

VALIDATION OF MICROSATELLITE MARKERS FOR USE IN GENOTYPING POLYCLONAL *PLASMODIUM FALCIPARUM* INFECTIONS

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Abstract. Genotyping methods for *Plasmodium falciparum* drug efficacy trials have not been standardized and may fail to accurately distinguish recrudescence from new infection, especially in high transmission areas where polyclonal infections are common. We developed a simple method for genotyping using previously identified microsatellites and capillary electrophoresis, validated this method using mixtures of laboratory clones, and applied the method to field samples. Two microsatellite markers produced accurate results for single-clone but not polyclonal samples. Four other microsatellite markers were as sensitive as, and more specific than, commonly used genotyping techniques based on merozoite surface proteins 1 and 2. When applied to samples from 15 patients in Burkina Faso with recurrent parasitemia after treatment with sulphadoxine-pyrimethamine, the addition of these four microsatellite markers to *msp1* and *msp2* genotyping resulted in a reclassification of outcomes that strengthened the association between *dhfr* 59R, an anti-folate resistance mutation, and recrudescence ($P = 0.31$ versus $P = 0.03$). Four microsatellite markers performed well on polyclonal samples and may provide a valuable addition to genotyping for clinical drug efficacy studies in high transmission areas.

INTRODUCTION

Clinical trials of anti-malarial drug efficacy against *Plasmodium falciparum* often require genotyping techniques to differentiate whether recurrent parasitemia after therapy is caused by recrudescence of the original infection or to newly infecting parasites. The distinction is important, because recrudescence implies failure of the drug treatment, whereas a new infection does not. Diversity within the *P. falciparum* genome enables useful comparison of genotypes from pre-treatment samples with samples collected at the time of recurrent parasitemia. If paired samples have the same genotypes, the treatment outcome is classified as a recrudescence. If they have different genotypes, the outcome is classified as a new infection. Different methods have been used to classify outcomes when paired samples have both the same and different alleles. No standardized approach to genotyping exists, and different strategies may have a large effect on the results of efficacy trials.¹

The most commonly used genotyping method involves amplifying family-specific alleles of the polymorphic genes merozoite surface protein 1 (*msp1*) and merozoite surface protein 2 (*msp2*) using nested polymerase chain reaction (PCR) and gel electrophoresis.² Interpretation of agarose gels has been shown to vary significantly even when performed repeatedly by the same individual.³ When pretreatment and recurrent parasitemia samples are compared with each other, genotypes of two unrelated strains may appear the same by chance alone, leading to the misclassification of a new infection as a recrudescence. Such misclassification is dependent on the number of molecular markers used in genotyping, the population diversity of the molecular markers, and the number of strains present in an individual infection. Misclassification may also occur when minority strains are not detected or when false-positive alleles are detected. Compared with low

transmission areas such as Southeast Asia, where genotyping techniques were first developed, and patients are typically infected with one or a few strains, misclassification is more likely to occur in areas of high transmission intensity where infections with multiple strains are common.¹ Misclassification of genotyping outcomes will lead to inaccurate estimates of drug efficacy, compromise the ability to compare results from different studies, and limit studies aimed at identifying risk factors for drug resistance.

The accuracy of genotyping may be improved by increasing the number of molecular markers assessed. Other polymorphic genes with large repeats that have been evaluated for this purpose, such as circumsporozoite protein and glutamine-rich protein, can be characterized with gel electrophoresis, but show less diversity than *msp1* and *msp2*. Microsatellites, or simple sequence repeats, have been used for studies of population structure⁴ and are good candidates for additional markers. Hundreds have been described,⁵ they are generally not under immune selection, and the sizes of alleles fall at predictable, discrete lengths that may enable easy comparison across multiple samples and laboratories. By measuring the size of microsatellites with capillary electrophoresis, which has a resolution of one nucleotide and is highly reproducible, the full diversity of length polymorphisms present in a population can be used. The use of microsatellite markers has been explored in two drug efficacy trials,^{6,7} but has not, to our knowledge, been validated using polyclonal controls.

We designed a new method for genotyping *P. falciparum* microsatellites, including an automated algorithm for interpreting results, and optimized the technique for conditions likely to be encountered in clinical samples from highly endemic areas. We validated this method on genomic DNA samples of *P. falciparum* laboratory clones prepared from dried blood spots on filter paper and compared the results to a commonly used genotyping method using *msp1* and *msp2*. Finally, we applied this method to a small set of field samples and showed that adding microsatellite genotyping to *msp1* and *msp2* alone strengthened the association between a known molecular marker of drug resistance and recrudescence.

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MATERIALS AND METHODS

Controls containing mixtures of laboratory clones. Six *P. falciparum* clones (3D7, FCR3, HB3, K1, W2, and V1/S) were cultured using standard methods and synchronized at the ring stage with 5% D-sorbitol.⁸ Parasitemia was determined using flow cytometry,⁹ and the concentration of red blood cells was measured with a hemocytometer. Red blood cells from cultures were mixed with uninfected whole blood to yield standardized parasite densities for each clone. Samples (35 μ L) containing single clones or mixtures of up to six clones at various parasite densities (50–50,000 parasites/ μ L) were spotted onto filter paper (Whatman 3MM; Whatman, Clifton, NJ) and allowed to air dry. After 1 month of storage at ambient temperature, the center of each spot was removed using a 6-mm hole punch. DNA was extracted with Chelex 100 Resin (Bio-Rad Laboratories, Hercules, CA) as previously described.¹⁰

Genotyping of *msp1* and *msp2*. The surface antigen loci *msp1* and *msp2* were amplified using previously described primers.¹¹ Briefly, 2 μ L of template DNA was amplified using nested polymerase chain reaction (PCR), with second round primers specific to allelic families: K1, MAD20, and RO33 for *msp1* and IC3D7 and FC27 for *msp2*. PCR products were separated on a 2.5% agarose gel (UltraPure Agarose; Invitrogen, Carlsbad, CA) and stained with ethidium bromide. A technician blinded to the composition of the samples used GelCompar II software (Applied Maths, Sint-Martens-Latem, Belgium) to select alleles and estimate the size of PCR products using a standardized approach.¹²

Genotyping of microsatellites. Twelve trinucleotide repeat microsatellite loci have been previously described for use in genotyping filter-paper blood samples.¹³ Of these, the six loci located on separate chromosomes with the largest heterozygosity were chosen. Primers were designed for single-round PCR using Primer3 software¹⁴ (Table 1). Forward primers were labeled at the 5' end with either a HEX or 6-FAM fluorophore, and a 5' GTGTCTT "tail" was added to the reverse primers to promote addition of an extra adenosine base for more uniform PCR product size.¹⁵ Reaction and cycling conditions were optimized to minimize formation of stutter peaks and maximize detection of minor alleles. Annealing temperatures were varied from 55°C to 70°C, extension temperatures were varied from 62°C to 72°C, the lengths of the denaturing, annealing, and extension steps were varied, the number of cycles was varied, different concentrations of Mg²⁺ and dNTP were tested, and three different polymerases were evaluated. Final reactions contained 1 μ L of template DNA, 200 nmol/L of each primer, 40 μ mol/L of each dNTP, 1.5 mmol/L Mg²⁺, 50 mmol/L KCl, 10 mmol/L Tris-HCl, and 0.4 U of AmpliTaq Gold DNA Polymerase (Applied Biosys-

tems, Foster City, CA) in a total volume of 10 μ L. PCR was performed in a Dyad thermal cycler (MJ Research, Waltham, MA) under the following conditions: 5 minutes at 94°C, 10 cycles of 20 seconds at 94°C, 30 seconds at 60°C, decreasing 0.5°C/cycle, 25 seconds at 68°C, 30 cycles of 20 seconds at 94°C, 30 seconds at 55°C, 25 seconds at 68°C, and 10 minutes at 68°C.

Products of the PCR reactions were diluted in water and pooled for electrophoresis. Two microliters of pooled sample was mixed with 10 μ L Hi-Di formamide and 0.2 μ L Genescan 400HD ROX size standard (Applied Biosystems) and denatured for 5 minutes at 95°C. Capillary electrophoresis was performed using an Applied Biosystems 3730xl DNA Analyzer, and alleles were sized with GeneMapper software (Applied Biosystems). Only peaks that were spaced at intervals corresponding to the trinucleotide repeats (present in all six microsatellites studied) were considered as possible alleles.

In addition to the primary peak for each allele, distinct patterns of "stutter" peaks were observed for each microsatellite marker. Stutter peak formation is common during amplification of microsatellites and is primarily caused by DNA slippage during PCR at intervals equal to the size of the repeat.¹⁶ To create an objective algorithm for filtering out stutter peaks, the height ratio and relative location of observed stutter peaks to true peaks was calculated for each microsatellite. Thresholds were determined to minimize both background noise and stutter peaks while maintaining sensitivity (Appendix 1). These thresholds were automatically applied to data exported from GeneMapper using Microsoft Access (Microsoft Corp., Redmond, WA); peaks with heights below the thresholds were removed, and the remaining peaks were considered alleles. Because thresholds were set relative to the height of other peaks present in the sample, the filtering algorithm does not depend on knowledge of the sample composition and was applied independently of known sample composition.

Interpretation of alleles. Allele sizes for single-clone samples were used to guide the interpretation of alleles in polyclonal samples. For *msp1* and *msp2*, size ranges were used for each clone, taking into account variation within and between gels. A range of 1 base was used for microsatellite alleles because sizes varied by < 0.5 base across all samples. A laboratory clone present in a sample was classified as detected if an allele of the correct size (and correct allelic family for *msp1* and *msp2*) was recorded for that sample. Any alleles recorded for a sample that did not correspond to clones present were designated false-positive alleles.

Genotyping of field samples. To evaluate these methods on clinical isolates, we genotyped filter-paper blood samples from a previously completed clinical trial in Burkina

TABLE 1
Primers used to amplify microsatellites

Marker	Forward primer (5'-3')	Reverse primer (5'-3')*
TA40	6FAM-TTTTGGTTTCCAAGGGATTG	gtgtcttTTAAGGCCACGAGGAAATTG
TA60	HEX-CCAAGAGAAAGCGATCCTCA	gtgtcttTTTTCCATCATATAAATFGGTATCT
TA81	HEX-AGGGAAGGTGAGGAAAAGGA	gtgtcttTTCATACATTTACACAACACAGG
TA87	6FAM-AATGGCAACACCATCAACC	gtgtcttTTATCGCGAAGGGATGTA
PfPK2	HEX-TCCTCAGACTGAAATGCATGA	gtgtcttCCTTTCATCGATACTACGATTATTTG
Poly α	HEX-TTGGGTCCATCGTCAGAAAT	gtgtcttGAACAGACCAGCCAAAAAT

* Lowercase letters indicate 5' sequence added to promote addition of a non-templated adenosine.

Faso.¹⁷ Paired samples from patients with recurrent parasitemia within 28 days after therapy with sulphadoxine-pyrimethamine were extracted and genotyped using the methods described above for *msp1*, *msp2*, and microsatellites. For *msp1* and *msp2*, alleles from original and recrudescence samples were considered a match if they were within 10 bp in size. For microsatellites, alleles were considered a match if they were within 1 base in size. A recurrent parasitemia sample was considered a recrudescence if at least one allele matched at every locus tested, and a new infection if no allele matched in at least one locus tested. Outcomes were classified using *msp1* and *msp2* alone and also with the addition of microsatellites. In addition, all pretreatment samples were evaluated for the presence of the *dhfr* 59R mutation using previously published methods.¹⁸ The association between *dhfr* 59R and recrudescence was measured using Fisher exact test.

RESULTS

Performance on polyclonal controls. All six microsatellite markers worked well in identifying the correct alleles in samples containing a single laboratory clone. However, two of the six markers were unreliable in situations where more than one clone was present. The marker Poly α was often unable to detect more than two alleles present in a sample; TA87 frequently produced false-positive alleles when multiple clones were present (data not shown). These two markers were therefore discarded, and the data are presented are for *msp1*, *msp2*, and the remaining four microsatellite markers (TA40, TA60, TA81, and PfPK2).

Ability to detect clones in controls. For each DNA sample, observed alleles were compared with the known composition of laboratory clones using two methods: 1) nested amplification of *msp1* and *msp2* followed by gel electrophoresis of amplified fragments and 2) amplification of four microsatellites followed by capillary electrophoresis (Table 2; more detailed results are available in Appendix 2).

msp1 and *msp2*. When only one clone was present per sample, genotypes of both *msp1* and *msp2* detected the correct allele in all samples at concentrations as low as 50 parasites/ μ L (0.001% parasitemia). When multiples clones were present, *msp1* genotyping sometimes missed one allele, whereas *msp2* genotyping sometimes missed one to two alleles. Missed alleles frequently corresponded to clones at the lowest density relative to others of the same allelic family.

Alleles with smaller sizes were preferentially detected. Thus, larger alleles present at low proportions were the most likely to be missed (Figure 1). Samples containing the same ratios of clones but different parasite densities generally gave similar results.

Microsatellites. The correct alleles of all single-clone samples were detected by all four microsatellite markers. When multiple clones were present, all four markers occasionally missed one allele (Table 2). A similar number of alleles were missed by the microsatellite markers and by *msp1* and *msp2* genotyping. The factors contributing to missed alleles were similar for the microsatellites as for *msp1* and *msp2*: a low proportion of parasites and larger allele size were associated with missing an allele (Figure 1).

Detection of false-positive alleles in controls. Observed alleles not expected based on the known composition of laboratory clones were designated false-positive alleles.

msp1 and *msp2*. False-positive alleles were frequently detected using *msp1* and *msp2* genotyping (Table 3; Figure 2; more detailed results are in Appendix 3). In single-clone samples, *msp1* genotyping detected no false-positive alleles, and *msp2* genotyping detected zero to two false-positive alleles. When multiple clones were present, *msp1* genotypes contained up to one false-positive allele, and *msp2* genotypes always contained one to three false-positive alleles. No pattern was detected that could reliably predict the occurrence of false-positive alleles.

Microsatellites. Fewer false-positive alleles were detected by the four microsatellite markers than by *msp1* and *msp2* genotyping. TA60 and TA81 produced no false-positive alleles in any sample. In single-clone samples, TA40 occasionally produced one false-positive allele. In samples containing multiple clones, TA40 occasionally detected one to two and PfPK2 detected one to four false-positive alleles. False-positive alleles included small random peaks and rare stutter peaks above defined thresholds (Figure 2).

Field samples. In Burkina Faso, *dhfr* 59R is the primary mutation associated with resistance to sulphadoxine-pyrimethamine. Patients without this mutation would not be expected to fail therapy. Classifying outcomes based on the results of *msp1* and *msp2* alone, four of seven patients presenting with parasites containing the *dhfr* 59R mutation experienced recrudescence versus two of eight patients presenting without the mutation (57% versus 25%, $P = 0.31$). When outcomes were reclassified adding microsatellite data, four of seven patients with *dhfr* 59R were considered recrudescence

TABLE 2
Ability to detect alleles of laboratory clones

Sample composition	Total parasite density	Correct laboratory clones detected per sample*					
		<i>msp1</i>	<i>msp2</i>	TA40	TA60	TA81	PfPK2
Single clone	50–50,000 parasites/ μ L	1	1	1	1	1	1
Three clones, ratio of 1:2:7	10,000 parasites/ μ L	2–3	3	3	3	3	2–3
	50,000 parasites/ μ L	2–3	3	3	3	2–3	2–3
Six clones, equal ratios	300 parasites/ μ L	5	5	6	6	6	6
	3,000 parasites/ μ L	6	5	6	6	6	6
	30,000 parasites/ μ L	6	5	6	6	6	6
Six clones, various ratios (individual clones present at 5% to 60% of total parasite density)	30,000–50,000 parasites/ μ L	5–6	4–6	5–6	5–6	5–6	5–6

* Bold, all clones present were detected in samples in that category.

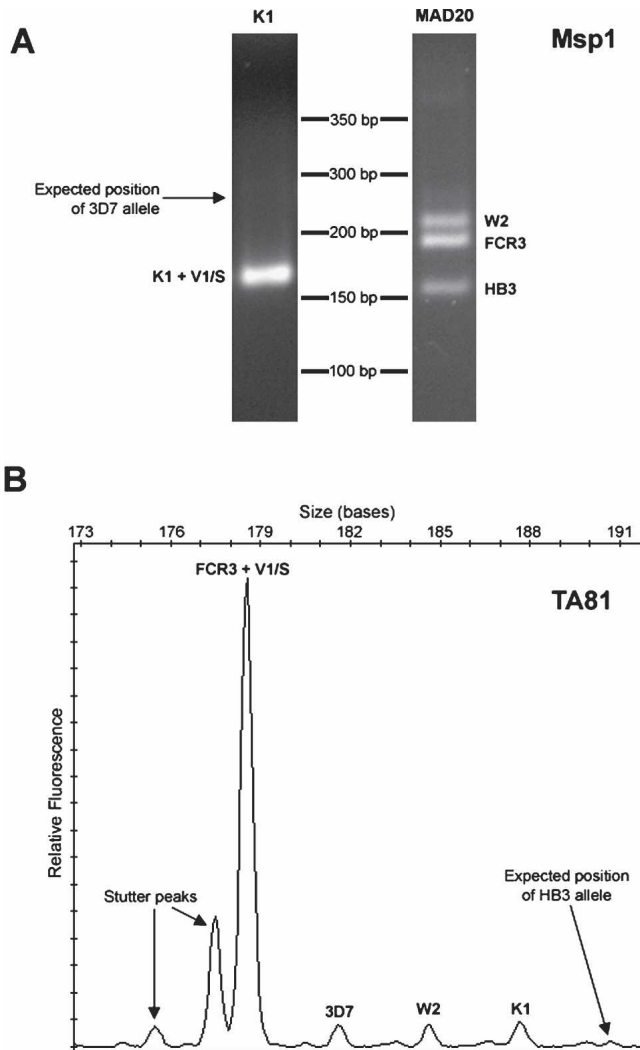


FIGURE 1. Examples of missed alleles for *msp1* and a microsatellite marker from a sample containing all six clones at the following parasite densities (parasites/ μ L): 3D7 (2,500), FCR3 (2,500), HB3 (2,500), K1 (10,000), W2 (2,500), V1/S (30,000). **(A)** Agarose gel images of K1- and MAD20-type family-specific PCR products for *msp1*. An arrow indicates the expected position of the missed 3D7 allele. **(B)** Electropherogram for microsatellite TA81. An arrow indicates the expected position of the missed HB3 allele. The stutter peaks were correctly filtered by the allele detection algorithm.

versus zero of eight patients without the mutation (57% versus 0%, $P = 0.03$).

DISCUSSION

Genotyping *P. falciparum* using surface antigen loci and agarose gel electrophoresis may be inadequate to accurately distinguish recrudescence from new infection in high transmission areas where polyclonal infections are common. Additional methods are needed and require validation under conditions similar to those encountered in field studies. In this study, *msp1*, *msp2*, and new methods for genotyping six microsatellite markers correctly detected the alleles of single-clone samples. Two of the microsatellite markers (Poly α and TA87) were inaccurate for polyclonal samples, showing the importance of validating new genotyping techniques on these

types of samples. The other four microsatellite markers performed at least as well as *msp1* and *msp2* in correctly identifying alleles present in filter-paper blood samples containing multiple strains of *P. falciparum* and produced fewer false-positive alleles. Finally, we showed that the addition of these four microsatellites to *msp1* and *msp2* genotyping in a small number of field samples strengthened the association between a known molecular marker of drug resistance and recrudescence.

Because our control samples were artificial mixtures of laboratory clones, we observed trends but cannot precisely estimate the probability of failing to detect alleles for field isolates collected from a clinical trial. Missed alleles occurred at a similar frequency for the four microsatellite markers as for *msp1* and *msp2*, suggesting that these microsatellite methods are as sensitive for detecting minority alleles in polyclonal samples. As seen previously,^{3,19} relative densities of clones strongly influenced detection at all six loci, and larger alleles were more likely to be missed.²⁰ The greater size differences between *msp1* and *msp2* alleles than those of microsatellites may have counterbalanced the sensitivity advantage of family-specific PCR,²¹ resulting in similar rates of detection overall. Although not observed in this study, it is also possible that our microsatellite genotyping method may miss a strain by inappropriately filtering out a minority allele that is at the same size as a stutter peak.

False-positive alleles were detected at *msp1*, *msp2*, and two of the four microsatellites (TA40 and PPK2), with *msp2* producing more false-positive alleles than other loci. For *msp1* and *msp2*, false-positive alleles may have been caused by primer or product carry-over from the first round of PCR, heteroduplex formations detected as additional bands,²² recombination during PCR resulting in formation of chimeric products,²³ DNA strand slippage during PCR, or PCR contamination. False-positive alleles at TA40 and PPK2 could have been created by the same factors except for carry-over, because our microsatellite PCR was not nested, and heteroduplex formation, because capillary electrophoresis was performed under denaturing conditions. Some or all of these factors are likely to influence the accuracy of any PCR-based genotyping system.

In choosing the most appropriate methods for genotyping a clinical drug efficacy trial, characteristics of both the genotyping techniques and the study site need to be considered. The probability of two independent strains having the same genotype depends on the diversity of the genetic loci assessed and the ability of the genotyping technique to measure that diversity. When unrelated strains appear the same, new infections may be misclassified as recrudescence. When more strains are simultaneously present, such as in sub-Saharan Africa, the probability of this misclassification increases. One way we have attempted to adjust for this problem in the past has been to classify sample pairs containing both matched and unmatched alleles as new infections.¹² However, the persistence of even one of a number of strains originally present may suggest drug failure and would ideally define recrudescence.² When using multiple loci, a recrudescence should be defined as the presence of at least one matched allele at every locus; if at least one locus shows only unmatched alleles, the outcome should be classified as a new infection. By increasing both the resolution of the genotyping method and the number

TABLE 3
Detection of alleles not corresponding to clones present in samples

Sample composition	Total parasite density	False-positive alleles detected per sample*					
		<i>msp1</i>	<i>msp2</i>	TA40	TA60	TA81	PfPK2
Single clone	50–50,000 parasites/ μ L	0	0–2	0–1	0	0	0
Three clones, ratio of 1:2:7	10,000 parasites/ μ L	0–1	1–3	0–1	0	0	0–3
	50,000 parasites/ μ L	0–1	3	0–1	0	0	0–3
Six clones, equal ratios	300 parasites/ μ L	0	3	0	0	0	1
	3,000 parasites/ μ L	1	3	0	0	0	0
	30,000 parasites/ μ L	1	3	0	0	0	0
Six clones, various ratios (individual clones present at 5% to 60% of total parasite density)	30,000–50,000 parasites/ μ L	0–1	1–2	0–2	0	0	0–4

* Bold, no false-positive alleles were detected in samples in that category.

of loci assessed, discriminatory power may be increased enough to enable use of these definitions. Increased resolution enables the detection of small differences between strains at a given locus, and adding additional loci further increases the probability of detecting genetic differences between

strains because they may have the same genotype at one locus but have detectable differences at another.

Using these definitions, missing alleles in genotypes from drug efficacy trials can lead to the misclassification of a recrudescence as a new infection. For example, a minority allele present in a pretreatment sample may be missed, especially if it is larger in size than the dominant allele(s). If the corresponding parasite strain is the only one able to survive drug therapy and causes recurrent parasitemia, the recurrence will be classified as a new infection, even if the allele is only missed at one locus. For this reason, the increase in discrimination afforded by adding additional loci needs to be balanced with the increased risk of misclassifying a recrudescence as a new infection if alleles are missed at those loci. False-positive alleles, on the other hand, can lead to misclassification of a new infection as a recrudescence at a particular locus. Adding an additional locus, however, will not increase the chance of misclassifying a new infection as a recrudescence, even if false-positive alleles are present at that locus.

By adding four microsatellite loci to *msp1* and *msp2* genotyping in a small set of field samples, we reclassified two outcomes from recrudescence to new infection. Both patients with reclassified outcomes presented with wild-type alleles at the *dhfr* 59 locus and would not have been expected to fail therapy with sulphadoxine-pyrimethamine. The increase in strength of association between the mutant allele *dhfr* 59R and recrudescence after adding the microsatellite loci ($P = 0.31$ versus $P = 0.03$) shows that a change in even a small number of genotyping outcomes may improve the ability to identify risk factors for drug resistance.

Our microsatellite genotyping method offers a number of advantages over genotyping using surface antigen loci and agarose gel electrophoresis. Paired clinical samples genotyped using agarose gels need to be run in adjacent lanes on the same gel to make meaningful comparisons, given the large variability in size measurements across gels. The precise sizing of capillary electrophoresis and discrete allele sizes of microsatellites make comparisons between multiple samples evaluated at different times and across different laboratories possible. The high resolution of capillary electrophoresis also enables measurement of the full diversity present at these loci; in contrast, with agarose gels, a resolution of 6 bp⁷ limits the ability to measure allelic diversity. Analysis of agarose gel results is inherently subjective³; our method, relying on a simple algorithm for determining true alleles, is more objective and minimizes investigator bias. Finally, our method dif-

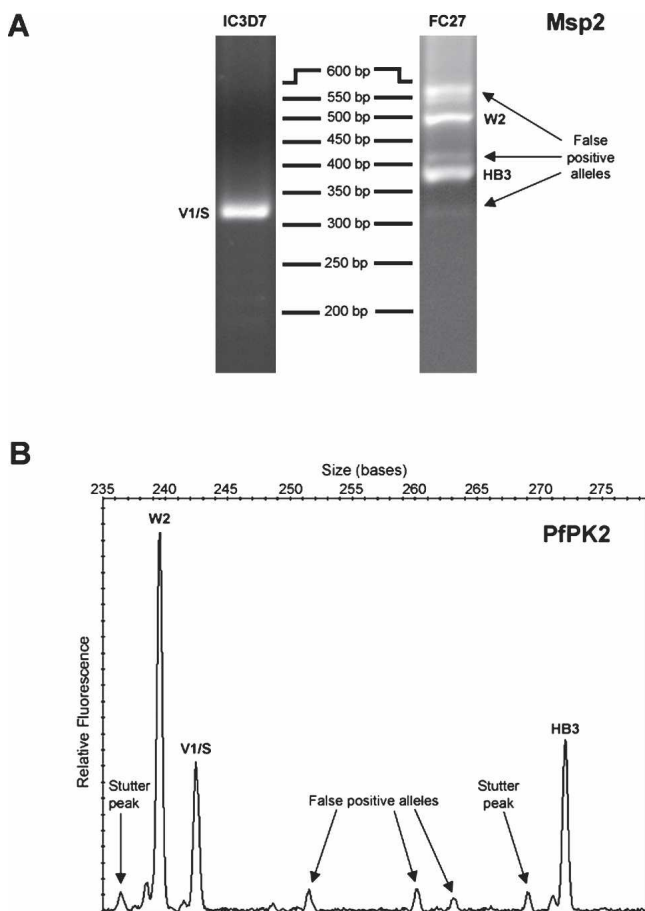


FIGURE 2. Examples of false positive alleles for *msp2* and a microsatellite marker from a sample containing three clones at the following parasite densities (parasites/ μ L): HB3 (35,000), W2 (10,000), V1/S (5,000). (A) Agarose gel images of IC3D7- and FC27-type family-specific PCR products for *msp2*. (B) Electropherogram for microsatellite Pfpk2. The stutter peaks were correctly filtered by the allele detection algorithm. Arrows in both A and B indicate PCR products identified as false-positive alleles that did not correspond to any clones present in the sample.

fers from most surface antigen and microsatellite genotyping techniques in that it does not depend on nested PCR. This decreases the probability of contamination, eliminates the problem of artifact from first-round primer or product carry-over, and saves on time and reagent costs. Capillary electrophoresis has the disadvantage of not being available in many resource-poor settings. However, it is available in most research centers that perform automated DNA sequencing, and at our institution, the cost of analyzing four microsatellite loci is similar to that of genotyping *msp1* and *msp2* when all reagent costs are considered. The additional cost we incurred in genotyping four microsatellites for our field samples was \$1.51/sample.

Choosing the optimal method of genotyping samples from a *P. falciparum* clinical drug efficacy trial requires knowledge of the performance and limitations of genotyping techniques when applied to a particular setting. In areas where polyclonal infections are common, additional loci may need to be added to commonly used genotyping techniques to accurately distinguish recrudescence from new infection. We have proposed an efficient and objective method for genotyping four independent loci that compares favorably with existing techniques and may aid in the interpretation of results from drug efficacy trials, especially in regions of high endemicity where rates of both polyclonal infections and new infections during follow up are high. We are currently in the process of applying this method to a large number of field samples from different sites to determine the optimal combination of markers, including microsatellites, *msp1*, and *msp2*, for use in different epidemiologic settings.

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