January 2007

Influence of consecutive-day blood sampling on polymerase chain reaction-adjusted parasitological cure rates in an antimalarial-drug trial conducted in Tanzania

Andreas Martensson  
*Karolinska University Hospital*

Johan Ursing  
*Karolinska University Hospital*

M. Isabel Veiga  
*University of Algarve*

Lisa Wiklund  
*Karolinska University Hospital*

Christopher Membi  
*Muhimbili Univerity College of Health Sciences*

See next page for additional authors

Follow this and additional works at: [http://ecommons.aku.edu/eastafrica_fhs_mc_pathol](http://ecommons.aku.edu/eastafrica_fhs_mc_pathol)

Part of the Pathology Commons

Recommended Citation


Available at: [http://ecommons.aku.edu/eastafrica_fhs_mc_pathol/70](http://ecommons.aku.edu/eastafrica_fhs_mc_pathol/70)
Influence of Consecutive-Day Blood Sampling on Polymerase Chain Reaction–Adjusted Parasitological Cure Rates in an Antimalarial-Drug Trial Conducted in Tanzania

Andreas Mårtensson,1,2 Billy Ngasala,1,2 Johan Ursing,1 M. Isabel Veiga,13 Lisa Wiklund,1 Christopher Membli,2 Scott M. Montgomery,14 Zul Premji,1 Anna Färnert,1 and Anders Björkman1

1Infectious Diseases Unit, Department of Medicine, and 2Clinical Epidemiology Unit, Karolinska University Hospital, Karolinska Institutet, Stockholm, 3Emergency Medicine Unit, Department of Medicine, Kulaberga Hospital, Katrineholn, and 4Clinical Research Centre, Örebro University Hospital, Örebro, Sweden; 5Department of Parasitology, Muhimbili University College of Health Sciences, Dar es Salaam, Tanzania; 6Centre of Molecular and Structural Biomedicine, University of Algarve, Faro, Portugal

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 190% [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
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 Ka-
rolinska 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. fal-
ciparum at a density of 2000–200,000 asexual parasites/mL of
blood, age;?6 months, body weight;?6 kg, and either axillary
temperature ;?37.5°C or a history of fever during the preceding
24 h; exclusion criteria included symptoms/signs of severe ma-
laria, hemoglobin level ;50g/L, and any serious underlying dis-
ease or known allergy to study drugs.

Enrolled children were assigned randomly to receive a fixed
combination of either (1) sulfadoxine-pyrimethamine (Fan-
sidar; Roche) as a single dose under supervision or (2) arte-
mether-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 re-
current 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-ad-
justed 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 de-
scribed 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 elec-
trophoresis, 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 paras-
temia. 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 neg-
ative PCR amplification at enrollment and/or during recurrent
parasitemia were considered to have nonconclusive PCR out-
comes. The primary objective was to evaluate the different
sampling protocols, and the study was considered to be ex-
ploratory. A sample of 100 children, with a minimum of 50 in
each treatment arm, was predefined. Data were entered, vali-
dated, and analyzed by use of SPSS (version 14.0) software.

Proportions were compared by x2 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<.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 de-
nominator in the calculations performed.

Results. A total of 434 children were screened for eligi-
ity. Of the 175 (40%) who had a blood-slide sample positive
for P. falciparum, 69 were excluded (61 had a parasite density
>2000/mL, 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 geo-
metric mean parasite count at enrollment was ~21,600/mL, 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 de-
tected on days 1, 2, and 3, respectively. When these additional
32 genotypes were included as part of the initial infection, the
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.
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>
<thead>
<tr>
<th>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.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Day, protocol</strong></td>
</tr>
<tr>
<td><strong>Initial msp2 genotyping</strong></td>
</tr>
<tr>
<td>14</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>28</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>42</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td><strong>Additional msp1 genotyping of recrudescences defined by msp2 genotyping (n = 33)</strong></td>
</tr>
<tr>
<td><strong>Day, protocol</strong></td>
</tr>
<tr>
<td><strong>PCR-APCR</strong></td>
</tr>
<tr>
<td>14</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>28</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>42</td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

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

* 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.
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.

**Acknowledgments**

We sincerely thank all of the children, parents/guardians, and staff members at Fukayosi Health Care Centre for their participation in the study.

**References**