; children with negative PCR amplification at enrollment and/or during recurrent parasitemia were considered to have nonconclusive PCR outcomes. The primary objective was to evaluate the different sampling protocols, and the study was considered to be exploratory. A sample of 100 children, with a minimum of 50 in each treatment arm, was predefined. Data were entered, validated, and analyzed by use of SPSS (version 14.0) software.

Proportions were compared by $\chi^2$ test, Fisher's exact test, and relative risk (RR), as appropriate. Continuous variables were compared by paired-sample *t* test. Statistical significance was defined as $P \leqslant .05$. The intention-to-treat approach was used to analyze the primary efficacy outcome in all children enrolled. Children lost to follow-up who did not have a defined primary efficacy outcome were kept in the analyses until their day of exit, after which they no longer contributed to the denominator in the calculations performed.

***Results.*** A total of 434 children were screened for eligibility. Of the 175 (40%) who had a blood-slide sample positive for *P. falciparum*, 69 were excluded (61 had a parasite density <2000/$\mu$L, 4 had severe malaria, 2 lived outside the study area, 1 left the clinic without notice, and 1 had a severe concomitant disease); the remaining 106 children were enrolled, and 56 of them were randomly assigned to treatment with sulfadoxine-pyrimethamine, 50 to treatment with artemether-lumefantrine.

Baseline clinical and demographic data showed no significant differences between the 2 treatments: in both groups, the geometric mean parasite count at enrollment was ~21,600/$\mu$L, and no deaths occurred. Because 3 of the 56 children treated with sulfadoxine-pyrimethamine traveled outside the study area and were lost to follow-up (1 each on days 28, 35, and 42 after treatment), a total of 103 (97%) of the 106 enrolled children were available for analysis of primary efficacy outcome by day 42 after treatment.

PCR-based *msp2* genotyping of blood samples collected on days 1–3 identified 32 additional (i.e., compared with those seen at day 0) parasite genotypes in 26 (25%) of the 106 enrolled children; of these additional genotypes, 21, 8, and 3 were detected on days 1, 2, and 3, respectively. When these additional 32 genotypes were included as part of the initial infection, the



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proportion of children with multiple genotypes increased from 60/106 (57%) to 73/106 (69%), although there was no effect on the number of genotypes detected (median, 2 [range, 0–7]). Similarly, in 10/78 (13%) children with recurrent parasitemia, a total of 11 parasite genotypes were detected not on the initial day but on the following day. When these 11 genotypes were included as part of the recurrent parasitemia, the proportion of children with multiple genotypes increased from 45/78 (58%) to 48/78 (62%), although there was no effect on the number of genotypes detected (median, 2 [range, 0–5]).

The PCR outcome for the entire cohort, as established by stepwise genotyping of *msp2* and *msp1* for the 2 sampling protocols, is presented in figure 1. Of the 33 recrudescences identified by the initial *msp2* analysis, 6 (18%) were identified exclusively by the enhanced protocol with consecutive-day sampling, meaning that the sensitivity of the standard protocol was 27/33 (82% [95% confidence interval {CI}, 68%–96%]). When PCR results from days 3 and 4 after enrollment were considered, only 1 of the 6 additional recrudescences identified exclusively by the enhanced protocol was detected.

After the additional *msp1* analysis, 21 episodes were still classified as recrudescences (treatment failures); 4 (19%) of these 21 were so classified on the basis of the enhanced protocol, all 4 by combining PCR results from the first 2 consecutive days after enrollment and from 2 days of recurrent parasitemia; the sensitivity of the standard protocol in the second genotyping step was therefore 17/21 (81% [95% CI, 63%–99%]).

When *msp1* was used as the initial marker for genotyping, the distribution of PCR results in all 78 children with recurrent parasitemia was similar to that when *msp2* was used as the

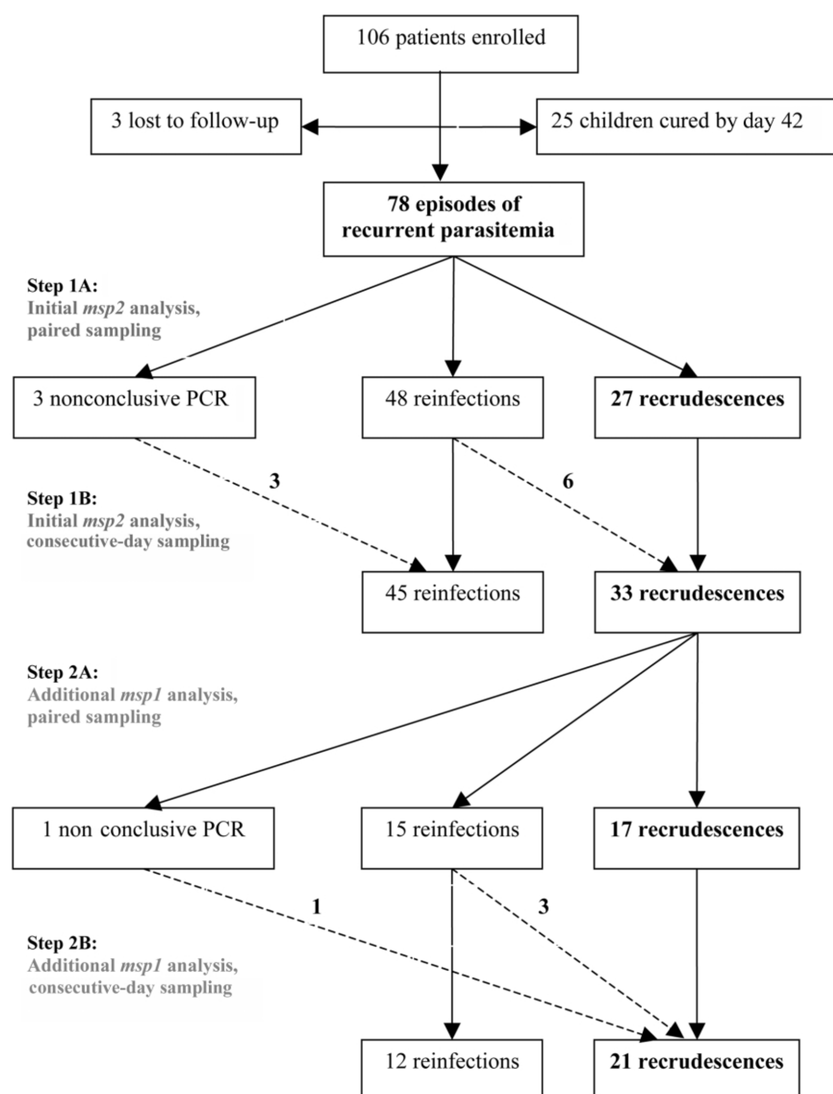


**Figure 1.** Flow chart of outcome of polymerase chain reaction (PCR), using stepwise genotyping consisting of initial *msp2* genotyping followed by *msp1* genotyping, for the single-day– and consecutive-day–sampling protocols.



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initial marker: for the paired and consecutive-day samples, respectively, 26 and 32 episodes were defined as recrudescences, 46 and 44 as reinfections, and 6 and 2 as nonconclusive PCR outcomes. When the 6 episodes initially defined as nonconclusive by the paired-samples protocol were analyzed by the enhanced protocol, 3 were classified as recrudescences, 1 as a reinfection, and 2 as nonconclusive.

For the entire cohort, the crude cure rates and PCR-APCRs by days 14, 28, and 42 of follow-up, as well as the respective analyses using the 2 different genotyping steps and sampling protocols, are presented in table 1.

***Discussion.*** The results of this clinical trial of uncomplicated childhood malaria, conducted during a highly intensive transmission period in Bagamoyo District, Tanzania, indicate that a single blood sample may not provide a complete picture of all parasite subpopulations present in an individual. The similar proportion of recrudescences that were identified exclusively by consecutive-day (4+2 days) sampling but were defined as either reinfections or nonconclusive PCR results when the standard single-day (1+1 day) protocol was used in both genotyping steps suggests that PCR protocols using paired blood samples may overestimate the efficacy that antimalarial drugs have in high-transmission areas.

The additional genotypes identified by consecutive-day sampling but not detectable at the day of enrollment (i.e., not detectable by single-day sampling) could be a reflection of either

**Table 1. Crude and polymerase chain reaction (PCR)–adjusted cure rates, and relative risk (RR) for the association between treatment type and cure rate, both after initial *msp2* genotyping and after additional *msp1* genotyping.**

| Day, protocol[a] | Cure rate, proportion (%): Entire cohort ($n$ = 106) | Treatment with SP ($n$ = 50) | Treatment with AL ($n$ = 56) | Relative risk, SP vs. AL |
|---|---|---|---|---|
| | Initial *msp2* genotyping | | | |
| 14 | | | | |
| PCR unadjusted | 97/106 (92) | 48/56 (86) | 49/50 (98) | 1.80 (1.32–2.44) |
| PCR-APCR | | | | |
| Standard | 98/106 (92) | 49/56 (88) | 49/50 (98) | 1.75 (1.26–2.43) |
| Enhanced | 97/106 (92) | 48/56 (86) | 49/50 (98) | 1.80 (1.32–2.44) |
| 28 | | | | |
| PCR unadjusted | 48/105 (46) | 24/55 (44) | 24/50 (48) | 1.09 (0.75–1.57) |
| PCR-APCR | | | | |
| Standard | 83/105 (79) | 39/55 (71) | 44/50 (88) | 1.55 (1.10–2.18) |
| Enhanced | 78/105 (74) | 36/55 (66) | 42/50 (84) | 1.52 (1.08–2.15) |
| 42 | | | | |
| PCR unadjusted | 25/103 (24) | 13/53 (24) | 12/50 (24) | 0.99 (0.64–1.52) |
| PCR-APCR | | | | |
| Standard | 76/103 (74) | 33/53 (62) | 43/50 (86) | 1.71 (1.21–2.40) |
| Enhanced | 70/103 (68) | 29/53 (55) | 41/50 (82) | 1.76 (1.24–2.49) |
| | Additional *msp1* genotyping of recrudescences defined by *msp2* genotyping ($n$ = 33) | | | |
| 14 | | | | |
| PCR-APCR | | | | |
| Standard | 99/106 (93) | 49/56 (88) | 50/50 (100) | 2.02 (1.66–2.46) |
| Enhanced | 98/106 (92) | 49/56 (88) | 49/50 (98) | 1.75 (1.26–2.43) |
| 28 | | | | |
| PCR-APCR | | | | |
| Standard | 90/105 (86) | 41/55 (74) | 49/50 (98) | 2.05 (1.58–2.67) |
| Enhanced | 87/105 (83) | 40/55 (73) | 47/50 (94) | 1.81 (1.33–2.46) |
| 42 | | | | |
| PCR-APCR | | | | |
| Standard | 86/103 (84) | 37/53 (70) | 49/50 (98) | 2.19 (1.67–2.87) |
| Enhanced | 82/103 (80) | 35/53 (66) | 47/50 (94) | 2.01 (1.48–2.73) |

**NOTE.** AL, artemether-lumefantrine; PCR-APCR, PCR-adjusted parasitological cure rate; SP, sulfadoxine-pyrimethamine.

[a] In the standard protocol, PCR adjustment was performed with paired blood samples collected on the day of enrollment and the day of recurrent parasitemia; in the enhanced protocol, PCR adjustment was performed with paired blood samples collected on 4 consecutive days after enrollment and on 2 consecutive days of recurrent parasitemia.



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the parasite population dynamics of *P. falciparum* [3] or the PCR technique's limitations, which are due to template competition in complex infections, in detecting all minority genotypes that are present [2].

The handling and interpretation of nonconclusive PCR results is a dilemma in antimalarial-drug trials, because such results can represent either recrudescences or reinfections. Thus, an advantage of the enhanced protocol used in the present study was the improved ability to retrieve PCR-adjusted outcomes in children for whom paired blood samples showed nonconclusive PCR results.

In the present study, we have used stepwise genotyping of 2 highly polymorphic loci to distinguish between treatment failure and new infection, an approach recently proposed by Mugittu et al. [8]. Interestingly, the distribution of PCR-adjusted outcomes was similar when the initial marker used for genotyping was either *msp2* or *msp1*. However, when the 2 markers were combined, the PCR-adjusted outcome changed significantly, with a substantial number of recrudescences being reclassified as reinfections. This finding is consistent with the results reported by Mugittu et al. [8] and indicates that the use of a single genetic marker to establish the PCR-adjusted cure may result in an underestimation of drug efficacy. On the other hand, the increased specificity achieved with incorporation of multiple genetic markers into the PCR analysis will simultaneously reduce the sensitivity of detection of true recrudescences arising from minority clones, which may not be detected by all markers, a result that may, instead, underestimate treatment failure.

Because a majority of both of the additional genotypes used in the present study were detected on the first day after enrollment, and because the additional recrudescences were identified by combining PCR results from 2 consecutive days both at enrollment and during recurrent parasitemia, we suggest that this sampling model (2+2 days) should be the primary target for future studies assessing the efficiency of consecutive-day sampling in different endemic settings in Africa.

In conclusion, the range of possible PCR-APCRs for the entire cohort varied from 86% by day 28, when stepwise genotyping with *msp2* and *msp1* and the standard paired-samples protocol were used, to 68% during an extended follow-up to day 42, when a single genetic marker *(msp2)* and the enhanced protocol were used. These results underscore how sensitive the assessment of PCR-adjusted cure is to the choice of methodology, and this has critical implications in the interpretation of data from in vivo studies, particularly in the context of evidence-based decisions on new antimalarial-drug policies in Africa.

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