

## DISTINGUISHING RECRUDESCENCE FROM REINFECTION IN A LONGITUDINAL ANTIMALARIAL DRUG EFFICACY STUDY: COMPARISON OF RESULTS BASED ON GENOTYPING OF *MSP-1*, *MSP-2*, AND *GLURP*

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**Abstract.** Genotyping frequently is used to distinguish recrudescence from new infections in antimalarial drug efficacy trials, but methodology and interpretation of results have not been standardized. We compared the utility of polymorphisms within 3 *Plasmodium falciparum* genes during a longitudinal trial in Kampala, Uganda. Merozoite surface protein-1 (*mSP-1*) and merozoite surface protein-2 (*mSP-2*) revealed greater diversity than glutamate-rich protein. Genotypes based on *mSP-1*, *mSP-2*, and all 3 genes combined were compared for 394 initial and subsequent isolates. Classification of most episodes as due to recrudescence or reinfection was straightforward. In 24% (*mSP-1*), 16% (*mSP-2*), and 62% (3 genes combined) of samples, subsequent episodes contained identical and new alleles, however. Our analysis suggested that such episodes should be classified as reinfections and not recrudescence. Comparing the 3 studied genes, *mSP-2* results were most accurate, and analysis of this single gene effectively distinguished recrudescence from reinfection in our study population.

### INTRODUCTION

Resistance to antimalarial therapy is a major concern in Africa.<sup>1</sup> Decisions regarding drug policy rely largely on the results of *in vivo* studies that assess clinical and parasitologic outcomes after therapy. Such studies are limited by their inability to determine whether disease that recurs after therapy is due to recrudescence of resistant parasites or reinfection with new parasite strains. Many studies (and World Health Organization [WHO] guidelines) attempt to minimize this limitation by restricting follow-up to 14 days because during this time reinfection is considered unlikely.<sup>2</sup> This approach may underestimate significantly, however, the true risk of treatment failure.<sup>3</sup> This underestimation may be especially important when studying drugs with short half-lives, such as artesunate,<sup>4</sup> and effective combination regimens,<sup>5,6</sup> where a majority of treatment failures may occur after 14 days.

Molecular genotyping is used increasingly to help distinguish recrudescence from reinfection in antimalarial drug efficacy studies.<sup>3</sup> Infecting strains of malaria parasites can be “fingerprinted” through polymerase chain reaction (PCR) amplification of polymorphic genes.<sup>7</sup> The “fingerprint” patterns of isolates causing successive episodes of malaria can then be compared to distinguish recrudescence from newly infecting parasites. Although the principle behind molecular genotyping is straightforward, the methods used to interpret results have varied widely. In particular, the definitions used to classify treatment failures as recrudescence or reinfection have not been consistent. This underestimation may be especially important when studying drugs with short half-lives, such as artesunate, and effective combination regimens, where a majority of treatment failures may occur after 14 days.

In the present study, we analyzed polymorphisms within the 3 genes—merozoite surface protein-1 (*mSP-1*), merozoite surface protein-2 (*mSP-2*), and glutamate-rich protein (*glurp*)—most commonly evaluated to distinguish recrudescence from reinfection in antimalarial drug efficacy studies. Our database was from a longitudinal comparison of 3 antimalarial regimens for the treatment of uncomplicated ma-

laria in Kampala, Uganda. Statistical techniques were used to compare different definitions of recrudescence and reinfection and to evaluate the utility of results obtained with each gene and all 3 genes combined. We discuss the impact of different approaches and offer a simple genotyping system using *mSP-2* alone to type adequately infecting parasites from our study population.

### MATERIALS AND METHODS

**Clinical trial.** The clinical study took place between July 2000 and August 2001 at the outpatient department of Mulago Hospital, Kampala, Uganda. The full details of the clinical trial were published previously.<sup>6</sup> Briefly, 316 healthy children between the ages of 6 months and 5 years were enrolled from the community and randomly assigned to 1 of 3 regimens, which then was given for all episodes of uncomplicated malaria diagnosed during 12 months of follow-up. These regimens were sulfadoxine/pyrimethamine (SP) plus placebo, SP plus amodiaquine, and SP plus artesunate. For each episode of uncomplicated malaria diagnosed, patients were treated with their same preassigned regimen, and outcomes were assessed using a standard WHO 14-day follow-up protocol.<sup>2</sup> Clinical treatment failures occurring within 14 days were treated with standard doses of quinine. Any case of malaria diagnosed >14 days after a previous episode was classified as a new episode for treatment purposes.

**Sample selection and polymerase chain reaction genotyping.** Blood was collected on filter paper (Whatman No. 3, Whatman Inc., Clifton, NJ) each time a new episode of malaria was diagnosed and when treatment failure occurred. Molecular genotyping was performed for all successive episodes of uncomplicated *Plasmodium falciparum* malaria treated with study drugs except that failures within 6 days of treatment were classified as recrudescence, episodes caused by *P. falciparum* followed by nonfalciparum malaria were classified as reinfections, and episodes after quinine therapy (for severe malaria or clinical failure within 14 days) were considered new infections.

Parasite DNA was extracted with chelex 100 Resin (Bio-Rad Laboratories, Hercules, CA) as previously described.<sup>8</sup> The polymorphic regions of *msp-1* (block 2), *msp-2* (block 3), and *glurp* (R2 repeat region) were amplified by nested PCR. First-round PCR primers corresponded to conserved sequences flanking these regions.<sup>9</sup> Second-round PCR primers were used to amplify the K1, MAD20, and RO33 allelic families of *msp-1*<sup>10</sup>; the IC3D7 and FC27 allelic families of *msp-2*<sup>11</sup>; and the repeat region of *glurp*.<sup>12</sup> For controls, genomic DNA from HB3 and 3D7 laboratory strains was isolated by standard techniques.

Nested PCR products were analyzed by electrophoresis using 2% agarose for *msp-1* and *msp-2* and 1.5% agarose for *glurp*. Samples from an individual patient were run in adjacent lanes. If there was no amplification for any allelic family, PCR was repeated with 5 times the quantity of template DNA. If no amplification was detected after this second reaction, amplification was classified as unsuccessful. Gel images were digitized and molecular weights were assigned to bands using GelCompar II software (Applied Maths, Sint-Martens-Latem, Belgium). Densitometric curves were calculated for each gel lane, and bands with >2% of the density of the dominant band in each lane were assigned molecular weights.

**Data interpretation.** Genotyping patterns were compared for successive episodes of uncomplicated *P. falciparum* malaria. Based on analysis of the HB3 strain, alleles were considered the same if molecular weights were within 10 bp for *msp-1* and *msp-2* and within 20 bp for *glurp*. It was assumed that after a patient initially was treated for malaria, a subsequent episode was caused by either parasite strains present before treatment (e.g., recrudescence) or parasite strains acquired after treatment (e.g., reinfection). An outcome was defined as recrudescence if a subsequent sample contained identical alleles or a subset of the alleles present in the first sample. An outcome was defined as reinfection if a subsequent sample contained only new alleles. If a subsequent sample contained alleles present in the first sample and

new alleles, the outcome initially was considered indeterminate.

**Statistical analysis.** Different criteria for classifying indeterminate outcomes were compared using a statistical modeling approach. Treatment regimen and pretreatment parasite density were identified as strong independent predictors of treatment outcome using a Cox proportional hazards model. Measures of association (hazard ratios) for these predictor variables first were estimated only for episodes in which the outcomes were defined clearly as recrudescence or reinfection. These parameter estimates were considered the reference standards. The analysis was repeated after including episodes with indeterminate outcomes, using varied classification criteria. The best method for classifying indeterminate outcomes was considered that in which the new parameter estimates were closest to our reference standards. In our comparisons of different genes and the application of genotyping to clinical results, the risks of recrudescence and reinfection were estimated using Kaplan-Meier survival analysis techniques. Survival functions were compared using the log-rank test.

## RESULTS

**Performance of polymerase chain reaction genotyping.** Our clinical study included 661 treatments for malaria. PCR genotyping was performed on 534 samples for which the results could affect the classification of treatment outcome (Figure 1). Genotyping was not performed if an episode was not preceded or followed by a study drug treatment (n = 59), subsequent treatment was <7 days after initial treatment (n = 23), the episode was followed by nonfalciparum malaria (n = 2), or the sample was missing (n = 4). PCR amplification was successful in 99%, 96%, and 98% of samples for *msp-1*, *msp-2*, and *glurp* and in 96% of samples when all 3 genes were combined. Of a possible 392 paired primary and subsequent samples, PCR results were available for 386, 371, 379, and 367 pairs for *msp-1*, *msp-2*, *glurp*, and when all 3 genes were combined.

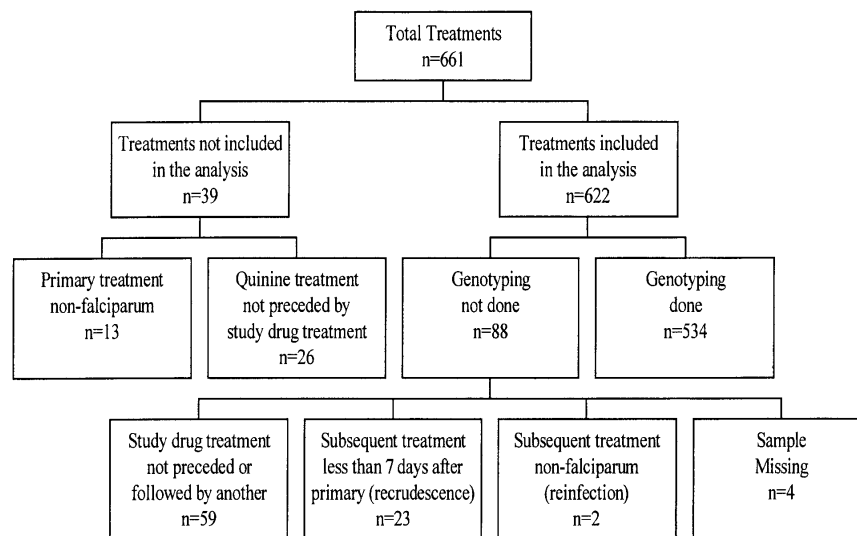
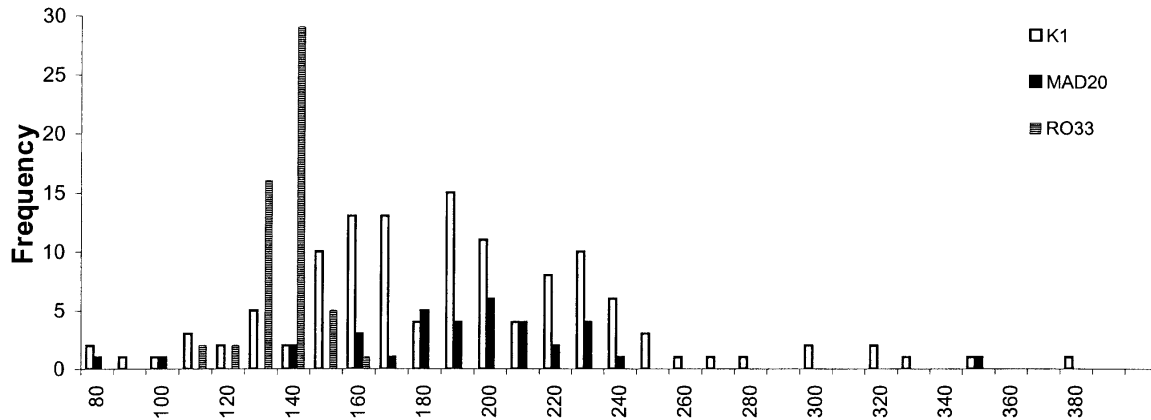
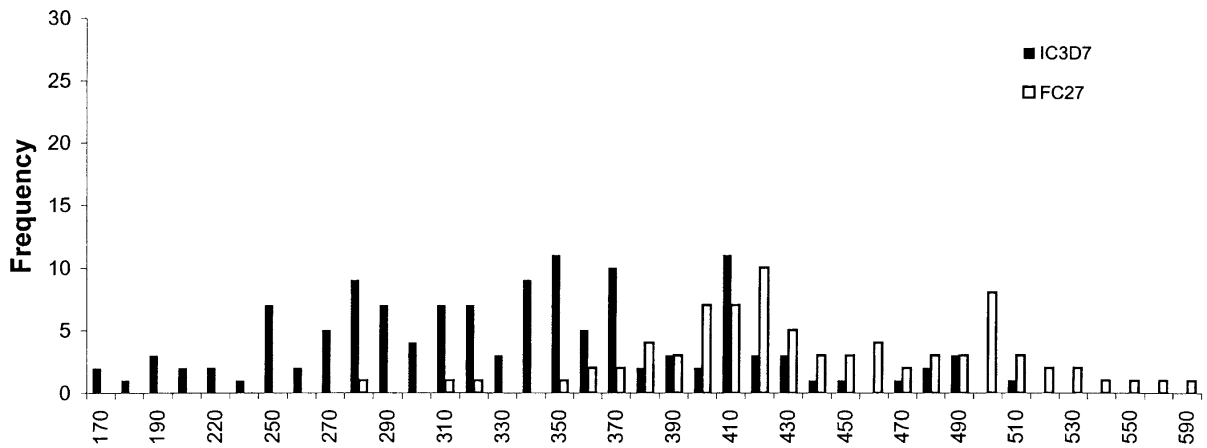


FIGURE 1. Sample selection. Decision tree that led to the selection of 534 samples for genotyping analysis. The primary outcome of interest was risk of recrudescence for all episodes of uncomplicated falciparum malaria treated with study drugs (sulfadoxine/pyrimethamine [SP] plus placebo, SP plus amodiaquine, and SP plus artesunate).

**A. MSP-1**



**B. MSP-2**



**C. GLURP**

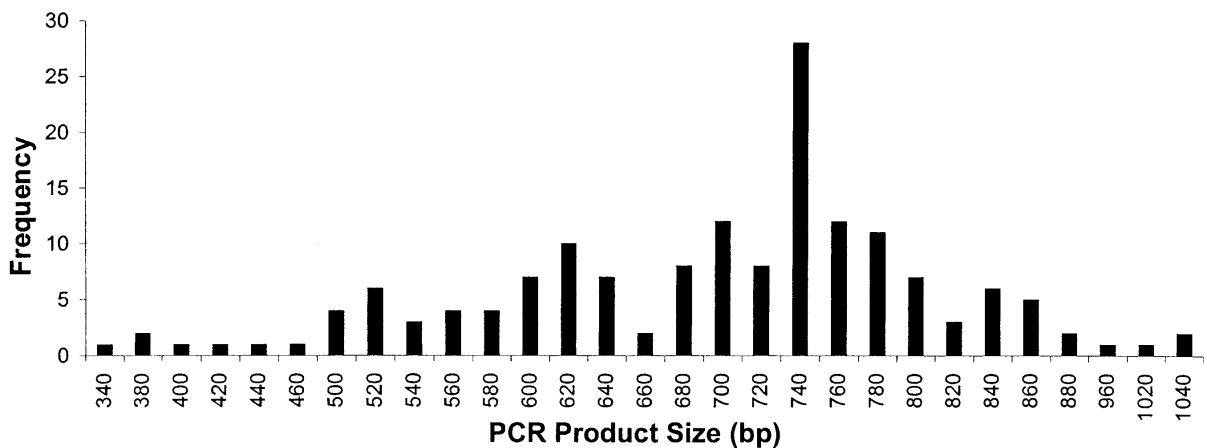


FIGURE 2. Parasite genetic diversity detected by *msp-1*, *msp-2*, and *glurp*. Polymerase chain reaction (PCR) products were categorized into molecular weight groups differing by 10 bp for *msp-1* and *msp-2* and by 20 bp for *glurp*. (A) *msp-1*. White bars represent K1; black bars, MAD-20; and checked bars, RO33 allelic families. The 123 K1 alleles ranged in size from 80 to 380 bp; the 35 MAD20 alleles, 80 to 350 bp; and the 55 RO33 alleles, 110 to 160 bp. (B) *msp-2*. White bars represent FC27 and black bars represent IC3D7 allelic families. The 81 FC27 alleles ranged in size from 280 to 590 bp, and the 130 IC3D7 alleles ranged in size from 170 to 510 bp. (C) *glurp*. The *glurp* alleles ranged in size from 340 to 1,040 bp.

**Population diversity.** To estimate the genetic diversity of *msp-1*, *msp-2*, and *glurp* in the parasite population, the frequency distribution of alleles was determined for the first episode of malaria of each study subject ( $n = 115$ ) (Figure 2). *msp-1* (45 alleles) and *msp-2* (57 alleles) revealed considerably greater parasite diversity than *glurp* (28 alleles). Based on a simple multinomial probability equation, the likelihood of 2 random infections having identical alleles was 2% for *msp-2*, 5% for *msp-1*, and 7% for *glurp*. The mean number of alleles per sample (complexity of infection) also was greater with *msp-1* (1.7) and *msp-2* (1.7) than with *glurp* (1.4). Because of the lesser degree of polymorphism detected by *glurp*, it was not considered further as a single genetic marker.

**Classification of indeterminate outcomes.** The percentage of indeterminate outcomes (in which a subsequent episode contained alleles present in the previous episode and new alleles) was 24% (93 of 386) for results based on *msp-1* alone, 16% (58 of 371) for results based on *msp-2* alone, and 62% (226 of 367) for results based on all 3 genes. To classify best outcomes initially considered indeterminate, parameter estimates (hazard ratios) for samples with only definitive outcomes (our reference standards) were compared with estimates obtained when indeterminate outcomes were included (Table 1). Three classification schemes for indeterminate outcomes were considered: (1) all indeterminate outcomes considered recrudescence, (2) indeterminate outcomes considered reinfection if  $\geq 50\%$  of the alleles in the subsequent sample were new (in this scheme, 90%, 91%, and 71% of episodes were classified as reinfections using results based on *msp-1*, *msp-2*, and all 3 genes), and (3) all indeterminate outcomes considered reinfections. Compared with our reference standards, parameter estimates changed considerably when indeterminate outcomes all were considered recrudescence (scheme 1). In contrast, parameter estimates were similar when all indeterminate outcomes were classified as reinfections (scheme 3) or most were classified as reinfections (scheme 2). Based on our prediction model, episodes with indeterminate outcomes were much more consistent with reinfection than recrudescence.

**Comparison of analyses using *msp-1*, *msp-2*, or all 3 genes.**

Reinfection survival functions were used to compare genotyping results based on either *msp-1* or *msp-2* alone, with indeterminate outcomes classified by scheme 2, as detailed earlier. Outcomes were concordant (classified as either reinfection or recrudescence by *msp-1* and *msp-2* analyses) in 79% (294 of 370) and discordant in 21% (76 of 370) of comparisons. Reinfection rates were not associated with treatment group or pretreatment parasite densities (data not shown). We assumed that reinfection survival functions should be similar whether results were concordant or discordant. The survival functions based on *msp-2* results fit this assumption (Figure 3). In contrast, survival functions based on *msp-1* results showed significant differences between concordant and discordant outcomes, suggesting a less accurate estimate of true outcomes. For the same comparison between *msp-2* and all 3 genes, outcomes were concordant in 89% (327 of 367) and discordant in only 11% (40 of 367) of comparisons, and survival functions for concordant and discordant data were not significantly different (Figure 3).

**Application of genotyping to clinical results.** The risk of recrudescence is the primary outcome of interest for antimalarial drug efficacy studies. Using survival analysis techniques

with the follow-up period extended to 42 days, we evaluated how using different genes and different methods of classifying indeterminate outcomes affected the calculated risk of recrudescence in our clinical study (Table 2). When comparing different classification schemes for indeterminate outcomes, scheme 2 provided the most robust results across the different genes (*msp-1* alone, *msp-2* alone, and all 3 genes combined). Compared with results based on *msp-1* or *msp-2* alone, the use of 3 genes combined showed the greatest variation in results across different indeterminate outcome classification schemes because of the larger number of indeterminate outcomes.

## DISCUSSION

Molecular genotyping is a simple and practical tool to distinguish between recrudescence and reinfection in antimalarial drug efficacy studies. Genotyping is particularly relevant in high transmission areas<sup>13</sup> and when follow-up is  $>14$  days.<sup>5,6,14-17</sup> Although genotyping analyses have been reported in many trials, the methodology and interpretation of results have not been standardized. In particular, the choice and number of genetic markers and the definitions used to classify outcomes as recrudescence or reinfection have not been consistent.

We have used results from a longitudinal study of antimalarial drug efficacy to compare methodologies critically and offer a standardized genotyping approach. In this study, we had a large sample size, extended follow-up covering multiple episodes of disease, and 3 different treatment groups covering a wide range of risk for recrudescence. We found that polymorphisms in either *msp-1* or *msp-2* were sufficiently diverse for us to distinguish adequately recrudescence from reinfection in our study population. In a direct comparison of analyses based on *msp-1* and *msp-2* polymorphisms, *msp-2* provided a more accurate measure of treatment outcomes. Analyses considering polymorphisms in *glurp* and all 3 genes combined provided no additional utility. We propose a standard approach in which the analysis of *msp-2* polymorphisms is used to distinguish recrudescence from reinfection.

TABLE 1  
Comparison of indeterminate outcome classification schemes

Gene	Model*	Hazard ratios for independent predictors <sup>†</sup>		
		Treatment with SP <sup>‡</sup>	Treatment with SP/AS <sup>‡</sup>	Log parasite density
<i>msp-1</i>	Reference standard	7.0	3.3	1.23
	Scheme 1	3.8	2.0	1.17
	Scheme 2	7.0	3.1	1.23
	Scheme 3	6.7	3.3	1.24
<i>msp-2</i>	Reference standard	6.7	2.9	1.31
	Scheme 1	3.9	1.8	1.18
	Scheme 2	6.8	2.8	1.31
	Scheme 3	7.0	3.0	1.33
All 3 genes	Reference standard	10.5	4.1	1.32
	Scheme 1	2.4	1.4	1.16
	Scheme 2	6.7	2.6	1.24
	Scheme 3	12.0	4.2	1.33

SP, sulfadoxine/pyrimethamine; AS, artesunate.

\* Reference standard includes only outcomes clearly considered recrudescence or reinfection. Other models include indeterminate outcomes based on different classification schemes. Scheme 1: All indeterminate outcomes considered recrudescence. Scheme 2: Indeterminate outcomes considered reinfection if half or more of the alleles in the subsequent sample were new. Scheme 3: All indeterminate outcomes considered reinfection.

<sup>†</sup> All  $P < 0.03$ .

<sup>‡</sup> Compared with baseline of treatment with SP/AQ (amodiaquine).

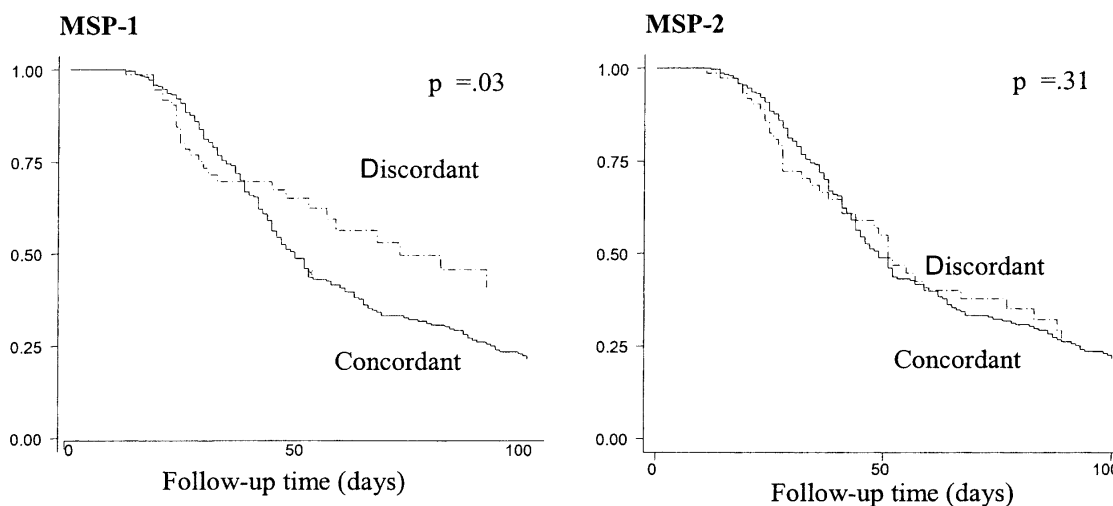
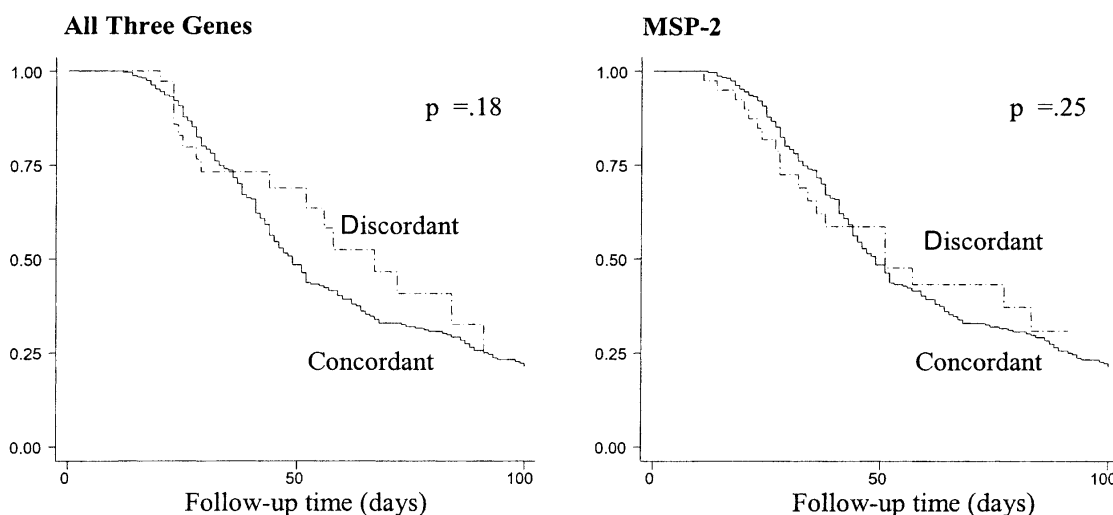
**A. MSP-1 versus MSP-2****B. All Three Genes versus MSP-2**

FIGURE 3. Comparison of reinfection survival functions for concordant and discordant genotyping outcomes. Survival analysis techniques were used to compare the accuracy of results based on *mSP-1* and *mSP-2* (A) and *mSP-2* and all 3 genes combined (B). For each comparison pair, the risk of reinfection over 100 days of follow-up based on discordant outcomes (dotted lines) was compared with the same standard based on concordant results (solid lines).  $P < 0.05$  represents a significant difference in the 2 reinfection survival functions compared. It is assumed that reinfection rates should be the same for concordant and discordant outcomes. Compared with *mSP-1*, *mSP-2* analysis seems to offer a more accurate representation of outcomes, and there was no significant difference between analyses based on *mSP-2* and all 3 genes.

Our genotyping results agreed with expected outcomes. Most recrudescences occurred within 28 days after treatment. The risk of recrudescence was highest in patients treated with SP monotherapy, the regimen that also was the least effective based on clinical and parasitologic criteria. Most reinfections occurred >28 days after treatment. The rate of reinfection was relatively constant after 42 days, when levels of all study drugs were expected to be low.<sup>18</sup>

Other investigators have suggested that in areas of high transmission, evaluation of recrudescence is achieved best by detailed analysis of a single, highly polymorphic locus.<sup>19</sup> It also has been argued that the use of multiple markers may enhance the detection of diversity and decrease the chance of misclassifying new infections as recrudescence.<sup>3</sup> In this study,

we detected considerable parasite diversity with analyses of either *mSP-1* or *mSP-2*, resulting in an acceptably low probability of outcome misclassification. When results from all 3 genes combined were used, the level of diversity increased but at the cost of a considerably larger proportion of outcomes being classified as indeterminate.

It was important to determine how best to classify outcomes initially classified as indeterminate. In these cases, patients developed recurrent malaria, and infecting parasites contained alleles present in the prior episode and new alleles. In our sample, the proportion of outcomes fitting this category was 24% using *mSP-1*, 16% using *mSP-2*, and 62% using all 3 genes. Approaches considered in previous studies have included omitting indeterminate outcomes from the analy-

TABLE 2  
Risk of recrudescence after 42 days of follow-up among treatment groups

Treatment group	<i>m</i> sp-1			<i>m</i> sp-2			All 3 genes		
	Scheme 1	Scheme 2	Scheme 3	Scheme 1	Scheme 2	Scheme 3	Scheme 1	Scheme 2	Scheme 3
SP	47%	40%	38%	47%	39%	38%	52%	42%	29%
SP/AQ	12%	5%	5%	10%	6%	5%	20%	6%	3%
SP/AS	30%	22%	22%	25%	21%	20%	37%	20%	14%

Note. Schemes as defined in Table 1.  
SP, sulfadoxine/pyrimethamine; AQ, amodiaquine; AS, artesunate.

sis,<sup>20,21</sup> considering them all as either recrudescences or reinfections,<sup>17,22,23</sup> or other more complex methods.<sup>13,15</sup> In many studies, it is unclear what method was used. In this study, we compared 3 different classification schemes, using as the reference standard the subset of outcomes that were definitive (either recrudescence, when subsequent isolates contained only alleles present in the prior episode, or reinfection, when a subsequent isolate contained only new alleles). Our analysis showed that the episodes initially classified as indeterminate were much more likely to be caused by reinfection than by recrudescence. Based on this result, we hypothesize that if residual and new parasite strains cocirculate, clinical symptoms are more likely to be caused by the new strain. This hypothesis is consistent with data from Gabon, in which the time to reappearance of parasites was similar in distribution when subsequent episodes contained only new alleles or when subsequent episodes contained new and old alleles.<sup>23</sup>

PCR-based genotyping is potentially limited by the sensitivity of the technique for detecting all allelic variants present in an infection.<sup>3</sup> This can be especially problematic with low-level parasitemia or when one strain is a minor component of a multistrain infection.<sup>24</sup> The problem of sensitivity for multiple alleles is relatively unimportant in drug efficacy studies, however, in which strains responsible for clinical illness are likely to be dominant and detected by PCR. Consistent with this conclusion, in our study 92% of our samples had a parasite density  $\geq 2,000$  asexual parasites/ $\mu$ l (96%  $\geq 500$  asexual parasites/ $\mu$ l), and geometric mean parasite density was not associated with the number of strains detected ( $P = 0.23$ ).

The presence of gametocytes in blood samples can confound genotyping analyses.<sup>3</sup> Outcomes can be misclassified as recrudescence if asexual parasites are cleared but gametocytes originating from the primary episode are detected at the time that a subsequent episode is diagnosed. Although the presence of gametocytes in subsequent samples was associated with recrudescence ( $P = 0.001$ ), gametocytes occurred in only 16 of 371 paired samples (11 of these in the group treated with SP alone), suggesting that confounding as a result of gametocytemia was not a significant problem in our study.

Genotyping is an important tool for antimalarial drug efficacy studies, but its value has been limited by inconsistent methodology. We have shown that the results of our clinical trial varied considerably depending on the choice of genes for analysis and the interpretation of genotyping results. We have shown that in this high-transmission area, a single, highly polymorphic genetic marker adequately distinguished between recrudescence and reinfection. We support the analysis of *m*sp-2 polymorphisms because this genetic marker provided more accurate results compared with *m*sp-1 and equiva-

lent results compared with all 3 genes combined. More studies are needed, however, to determine if *m*sp-2 or another marker is the appropriate choice in other high-transmission areas. In low-endemicity regions, multiple genetic markers may be needed to detect sufficient parasite diversity. In addition, we recommend that when recurrent isolates contain old and new alleles, outcomes should be classified as reinfection. Our hope is that additional studies will allow the development of a simple, standardized approach to genotyping analyses in endemic areas of Africa. This approach should allow for more accurate estimation of treatment failure risk and improve comparability among studies.

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