Estimating True Antimalarial Efficacy by Heteroduplex Tracking Assay in Patients with Complex *Plasmodium falciparum* Infections[▽]

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Heteroduplex tracking assays (HTAs) of *Plasmodium falciparum* merozoite surface protein 1 block-2 were used to assess complexity of infection and treatment efficacy in a trial of three antimalarial treatments in 141 Malawian pregnant women. An elevated complexity of infection (COI) was associated with anemia, parasite burden, and human immunodeficiency virus infection but was not associated with age or gravidity. Comparisons of HTA patterns before and after treatment allowed the classification of 20 of 30 (66%) recurrent episodes as either definite treatment failures or reinfections. An elevated COI was strongly associated with treatment failure ($P = 0.003$). An algorithm was developed to assign a probability of failure for the 10 indeterminate participants, some of whose infections shared a single variant of high prevalence ($>10\%$). By summing these probabilities, treatment efficacy was estimated.

More than 500 million people suffer from *Plasmodium falciparum* malaria each year. The majority of all malaria infections occur in sub-Saharan Africa, where there is often intense year-round exposure to infected mosquitoes (20). Probably as a result of frequent infectious bites, malaria infections in sub-Saharan Africa are usually polyclonal (2, 3, 13). Individual patients have been infected with as many as 14 genetically distinct parasite subpopulations or "variants" (3, 6, 11). The complexity of infection (COI), which is the number of variants in an individual host, has been measured by a variety of molecular methods. These methods include nested PCR, restriction fragment length polymorphism, microsatellite analysis, pyrosequencing, and PCR with a fluorescence primer followed by capillary electrophoresis (7, 14, 19, 21).

Using these molecular tools, a higher COI has been associated with symptomatic malaria manifestation in pregnant women (3). In a recent study, the risk of treatment failure in children was positively correlated with the COI (13).

The current World Health Organization protocol recommends that all clinical efficacy studies with more than 14 days of follow-up use PCR-based methods to determine whether recurrent infection is due to drug failure (recrudescence) or a newly acquired infection (reinfection) (25). The inherent limitations of conventional nested PCR genotyping, such as sensitivity only to size differences, and different interpretations of the PCR comparisons can both have a dramatic impact on the treatment failure rate (18). Therefore, more definitive methods are needed.

We have previously used the heteroduplex tracking assay (HTA) in an area of low malaria transmission to characterize the COI in Southeast Asia (15, 26). In the HTA, radiolabeled *P. falciparum* merozoite surface protein 1 (PfMSP1) probe is annealed to amplicons from a patient whose PfMSP1 block-2 region has undergone a single round of PCR amplification. Double-stranded complexes are formed between the probe and host amplicon(s); these complexes, termed heteroduplexes, migrate on a nondenaturing gel at various speeds based on the complementarity of the patient-probe complexes. Clustered base-pair mismatches, insertions, and deletions all change the migration speed of the heteroduplexes and, after electrophoresis on a nondenaturing gel, the host PfMSP1 sequence variants can be quantified. Because they are simple to perform, sensitive to size and clustered sequence polymorphisms, and allow quantification of each sequence variant, HTAs are commonly used by virologists to characterize polyclonal human immunodeficiency virus (HIV) infections (5, 10). We report here the first use of the HTA to characterize *P. falciparum* sequence diversity in an area of high malaria transmission and discuss a new approach to determine the rate of treatment failure.

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MATERIALS AND METHODS

Patient samples and information. A full description of the study describing the samples used in this report is in preparation (L. Kalilani et al., unpublished data). Briefly, 141 pregnant women with slide-confirmed *P. falciparum* infections and estimated gestational ages of 14 to 26 weeks were recruited into a randomized, open-label study conducted at the Mpemba and Madziabango Health Centers in Blantyre District, Malawi. Women were excluded from the study if they met any of the following conditions: multiple gestation; history of tuberculosis or diabetes; mental disorder; known allergies to sulfonamides, macrolides, or pyrimethamine; and pregnancy complications at enrollment or if they had taken antimalarial drugs 28 days prior to enrollment. Women received directly observed treatments with either standard doses of sulfadoxine-pyrimethamine (SP; 1,500 mg of sulfadoxine and 75 mg of pyrimethamine), SP plus azithromycin (1 g/day for 2 days), or SP plus artesunate (200 mg/day for 3 days). Women returned to the clinic at least 28 days later for routine antenatal care, which included obtaining the body temperature, a malaria smear, and a second dose of the study treatment. After each treatment, thick smears were prepared on days 1, 2, 3, 7, and 14. Women with fever who returned for unscheduled visits were tested for

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malaria; women with parasites between days 7 and 28 posttreatment were given quinine (600 mg, three times/day, for 5 days).

Malaria was diagnosed by microscopic inspection of Giemsa-stained thick blood films. The total number of parasites detected per 200 leukocytes was multiplied by 30 to estimate the parasite count per microliter of blood (assuming a leukocyte count of 6,000 leukocytes/ml of blood). A thick film was considered negative if no parasites were detected after examining 100 microscopic fields. Parasite density was dichotomized at the median value (930 parasites/µl) into categories of high and low parasite density.

Women were offered counseling and testing for HIV and nevirapine according to the HIVNET-012 protocol if they were HIV positive. HIV testing was performed by using the Determine HIV-1/2 rapid test (Abbott Laboratories) and Unigold Test (Trinity Biotech, Dublin, Ireland). Discordant results were resolved with the Hemastrip Rapid Test (Saliva Diagnostic Systems).

The hemoglobin concentration was measured by using a HemoCue machine (HemoCue, Inc., Angelholm, Sweden). Women were considered anemic if their hemoglobin concentration was <11 g/dl.

DNA isolation. Venous blood was spotted onto filter paper and stored under desiccation. DNA was extracted from the filter paper by using the QIAMP DNA minikit (QIAGEN, Valencia, CA) according to the manufacturer's instructions.

PCR. Block-2 of PfMSP1 (22) was amplified with a Peltier thermal cycler (MJ Research, Waltham, MA) according to the protocol of Ngrenngarmlert et al. (15) using the primers C1F (5′-GAAGATGCAGTATTGACAGG-3′) and C3R (5′-TGATTGGTTAAATCAAAGAG-3′) (8); the C1F primer starts at nucleotide 106 in the 3D7 MSP1 gene (9). PCR products were run on a 1% agarose gel, stained with ethidium bromide, visualized by UV light, and sized against a 100-bp molecular weight marker (Promega, Madison, WI). Successful amplification resulted in a product of approximately 400 bp. All PCRs were done in duplicate.

Plasmids and probes. The COI was determined using a single, heterologously expressed PfMSP1 block-2 probe (MHP [described in reference 15]). Ten micrograms of the MHP-pT7Blue construct was digested with BamHI at 37°C for 1 h and then end labeled in the same buffer by filling in the BamHI overhang for15 min at 25°C (1× BamHI buffer [New England Biolabs], 10 mM dithiothreitol, 50 µM dGTP, 50 µCi of [α-^{35}S]dATP [1,250 Ci/mmol; ICN], 10 U of Klenow fragment). The reaction was stopped by the addition of EDTA (1 mM final concentration) and heat inactivation at 75°C for 15 min. The radiolabeled probe was released from the vector by digestion with PstI at 37°C for 1 h, separated on an agarose gel, excised, and gel purified with the QIAquick gel extraction kit (QIAGEN).

Heteroduplex tracking assay. A total of 8 µl of PCR-amplified block-2 of PfMSP1 from patient samples was mixed with 1.2 µl of 10× annealing buffer (1 M NaCl, 100 mM Tris-HCl [pH 7.5], 20 mM EDTA), 0.1 µM C1F primer, 0.1 µM C3R primer, 1 µl of ^{35}S-labeled probe, and 2 µl of 6× loading dye (15% Ficoll, 0.25% bromophenol blue, 0.25% xylene xylanol [Sigma, St. Louis, MO]) in a total volume of 12 µl. The mixture was denatured at 95°C for 2 min and allowed to reanneal at 25°C for 5 min. Heteroduplexes were electrophoresed on a 6% polyacrylamide gel (acrylamide-bisacrylamide, 37.5:1) in 1× Tris-borate-EDTA buffer at 17 mA per gel for 5.5 h. The gels were dried onto filter paper (Whatman) and exposed to BioMax MR X-ray film (Kodak, Rochester, NY). For quantitative data, the dried gels were exposed to a phosphorimager screen for 7 days, and the band intensities were determined by quantifying the area under the curve according to the manufacturer's instructions (Molecular Dynamics). HTA bands were considered unique PfMSP1 sequence variants if they were not in the probe alone lane and they were present in both PCR replicates. The relative migration distance (R_f) was calculated for each variant by dividing the distance migrated by the variant by the distance migrated by the probe homoduplex. To test the precision of the R_f measurement, a single variant was run on 16 separate gels and the R_f was calculated. The cloned variant had a coefficient of variation of 14% (the average R_f was 0.16, and the standard deviation was 0.022).

Colony PCR. The PfMSP1 amplicon from participant 1/019 was cloned into the pCR2.1TOPO by using the Invitrogen TOPO TA cloning kit and transformed into *Escherichia coli* (Invitrogen Corp., Carlsbad, CA). Then, 52 colonies were selected and PCR amplified using the primers and thermal cycler settings described above. PCR products from representative variants were cleaned by using the QIAquick PCR purification kit (QIAGEN) and sequenced using the C1F primer at the University of North Carolina-Chapel Hill Genome Analysis Facility. DNA sequences were hand cleaned, aligned with CLUSTAL X (http://www2.ebi.ac.uk/clustalw) and displayed with GeneDoc (http://www.psc.edu/biomed/genedoc/).

Statistics and modeling. The association between the COI and clinical features was tested by using the Wilcoxon rank-sum test. The relationship between the COI and treatment failure was monotonic (data not shown), and therefore
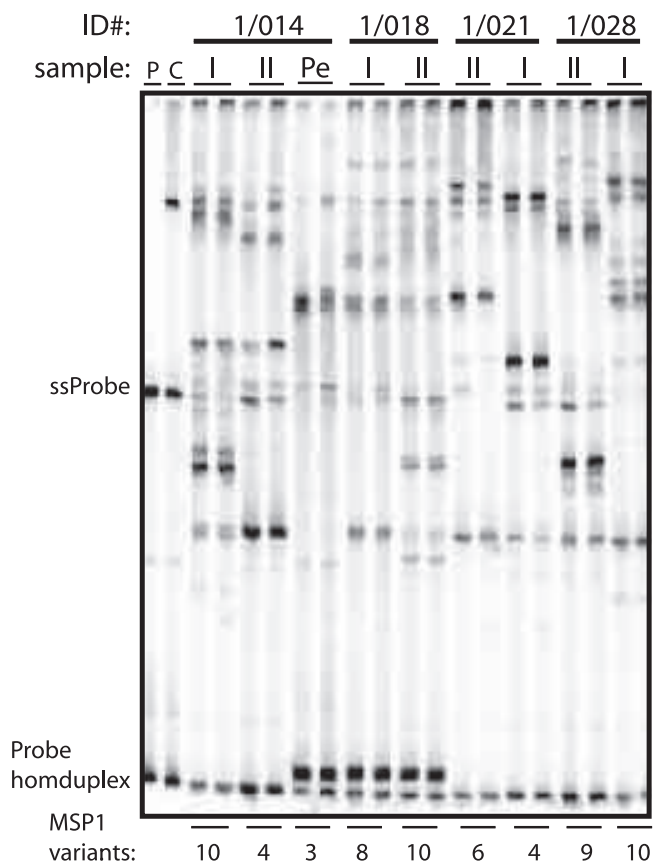

FIG. 1. Representative PfMSP1 HTA. The COI is shown for four women at enrollment (I), during their second visit (II), or during delivery (Pe). All samples were run in duplicate, with the number of unique PfMSP1 variants per sample listed at the bottom. ssProbe, single-stranded probe; P, probe alone; C, AF42 plasmid control.

COI was coded as a continuous variable in the multivariable regression. Primigravidity and parasite burden (dichotomized at the median) were used as covariates in the multivariable regression. All statistical analysis was done by using STATA v.8.2, (STATACorp, College Station, TX).

Ethical approval and consent. This study was approved by the College of Medicine Research Committee (University of Malawi, Blantyre, Malawi) and the Institutional Review Boards of the University of North Carolina (Chapel Hill, NC). Separate consent forms in the local language were administered for blood and/or data collection and for HIV testing.

GenBank accession numbers. Six MSP1 block-2 sequences from participant 1/019 are deposited in GenBank under accession numbers DQ855130 to DQ855135. The MHP probe is deposited under accession number DQ858166.

RESULTS

Clinical characteristics. We successfully PCR amplified the PfMSP1 block-2 in 125 of the 141 (88.7%) pregnant women enrolled. The average parasite density of the amplified samples was 1,815 parasites/µl (median = 930, range = 120 to 22,500). The parasite density of the samples that did not amplify (median = 705 parasites/µl) was similar to those that did amplify (P = 0.915 [Wilcoxon rank-sum test]). Of those with a positive PCR, the average age at enrolment was 21 years (median = 20, range = 15 to 39); 56% were primigravid, 72% were anemic, and 25 of the 80 (31%) women who accepted HIV testing were HIV infected.
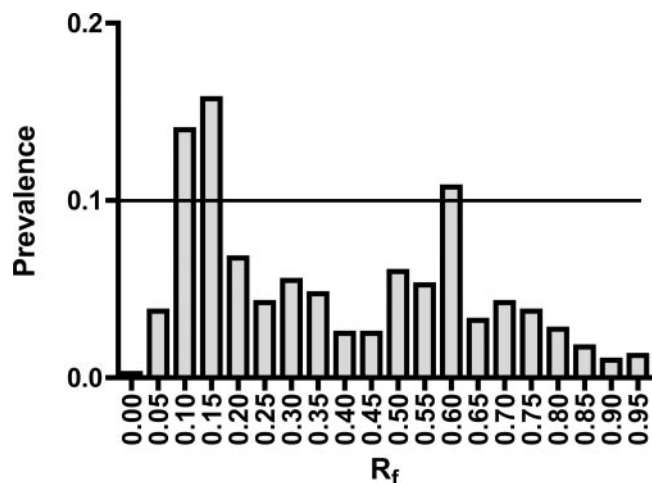
FIG. 2. Prevalence of PfMSP1 sequence variants at enrollment. The line indicates the cutoff point for abundant ("common") versus nonabundant variants.

PfMSP1 sequence variation. HTAs were run, in duplicate, on the 125 patients, and the numbers of variants were determined. A representative gel is shown in Fig. 1; sequence variants migrated both above and below the single-stranded probe. The participants had a mean COI of 3.82 (median = 3, range = 1 to 11). The distance migrated by each variant was measured, and the variants were divided into 20 bins of 0.05 R_f units. The prevalence of individual variants ranged from 0.03 to 15.75%, and three variants were present in >10% of the women (Fig. 2).

COI and clinical features. The relationship between the COI at enrollment (i.e., the first malaria episode) and clinical features was investigated. A small, positive association between the COI and the $\log_{10}$ parasite density was observed (correlation coefficient = 0.22, $P = 0.0117$). An increased COI was also associated with both anemia and HIV infection (Table 1). Women who failed the first treatment had a greater COI than those who did not fail (median = five versus three variants; $P = 0.0003$); the COI was similar among the three study arms ($P = 0.109$ [Kruskal-Wallis test]). After we adjusted for the parasite burden and primigravidity, we found that the COI remained independently associated with anemia, HIV status, and treatment failure (Table 1). A small association was observed between the COI and reinfection (median COI = 4 versus 3; $P = 0.106$), which weakened after we adjusted for parasite burden and primigravidity (Table 1).

Variant subpopulations within a single host. Using colony PCR as the reference standard, we assessed the ability of the HTA to accurately measure the proportion of each variant within a single host. The PfMSP1 amplicon from one participant with 10 variants was TA cloned, and 52 colonies were selected. The PfMSP1 block-2 from each of the colonies was PCR amplified and rescreened by the HTA. The proportion of colonies containing each variant (Fig. 3A, left panel) was compared to the phosphorimager-derived scan of the donor population (Fig. 3A, middle and right panels). There was a strong, positive correlation between phosphorimager intensity and the number of clones with the corresponding variant ($R^2 = 0.93$). Four HTA variants representing <3% of total population (variants 1, 3, 4, and 9) were not represented among the 52 colonies selected.

Next, 15 clones from the same participant were sequenced and all were identified as PfMSP1 block-2. Figure 3B shows the alignment of six representative MSP1 sequences; this alignment includes a variant that migrated rapidly through the gel, near the probe homoduplex (variant 10), four variants that migrated near the single-stranded probe (variants 5 to 8), and a variant that migrated very slowly (variant 2). The sequenced variants ranged from 307 to 391 nucleotides, and three of the variants were within 20 nucleotides of each other. Besides its sensitivity to size polymorphisms, the HTA was also sensitive to sequence polymorphisms and, as expected, the sequences that were most divergent from the probe migrated the farthest distance from the probe homoduplex (data not shown).

Classification of recurrences. One important potential use of HTA would be to distinguish new infections (reinfections) from treatment failures (recrudescences). Many reports use PCR to distinguish reinfection from treatment failure but do not specify the criteria used to distinguish each outcome. Often, if the initial and recurrent infections share any variant, then the recurrent infection is classified as a treatment failure (12, 17). However, in an area of high malaria transmission, such as Malawi, if specific PfMSP1 variants are frequent in the

TABLE 1. PfMSP1 diversity stratified by baseline features

Characteristic	No. of isolates (%) ($n = 125$)	No. of MSP1 variants (IQR)[a]	P[b]	Adjusted OR (95% CI)[c]
Subject age (yr)				
≤20	73 (58)	3 (2–4)	0.54	
>20	52 (42)	3 (2–5)		
Gravidity				
Primigravid	71 (57)	3 (2–5)	0.52	
Multigravid	54 (43)	3 (2–5)		
Parasitemia[d]				
Low	62 (50)	3 (2–5)	0.112	
High	63 (50)	3 (2–6)		
Anemia[e]				
No	35 (28)	3 (2–4)	0.052	Referent
Yes	90 (72)	3 (2–6)		1.21 (0.98–1.48)
HIV infection				
Negative	55 (69)	3 (2–4)	0.035	Referent
Positive	25 (31)	3 (3–6)		1.29 (1.01–1.65)
Treatment failure[f]				
No	104 (83)	3 (2–4)	0.0003	Referent
Yes	21 (17)	5 (4–8)		1.50 (1.08–2.08)
Reinfection[f]				
No	111 (89)	3 (2–5)	0.106	Referent
Yes	14 (11)	4 (3–6)		1.15 (0.87–1.53)

[a] IQR, interquartile range.
[b] As determined by the Wilcoxon rank-sum test.
[c] OR, odds ratio. The OR was adjusted for primigravidity and parasitemia (low or high). CI, confidence interval.
[d] That is, <930 parasites/μl of blood.
[e] That is, a hemoglobin level of <11.0 g/dl.
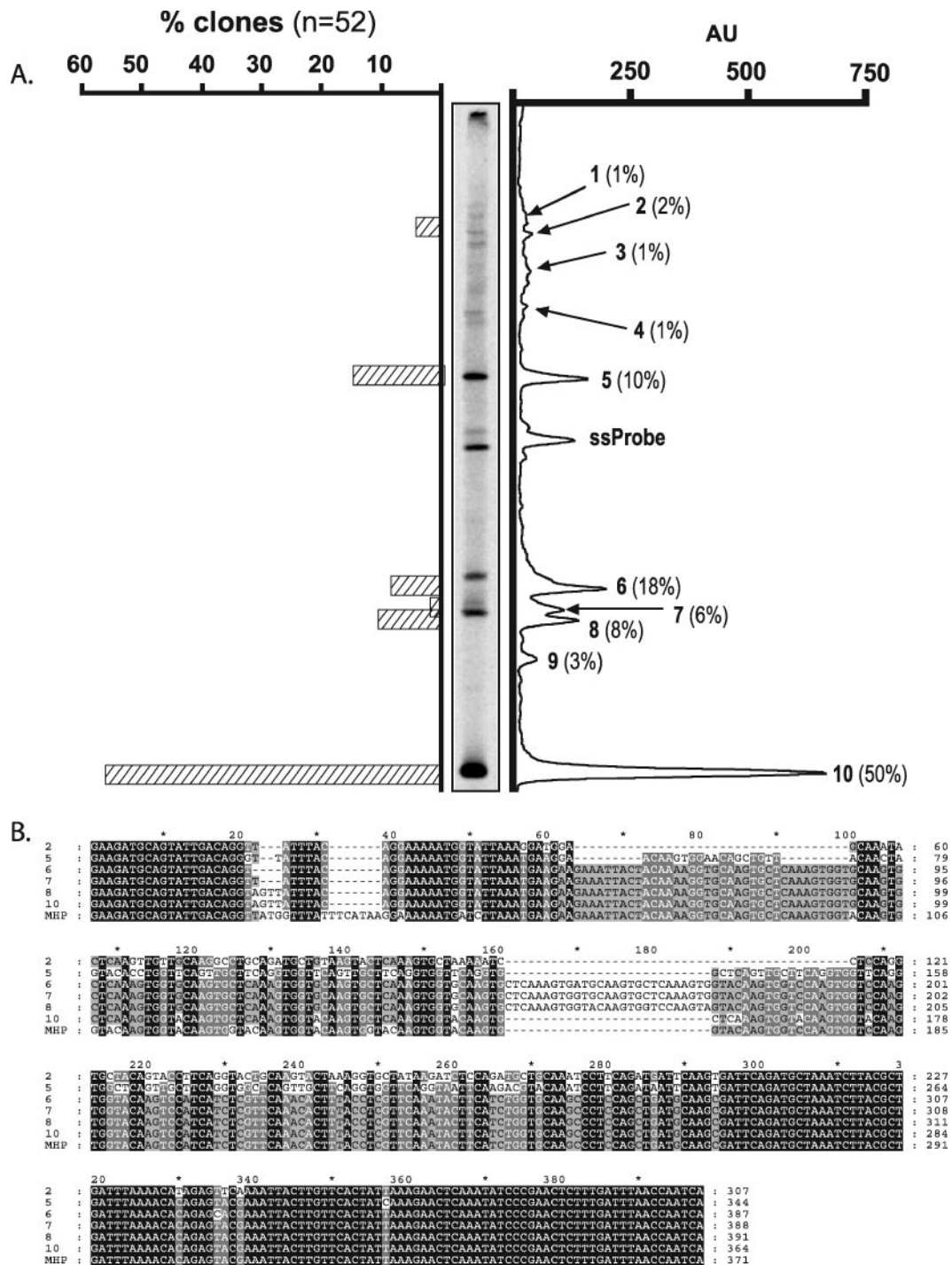[f] Failing first drug treatment only.

FIG. 3. Comparison of HTA and colony PCR. The PfMSP1 amplicon from patient 1/019 was TA cloned and subjected to colony PCR. Next, 52 colonies were picked and rescreened with the MHP HTA. (A) The relative abundance of each variant after colony PCR (left panel), autoradiography of the HTA complexes (middle panel), and the phosphorimager-quantified relative abundance of each of the 10 variants in the parent population (right panel) are shown. Variant numbers correspond to the sequences listed in panel B. The probe homoduplex is not shown but runs just below variant 10. (B) Pairwise alignment of six representative sequences isolated by colony PCR from patient 1/019.

parasite population, then a new infection containing one of these variants might be misclassified as a failure. In the present study, three variants were common, with prevalences between 11 and 16% (Fig. 2). Since the average infection comprised four variants, any recurrent infection had a 37 to 50% chance

of containing each of these variants coincidentally. To eliminate the possibility of misclassifying patients with these common variants as failures, we consider a second episode indeterminate if the initial and recurrent episodes only shared one band with a prevalence >10%.

TABLE 2. Characterization of PfMSP1 sequence variants among women with multiple *P. falciparum* infections[a]

Episode and patient	No. of variants			Diagnosis[b]
	Total	Shared (<10%)	Shared (>10%)	
Second episode				
1/004	2		1	IND
1/006	1			REI
1/008	4		1	IND
1/009	10	2	4	LTF
1/010	3	1	2	LTF
1/014	4	1	2	LTF
1/018				ETF
1/021				ETF
1/028	9		1	IND
1/030	4	4		LTF
1/034	6	3	1	LTF
1/041				ETF
1/050	4		1	IND
2/007	3		1	IND
2/008	4		3	LTF
2/011	6	2	3	LTF
2/012	6			REI
2/014				ETF
2/015	7		4	LTF
2/018	1		1	IND
2/019	4	1	3	LTF
2/020	3	1		LTF
2/022	5	2	2	LTF
2/025	1	1		LTF
2/027	2		1	IND
2/033	4	1	1	LTF
2/039	3		1	IND
2/040				ETF
2/041	6		1	IND
2/042	8	5		LTF
2/044	5		1	IND
2/047	3	1	2	LTF
2/054	2		2	LTF
2/058	2			REI
2/083	1			REI
Third episode				
1/006	1			REI
1/008	4		1	IND
1/009	11		1	IND
1/014	4		1	IND
1/030				ETF
2/012	5	1	1	LTF
2/014	5			REI
2/025				ETF
2/044	8	3	2	LTF
2/047	2		1	IND
2/058	2			REI

[a] Sequence variants in the second or third episode are shown.
[b] ETF, early treatment failure; LTF, PCR-corrected late treatment failure; REI, PCR-corrected reinfection; IND, indeterminate (PCR-corrected episode containing one variant present in >10% of all participants).

TABLE 3. Probability of reinfection in patients whose initial and recurrent episodes shared a single frequent variant (prevalence of >10%)

Treatment and patient	Total no. of bands	Shared band prevalence	Probability of reinfection
First treatment			
1/004	2	0.108	0.204
1/008	4	0.158	0.497
1/028	9	0.14	0.743
1/050	4	0.158	0.497
2/007	3	0.108	0.290
2/018	1	0.108	0.108
2/027	2	0.108	0.204
2/039	3	0.158	0.403
2/041	6	0.14	0.595
2/044	5	0.14	0.530
Second treatment			
1/008	4	0.140	0.453
1/009	11	0.108	0.716
1/014	4	0.140	0.453
2/047	2	0.158	0.291

with the first episode and were considered reinfections. The remaining 20 women had recurrent infections that both shared variants with the initial infection and contained new ones. For 10 of these participants, either the shared variant was uncommon (<10% prevalence) or two common variants were shared, and the second episode was classified as a late treatment failure. For the remaining 10 patients, the single shared variant was common (>10% prevalence), and the second episode was considered indeterminate.

Of the 35 women with second episodes, 11 experienced recurrences (third episodes) (Table 2). Two failed within 14 days and were classified as early treatment failures. Nine episodes occurred 14 to 28 days posttreatment; two of these were classified as late treatment failures, three were classified as reinfections, and four were classified as indeterminate by the criteria used for the second episodes.

Estimation of true failure rate. Thus, there were 10 second episodes and 4 third episodes that were classified as indeterminate. Fortunately, since we know the prevalence of the common bands and the number of variants in the recurrent episode, we can calculate a probability of reinfection by each variant. For the recurrent patient with x variants and sharing a single variant of prevalence y, the binomial probability (16) that this variant is found by chance in a recurrent infection is calculated as $1 - (1 - y)^x$ [and therefore the probability that it represents a failure is $(1 - y)^x$]. The probabilities of reinfection for the 14 indeterminate episodes are shown in Table 3. The mean probabilities of failure are 0.59 for the 10 indeterminate second episodes (or a predicted 5.93 patients) and 0.48 for the 4 indeterminate third episodes (or a predicted 1.91 patients). Thus, the estimated failure rates are (21 + 5.93)/125 or 21.5% for the first treatment and (4 + 1.91)/35 or 16.9% for the second treatment.

DISCUSSION

This is the first use of the PfMSP1 HTA to study COI in an area of high malaria transmission. In the present study, Mala-

Comparison of first and second malaria episodes. Table 2 summarizes the HTA patterns of 35 women with second malaria episodes. Five patients failed treatment within 14 days and were considered early treatment failures. Thirty patients had a second infection 14 to 56 days posttreatment. Six of these shared multiple and/or uncommon PfMSP1 variants with the first episode with no new variants and were considered late treatment failures. Four shared no variants

wian pregnant women carried a mean of 3.82 PfMSP1 sequence variants. Using colony PCR as the gold standard, we conclude that the HTAs can accurately measure the relative proportions of each PfMSP1 variant. Further, independent of parasite burden and primigravidity, the risk of treatment failure increased 23% with every unit increase in COI; this result is consistent with the findings of Lee et al. (13). To our knowledge, this is also the first report showing that HIV coinfection is associated with an elevated COI.

The HTA method has several advantages. First, it is more sensitive to minority variants than nested PCR and is less prone to cross-contamination (15). Second, as we demonstrate here, it is quantitative and capable of measuring variants representing as little as 1% of the parasite population in an individual host. Third, because it generates highly reproducible R_f values, one can determine the prevalence of individual variants in a given population. Fourth, the combination of HTA and colony PCR allows for the rapid cloning and sequencing of variants.

The major disadvantage of the HTA is that it requires the use of radioactive probes. However, once HTA methods are firmly established, related nonradioactive methods, such as the heteroduplex migration assay (HMA) can be developed (4, 24); whether the HMA can detect low-abundance variants as well as the HTA remains unknown. In addition, the HTA is also potentially limited in its resolution, in that multiple variants, with similar sequences, could run at the same position on the gel; these variants could only be detected by cloning and sequencing. A third disadvantage of the HTA is the possibility that observed variants are actually recombination artifacts. Because the probability of recombination increases with the size of the amplicon, the degree of recombination of a 400-bp PfMSP1 block-2 amplicon would most likely be less than the recombination observed by Tanabe et al. (23). However, this drawback holds for all PCR-based methods.

HTAs, combined with a binomial probabilistic approach, represent a new way to estimate drug efficacy. Alternative categorical methods for "PCR correction" suffer from several problems. First, since non-HTA methods may not be able to detect minority variants, sharing of such variants by enrollment and recurrent samples might be missed and failures misclassified as reinfections. Second, since specific variants may predominate in certain areas, reinfections, by these common variants, might be misclassified as failures. In contrast, HTAs can detect minority variants. Thus, inferring dichotomous outcomes (failure versus reinfection) for PCR correction methods can be misleading. However, by calculating the prevalence of each variant in a population, we can determine the probability that each individual patient originally scored as indeterminate failed and, by summation, the estimated overall failure rate.

There are several limitations to this method. First, if a treated patient were reinfected but bore gametocytes from his initial infection, then this patient might be misclassified as a failure (25). This is a problem facing all genotyping correction methods. A second potential limitation of the binomial method, which considers the prevalence of the recurrent variants, is the assumption that the variants are transmitted independently (i.e., a single mosquito bite only contains a single parasite variant). Although this assumption has not been directly assessed in Malawi, in 2003 in the Republic of Guinea

Bissau Arez et al. (1) found single PfMSP1/MSP2/GLURP alleles in ca. 50% of the *P. falciparum*-infected mosquitoes. A third potential limitation is the choice of bin size. Although it is possible that different variants may be counted within the same bin and smaller bin sizes might be preferable, in our study we observed only one individual out of 125 for whom this may have happened. A fourth limitation is that this method does not lend itself to survival analysis, a method commonly used in drug efficacy studies.

In summary, by HTAs the COI was found to be elevated in Malawian women with HIV and in those who failed therapy. Assigning a probability of failure to specific recurrent episodes mitigates misclassification for clinical trials in Africa and other high transmission areas. The method, developed here, will now be used to compare the efficacy of SP, SP plus artesunate, and SP plus azithromycin within this study population.

ACKNOWLEDGMENTS

REFERENCES

1. **Arez, A., J. Pinto, K. Palsson, G. Snounou, T. Jaenson, and V. do Rosario.** 2003. Transmission of mixed *Plasmodium* species and *Plasmodium falciparum* genotypes. Am. J. Trop. Med. Hyg. **68:**161–168.
2. **Babiker, H., L. Ranford-Cartwright, and D. Walliker.** 1999. Genetic structure and dynamics of *Plasmodium falciparum* infections in the Kilombero region of Tanzania. Trans R. Soc. Trop. Med. Hyg. **93:**(Suppl. 1)11–14.
3. **Beck, S., F. P. Mockenhaupt, U. Bienzle, T. A. Eggelte, W. N. Thompson, and K. Stark.** 2001. Multiplicity of *Plasmodium falciparum* infection in pregnancy. Am. J. Trop. Med. Hyg. **65:**631–636.
4. **Delwart, E., E. Shpaer, J. Louwagie, F. McCutchan, M. Grez, H. Rubsamen-Waigmann, and J. Mullins.** 1993. Genetic relationships determined by a DNA heteroduplex mobility assay: analysis of HIV-1 *env* genes. Science **262:**1257–1261.
5. **Delwart, E. L., H. W. Sheppard, B. D. Walker, J. Goudsmit, and J. I. Mullins.** 1994. Human immunodeficiency virus type 1 evolution in vivo tracked by DNA heteroduplex mobility assays. J. Virol. **68:**6672–6683.
6. **Farnert, A., K. Tengstam, I. B. Palme, U. Bronner, M. Lebbad, G. Swedberg, and A. Bjorkman.** 2002. Polyclonal *Plasmodium falciparum* malaria in travelers and selection of antifolate mutations after proguanil prophylaxis. Am. J. Trop. Med. Hyg. **66:**487–491.
7. **Jafari, S., J. Le Bras, O. Bouchaud, and R. Durand.** 2004. *Plasmodium falciparum* clonal population dynamics during malaria treatment. J. Infect. Dis. **189:**195–203.
8. **Kimura, M., O. Kaneko, A. Inoue, A. Ishii, and K. Tanabe.** 1995. Amplification by polymerase chain reaction of *Plasmodium falciparum* DNA from Giemsa-stained thin blood smears. Mol. Biochem. Parasitol. **70:**193–197.
9. **Kissinger, J., B. Brunk, J. Crabtree, M. Fraunholz, B. Gajria, A. Milgram, D. Pearson, J. Schug, A. Bahl, S. Diskin, H. Ginsburg, G. Grant, D. Gupta, P. Labo, L. Li, M. Mailman, S. McWeeney, P. Whetzel, C. Stoeckert, and D. Roos.** 2002. The *Plasmodium* genome database. Nature **419:**490–492.
10. **Kitrinos, K. M., N. G. Hoffman, J. A. Nelson, and R. Swanstrom.** 2003. Turnover of *env* variable region 1 and 2 genotypes in subjects with late-stage human immunodeficiency virus type 1 infection. J. Virol. **77:**6811–6822.
11. **Kobbe, R., R. Neuhoff, F. Marks, S. Adjei, I. Langefeld, C. von Reden, O. Adjei, C. G. Meyer, and J. May.** 2006. Seasonal variation and high multiplicity of first *Plasmodium falciparum* infections in children from a holoendemic area in Ghana, West Africa. Trop. Med. Int. Health **11:**613–619.
12. **Kyabayinze, D., A. Cattamanchi, M. Kamya, P. Rosenthal, and G. Dorsey.** 2003. Validation of a simplified method for using molecular markers to predict sulfadoxine-pyrimethamine treatment failure in African children with falciparum malaria. Am. J. Trop. Med. Hyg. **69:**247–252.
13. **Lee, S., A. Yeka, S. Nsobya, C. Dokomajilar, P. Rosenthal, A. Talisuna, and G. Dorsey.** 2006. Complexity of *Plasmodium falciparum* infections and antimalarial drug efficacy at 7 sites in Uganda. J. Infect. Dis. **193:**1160–1163.
14. **Mwangi, J. M., S. A. Omar, and L. C. Ranford-Cartwright.** 2006. Comparison of microsatellite and antigen-coding loci for differentiating recrudescing *Plasmodium falciparum* infections from reinfections in Kenya. Int. J. Parasitol. **36:**329–336.
15. **Ngrenngarmlert, W., J. J. Kwiek, D. D. Kamwendo, K. Ritola, R. Swanstrom,**

C. Wongsrichanalai, R. S. Miller, W. Ittarat, and S. R. Meshnick. 2005. Measuring allelic heterogeneity in *Plasmodium falciparum* by a heteroduplex tracking assay. Am. J. Trop. Med. Hyg. **72:**694–701.

16. Norman, G. R., and D. L. Streiner. 2000. Biostatistics: the bare essentials, 2nd ed. B. C. Decker, New York, NY.

17. Nyachieo, A., C. VAN Overmeir, T. Laurent, J. Dujardin, and U. D'Alessandro. 2005. *Plasmodium falciparum* genotyping by microsatellites as a method to distinguish between recrudescent and new infections. Am. J. Trop. Med. Hyg. **73:**210–213.

18. Slater, M., M. Kiggundu, C. Dokomajilar, M. R. Kamya, N. Bakyaita, A. Talisuna, P. J. Rosenthal, and G. Dorsey. 2005. Distinguishing recrudescences from new infections in antimalarial clinical trials: major impact of interpretation of genotyping results on estimates of drug efficacy. Am. J. Trop. Med. Hyg. **73:**256–262.

19. Snounou, G., and H. P. Beck. 1998. The use of PCR genotyping in the assessment of recrudescence or reinfection after antimalarial drug treatment. Parasitol. Today **14:**462–467.

20. Snow, R. W., C. A. Guerra, A. M. Noor, H. Y. Myint, and S. I. Hay. 2005. The global distribution of clinical episodes of *Plasmodium falciparum* malaria. Nature **434:**214–217.

21. Takala, S. L., D. L. Smith, O. C. Stine, D. Coulibaly, M. A. Thera, O. K. Doumbo, and C. V. Plowe. 2006. A high-throughput method for quantifying alleles and haplotypes of the malaria vaccine candidate *Plasmodium falciparum* merozoite surface protein-1 19 kDa. Malaria J. **5:**31.

22. Tanabe, K., M. Mackay, M. Goman, and J. G. Scaife. 1987. Allelic dimorphism in a surface antigen gene of the malaria parasite *Plasmodium falciparum*. J. Mol. Biol. **195:**273–287.

23. Tanabe, K., N. Sakihama, A. Farnert, I. Rooth, A. Bjorkman, D. Walliker, and L. Ranford-Cartwright. 2002. In vitro recombination during PCR of *Plasmodium falciparum* DNA: a potential pitfall in molecular population genetic analysis. Mol. Biochem. Parasitol. **122:**211–216.

24. Upchurch, D. A., R. Shankarappa, and J. I. Mullins. 2000. Position and degree of mismatches and the mobility of DNA heteroduplexes. Nucleic Acids Res. **28:**E69.

25. World Health Organization. 2005. Susceptibility of *Plasmodium falciparum* to antimalarial drugs: report on global monitoring: 1996–2004. World Health Organization, Geneva, Switzerland.

26. Yeramian, P., S. R. Meshnick, S. Krudsood, K. Chalermrut, U. Silachamroon, N. Tangpukdee, J. Allen, R. Brun, J. J. Kwiek, R. Tidwell, and S. Looareesuwan. 2005. Efficacy of DB289 in Thai patients with *Plasmodium vivax* or acute, uncomplicated *Plasmodium falciparum* infections. J. Infect. Dis. **192:**319–322.