

## Effects of *Plasmodium falciparum* Mixed Infections on *In Vitro* Antimalarial Drug Tests and Genotyping

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**Abstract.** Studying drug resistance in *Plasmodium falciparum* requires accurate measurement of parasite response to a drug. Factors such as mixed infection of drug-resistant and -sensitive parasites can influence drug test outcome. Polymorphic DNA sequences are frequently used to detect mixed infections; infections with a single genotype or having a minor allele smaller than a subjectively selected cut-off value are often considered single infection. We studied the effects of mixed parasite populations containing various ratios of parasites resistant and sensitive to chloroquine on outcomes of drug tests and how ratios of parasite mixtures correlated with genotypes using polymerase chain reaction-based methods. Our results show that a mixture with a resistant population as low as 10% could greatly impact a drug test outcome. None of the genotyping methods could reliably detect minor DNA alleles at  $\leq 10\%$ . Mixed infection presents a serious problem for drug tests, and genotyping using microsatellite or other methods may not reliably reflect true ratios of alleles.

### INTRODUCTION

Drug resistance in *Plasmodium falciparum* is one of the major health problems in malaria-endemic regions. In addition to widespread resistance to chloroquine (CQ) and pyrimethamine-sulfadoxine (PS), parasite resistance to mefloquine, quinine, and other antimalarial drugs have been reported.<sup>1</sup> Understanding the molecular mechanisms of drug resistance in malaria parasites may allow development of better drug treatment policies and strategies to overcome resistance.

Various methods have been developed to evaluate parasite responses to antimalarial drugs. All are based on measuring parasite growth or growth inhibition under various drug concentrations, including counting parasitemia,<sup>2</sup> measuring [<sup>3</sup>H] hypoxanthine incorporation,<sup>3</sup> parasite lactate dehydrogenase (LDH) activity<sup>4</sup> or signals from antibodies against histidine-rich protein II (HRPII)<sup>5,6</sup> or LDH,<sup>7</sup> and staining parasite DNA with SYBR green or DAPI dyes.<sup>8–12</sup> These assays have been widely used to study drug application efficacy, to monitor drug resistance, and to map drug-resistant genes.<sup>13–17</sup>

Many factors can affect drug test results. Because most tests evaluate parasite growth by measuring the amount of parasite proteins/DNA in a sample after drug treatment, differences in parasite growth rate or developmental state may affect the test results. *In vitro* drug tests are usually performed using either culture-adapted parasites or parasites in blood samples freshly isolated from patients. Although selection of a dominant clone during culture adaptation is a potential concern when using culture-adapted parasites, drug tests using parasites collected directly from patients also have various potential problems. First, it is well known that fresh isolates may not grow well initially in *in vitro* culture, and some parasites eventually die during culture because of problems in surviving *in vitro* culture conditions, not because of drug effect. Second, unknown factors in patient blood, such as medicines taken by

the patient before the tests and/or host antibodies against a particular “strain” can also contribute to inaccuracy in drug tests. Third, patient blood samples usually have very different parasitemia, and most drug assays are sensitive to variation in parasitemia. Fourth, limited supplies of patient blood will not allow repeated tests at different times, because bloods are usually drawn once and patients are treated with antimalarial agents thereafter. Repeated tests under similar conditions are critical for malaria drug assays, because variations from test to test are frequently observed. Finally, mixed infections of different genotypes are highly prevalent in malaria-endemic areas, particularly in Africa and Southeast Asia.<sup>18–21</sup> It is likely that mixed infections of both drug-sensitive and -resistant parasites in a patient will produce test readings not representative of a typical resistant or sensitive parasite.<sup>21</sup>

Another important issue related to testing parasites directly from patient bloods is how to define a mixed infection. Highly polymorphic genetic markers such as genes encoding surface antigens are frequently used for genotyping parasites.<sup>20,22,23</sup> Genotypes obtained using antigen genes could potentially overestimate parasite diversity, because antigen genes are often under host immune pressure to change, leading to overestimation of the percentage of mixed infection. Recently, more neutral microsatellites (MSs) were used, which may provide more realistic estimates of parasite populations, although MSs generally vary at higher rates than single nucleotide polymorphisms (SNPs).<sup>24–26</sup> Despite extensive efforts in developing genotyping methods, there are issues that have to be addressed regarding mixed infection and genotyping. First, how many markers are sufficient to detect and declare a mixed infection? Obviously, the more polymorphic markers are used to type parasites, the more likely different genotypes will be detected. Second, how strong a signal from a minor allele should be considered as a mixed infection in drug phenotype measurement? Should we discard a mixed genotype sample with a minor allele 5% (or 20%) of the major allele? Frequently, the numbers of markers and the cut-off values for a minor allele being called a mixed infection were subjective, depending on the investigators' preferences. Third, can a genetic marker or a method used in genotyping faithfully reflect true DNA proportion in a DNA samples?

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Accurate measurement of parasite drug responses is critical for many drug studies, particularly for mapping drug resistance controlled by quantitative trait loci (QTL). Ideally, a negligible minor allele should not have significant influences on phenotype measurement. To study the effects of mixed parasite populations on the measurement of parasite drug response, we evaluated the influence of parasite mixtures with various ratios of CQ-resistant (CQR) and CQ-sensitive (CQS) parasites on drug test outcomes. We also used three methods to type DNA samples from mixed parasite populations to study how different ratios of parasite mixtures correlated with genotyping signals. We show that parasite mixtures with a minor allele of  $\geq 10\%$ , despite being barely detectable in genotyping, could greatly impact parasite half-maximal inhibitory concentration ( $IC_{50}$ ) measurements. We conclude that samples with detectable mixed genotypes should be considered mixed infections in drug tests for association studies.

## MATERIALS AND METHODS

**Parasites and parasite culture.** *Plasmodium falciparum* parasite lines HB3, Dd2, FCB, and Dd2<sup>192</sup> used in this study have been described.<sup>13,27</sup> The parasites were cultured *in vitro* according to the methods of Trager and Jensen.<sup>28</sup> Briefly, parasites were maintained in RPMI-1640 medium containing 25 mmol/L HEPES, 5% human O<sup>+</sup> erythrocytes (5% hematocrit), 0.5% Albumax (GIBCO, Life Technologies, Grand Island, NY), 24 mmol/L sodium bicarbonate, and 10  $\mu$ g/mL gentamycin at 37°C with 5% CO<sub>2</sub>, 5% O<sub>2</sub>, and 90% N<sub>2</sub>.

**Drug assays.** Drug assays were performed using a SYBR green staining method modified from a procedure described previously.<sup>9</sup> Briefly, parasites were diluted to 1% parasitemia with 1% hematocrit shortly after thawing, and the diluted parasites (150  $\mu$ L) were added to wells in a 96-well plate containing 50  $\mu$ L properly diluted CQ. The drug was 2-fold diluted in triplicate with concentration ranging from 10,000 nmol/L to  $\sim 10$  nmol/L. The parasites were incubated with the drug at 37°C and measured at 72 hours. After incubation, DNA was released from the cultured parasite and stained with SYBR green dye as described.<sup>9</sup> The plate was kept in the dark for 30 minutes, and signals were read in a FLUOstar OPTIMA reader (BMG Labtech, Durham, NC). Data from the microplate reader were analyzed using Prism 4.0 software (GraphPad Software, San Diego, CA).

**MS typing.** The MS markers used in this study are listed in Table 1, with the forward primers of the markers fluorescently labeled with either Hex or 6FAM dyes (Applied Bio-

systems, Foster City, CA). DNA samples from various parasite mixtures were isolated from saponin-treated parasite pellets using BioRobot EZ1 genomic DNA kit (Qiagen, Valencia, CA). MS typing was performed essentially as described,<sup>29</sup> except individually labeled primers were used. Briefly, each 14- $\mu$ L polymerase chain reaction (PCR) mix contained 7.5  $\mu$ L H<sub>2</sub>O, 1.4  $\mu$ L 10 $\times$  buffer (200 mmol/L Tris-HCl [pH 8.4], 500 mmol/L KCl, and 50 mmol/L MgCl<sub>2</sub>), 0.3  $\mu$ L 10 mmol/L dNTP, 0.4  $\mu$ L each of the two primers (50 pmol/ $\mu$ L), 0.1 unit Taq polymerase, and 4  $\mu$ L diluted DNA ( $\sim 4$  ng). The reactions were denatured at 94°C for 2 minutes and cycled at 94°C for 20 seconds, 45°C and 40°C for 10 seconds each, and 60°C for 30 seconds for 40 cycles. Fluorescently labeled PCR products (2.5  $\mu$ L) were directly separated on an ABI 3100 Genetic Analyzer, and signals were scored and analyzed using GeneMapper v3.2 (Applied Biosystems, Foster City, CA).

**Real-time quantitative PCR.** Real-time quantitative PCR (qPCR) was performed in an iCycler multicolor real-time detection system using IQ SYBR green supermix (Bio-Rad, Hercules, CA). Allele-specific oligonucleotides from the gene encoding parasite apical membrane antigen 1 (PfAMA1) were selected based on the coding sequence alignment of HB3 and Dd2 parasites. Sequences of parasite specific primers are as follows: HB3-AMA1-F2, 5'-TTAGATCAA-ATGAGACATCTT-3'; HB3-AMA1-R2, 5'-CTTAAATATGTATAGTTTTCAAATA-3'; DD2-AMA1-F2, 5'-TTAGATGATATGAGACTTTTG-3'; and DD2-AMA1-R2, 5'-CTTAAATATGTATAGTTTTGAAATG-3'. The reactions were carried out according to manufacturer's instructions with the following cycling program: 5 minutes at 94°C for initial denaturation, 95°C for 30 seconds, 50°C for 30 seconds, 60°C for 30 seconds for 45 cycles, and a final extension at 60°C for 2 minutes. The quantity of DNA was calibrated using a serial-diluted plasmid DNA (PVLH1X) with the following primer sequences: R-LucF3, 5'-TTGTGTTTGTGGA-CGAAGTAC-3' and R-LucR3, 5'-TTCTTGGCCTTTATGAGGATC-3'.

**Pyrosequencing.** Mixed DNA samples (from 1:99 to 50:50) from different combinations of the parasites (Dd2:HB3, Dd2<sup>192</sup>:HB3, FCB:HB3) were sent to EpigenDX (Worcester, MA; <http://www.epigendx.com>) for pyrosequencing. Two SNPs (A/T at nucleotide position 3,100 and A/G at position 3,125) in the putative *P. falciparum* multiple drug-resistant gene 1 (*Pfmdr1*) were amplified and sequenced. Primers for PCR amplification and pyrosequencing were as follows: 5'-TTATTGTAAATGCAGCTTTATGGG-3', 5'-GGGACACCGCTGATCGTTTATAGATCCAAACCAAT-AGGCAAAC-3', and 5'-GCAGCTTTATGGGGATT-3'.

TABLE 1  
Microsatellite markers used in typing mixed DNA samples

Name	Chromosome	UniSTS number*	Allele size†				Forward primer (5'-3')	Reverse primer (5'-3')
			HB3	Dd2	Dd2 <sup>192</sup>	FCB		
C1M4	1	G38014	212	202	202	190	atatcctacaacgtaagca	ggcacataaataacatcac
BM17	6	G44440	142	140	140	138	aacgatatggtatgataaa	gcacacatgattatcatat
B5M5	3	G42731	207	213	213	225	taaataatacaactactaatga	atcatgctcttcagcattc
B5M124	11	G44571	226	194	194	176	taataagttaagaatatgga	aaacagagcaaaataaagcagt
C13M13	13	G44636	142	134	134	132	gtataagtatagcagcaga	taaatataaataacattacgg
C14M17	14	G41045	186	190	190	216	acacaaagagaataggtata	ggcaatctatggcttaac

\* UniSTS is a database of sequence tagged sites at NCBI website (<http://www.ncbi.nlm.nih.gov/sites/entrez?db=unists>).

† Product sizes were estimated using labeled primers and might not match the sizes in the genome or reported previously.<sup>27</sup>

RESULTS

**Measurement of parasite IC<sub>50</sub>.** We used a SYBR green drug assay modified from a protocol described previously<sup>9</sup> to measure parasite IC<sub>50</sub>. Parasite responses to CQ were tested at least three times to obtain reproducible IC<sub>50</sub> for each sample. The average IC<sub>50</sub> values for the parasites used in this study were 13.6 ± 2.2 (SD) nmol/L for HB3 (21 repeats); 237 ± 40.6 nmol/L for Dd2 (11 repeats); 391.6 ± 33.3 nmol/L for Dd2<sup>192</sup> (11 repeats), and 196.6 ± 23.0 nmol/L for FCB (11 repeats; Table 2). These results were comparable with those obtained using the [<sup>3</sup>H] hypoxanthine assay (Dd2, 404.1 nmol/L; HB3, 33.9 nmol/L; FCB, 492.2 nmol/L), although the absolute values were different.<sup>30</sup>

**Drug response curves from mixtures of drug-resistant and -sensitive parasites.** To evaluate how a minor population of a drug-resistant parasite affects drug test outcome from a sensitive parasite culture, we generated a series of mixtures with parasite ratios of 1:99 (Dd2: Hb3), 2.5:97.5, 5:95, 7.5:92.5, 10:90, 20:80, 30:70, 40:60, and 50:50. Mixed parasites were grown under CQ for 72 hours before addition of lysis buffer containing SYBR green. As shown in Figure 1A and Table 2, a parasite mixture with as little as 10% of resistant Dd2 parasite not only greatly affected IC<sub>50</sub> (shifting the curve left or approximately double the IC<sub>50</sub>), but also changed (flattened) the slope; a 50:50 mixture produced a curve close to that for a pure Dd2 CQR parasite. Whereas both Dd2 and HB3 had curves that showed sharp growth inhibition within a narrow range of drug concentration, mixed populations had curves of more gradual changes (Figure 1A) or a double-hump reflecting two parasite populations. Although slight shifts of curve positions could be seen, curves from mixtures with resistant parasites at a ratio of < 5% did not significantly change the positions or the shapes (Figure 1B). Similar results were obtained from parasite mixtures of Dd2<sup>192</sup>/HB3 and FCB/HB3, respectively (Figure 1C–F).

**Effect of a highly resistant parasite on drug IC<sub>50</sub> measurement.** Because patients can carry parasites that are resistant to a drug at different levels, we similarly tested a parasite (Dd2<sup>192</sup>) with higher IC<sub>50</sub> than that of Dd2 and studied how the parasite affected drug tests in mixtures with different ratios of resistant and sensitive parasites (Figure 1C and D). Parasite Dd2<sup>192</sup> was derived from Dd2 under continuous CQ pressure *in vitro*<sup>27</sup> and has an IC<sub>50</sub> higher than Dd2 (391 versus 237 nmol/L; Table 2). Drug test results from mixtures of various Dd2<sup>192</sup> and HB3 ratios showed that Dd2<sup>192</sup> indeed had a more profound effect on IC<sub>50</sub> measurements than Dd2 at the same parasite ratios, producing higher IC<sub>50</sub> at mixtures with 10% or higher resistant parasites (Table 2). Similarly, FCB parasite had an IC<sub>50</sub> (191 nmol/L) slightly lower than that of Dd2; and mixtures with 30–50% FCB parasites also had lower IC<sub>50</sub> than those of Dd2. The results showed that parasites with different resistance levels (IC<sub>50</sub>) could also have different impacts on IC<sub>50</sub> measurements of mixed parasite populations.

**Correlation of drug test IC<sub>50</sub> and genotype ratios using MSs.** Genotyping using genetic markers such as MSs is frequently used to detect mixed parasite populations in patient blood samples; however, it can be difficult to detect or to rule out a mixed infection. To study how well genotyping reflects the actual parasite (DNA) proportions in a mixture, we genotyped DNA mixtures with various ratios of CQR and CQS

TABLE 2  
CQ IC<sub>50</sub> of mixed parasite populations with various ratios and parasite combinations

Parasite ratio	100:0 ± SD	50:50 ± SD	40:60 ± SD	30:70 ± SD	20:80 ± SD	10:90 ± SD	7.5:92.5 ± SD	5:95 ± SD	2.5:97.5 ± SD	1:99 ± SD	0:100 ± SD
IC <sub>50</sub> (Dd2:HB3)	237.0 ± 40.6	180.7 ± 28.3	156.2 ± 39.1	134.0 ± 22.0	50.9 ± 18.7	20.1 ± 5.3	16.5 ± 3.3	13.4 ± 0.6	13.1 ± 0.6	13.1 ± 0.8	14.0 ± 2.3
IC <sub>50</sub> (Dd2 <sup>192</sup> :HB3)	391.6 ± 33.3	270.4 ± 25.9	224.4 ± 13.8	206.04 ± 21.8	146.1 ± 30.0	27.0 ± 6.7	16.5 ± 5.5	13.0 ± 0.7	12.9 ± 0.4	12.8 ± 0.5	13.8 ± 2.8
IC <sub>50</sub> (FCB:HB3)	196.6 ± 23.0	110.1 ± 50.1	92.6 ± 53.0	84.9 ± 29.5	70.2 ± 16.6	57.7 ± 50.0	17.3 ± 1.6	14.1 ± 1.2	12.7 ± 0.8	12.6 ± 0.2	13.0 ± 1.5

For ratios of 100:0, 50:50, and 0:100, the numbers (in nmol/L) were averages and SDs from 11 or more repeats. The rest of the measurements were from four to six repeats.

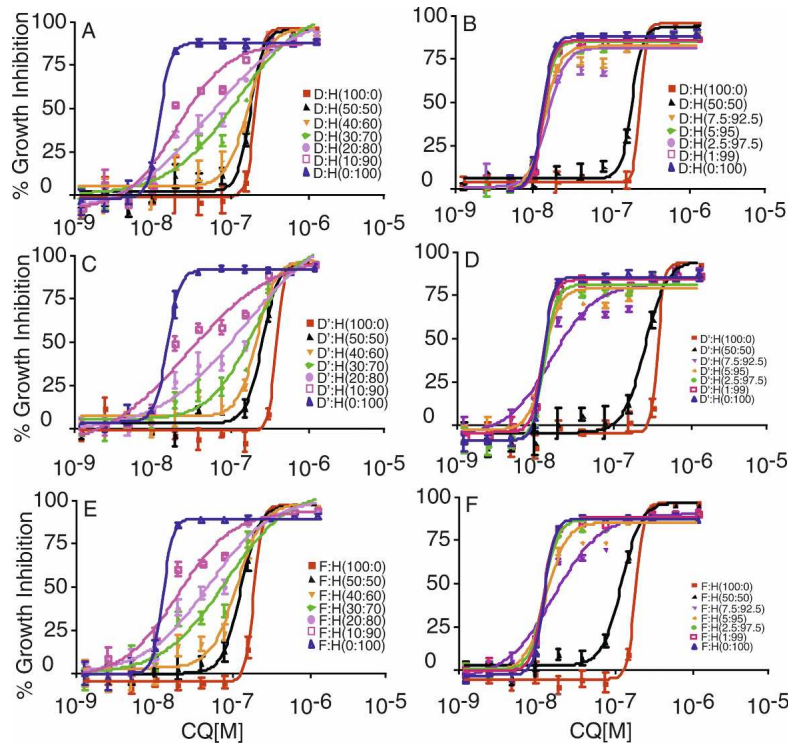


FIGURE 1. Growth inhibition drug assays of parasite mixtures. Chloroquine-resistant parasites (Dd2, Dd2<sup>192</sup>, and FCB) were mixed with chloroquine-sensitive parasite HB3 at various ratios. Inhibition data of Dd2:HB3 ratios from 10:90 to 50:50 were plotted in **A**; from ratios of 1:99 to 7.5:92.5 in **B**; Dd2<sup>192</sup> (D'):HB3 were plotted in **C** and **D**; and FCB:HB3 were plotted in **E** and **F**, respectively. All data points were repeated at least three times, and SD bars indicate variations between tests. Negative values were generated after subtraction from controls (no drug). A mixture of 10% of resistant parasites can greatly affect IC<sub>50</sub> of a sensitive parasite, whereas a mixture of 5% or less has minimal affects.

parasites using six MS markers. Our results showed that the ratios of MS signals were generally co-linear with the ratios of parasite (DNA) mixtures, i.e., as the ratios of minor parasite strains over the major strain (HB3) increased, the ratios of minor alleles over the major allele also increased (Figure 2). Additionally, the signals from the same DNA and MS marker were well repeated, because the SDs for the markers (except C1M4) were generally small, and the plots from DNA samples of Dd2:HB3 and Dd2<sup>192</sup>:HB3 mixtures were essentially the same. Quite different signal ratios for different MS markers (reflected in different slopes of the regression lines) were obtained from the same DNA mixture, however, suggesting differential amplification of DNA alleles by different MS markers (Figure 2). At higher proportions of minor parasite populations (Dd2, Dd2<sup>192</sup>, and FCB), signal ratios of more than one (the theoretical maximum ratio should be one because of 50:50 parasite mixture) were observed for several MS markers, suggesting preferential amplification of MS alleles from some parasites. For marker C1M4, signals from Dd2, Dd2<sup>192</sup>, and FCB were 4–6 times higher than that of HB3 when the parasite ratio was actually one (50:50 mixture). These results clearly showed that signals from randomly selected MS could not faithfully reflect the actual amount of DNA in a mixture. Some normalizations and/or selection of genetic markers are necessary to correctly estimate DNA ratios in mixed infections.

Further inspection of the MS sequences showed that the sizes of amplicons from different parasites might play a role in the preferential amplification of some alleles. Although there

was no clear correlation between MS allele sizes and the slopes of the regression lines in Figure 2, the two markers having smaller HB3 alleles (C14M17 and B5B5) also had regression lines with smaller slopes, whereas markers with larger HB3 alleles (C1M4, C13M13, and B5M124) had larger slopes, suggesting more efficient amplification of smaller alleles (Figure 2; Table 1).

**Real-time qPCR and pyrosequencing to quantitate allelic proportions in parasite mixtures.** Because many MS markers could not correctly estimate the proportions of DNA alleles in a mixture, we designed primers based on two SNP in the gene encoding PfAMA1 to estimate DNA alleles in the parasite mixtures using real-time qPCR. Real-time qPCR has been used to estimate allelic proportions in DNA mixtures from *Plasmodium chabaudi* clones.<sup>31</sup> Indeed, a regression line from real-time qPCR matched well with a line from known mixed DNA proportions (Figure 3A), although there were some variations between tests. We also used pyrosequencing to estimate the DNA ratios. Similar to MS markers, pyrosequencing produced parallel lines reflecting changes in DNA ratios but seemed to have a tendency to overestimate the minor allele at higher minor allele proportions (Figure 3B).

## DISCUSSION

This study investigated quantitative effects of mixed malaria parasite infections on drug test outcomes and the accuracy of various methods in estimating mixed DNA. Because

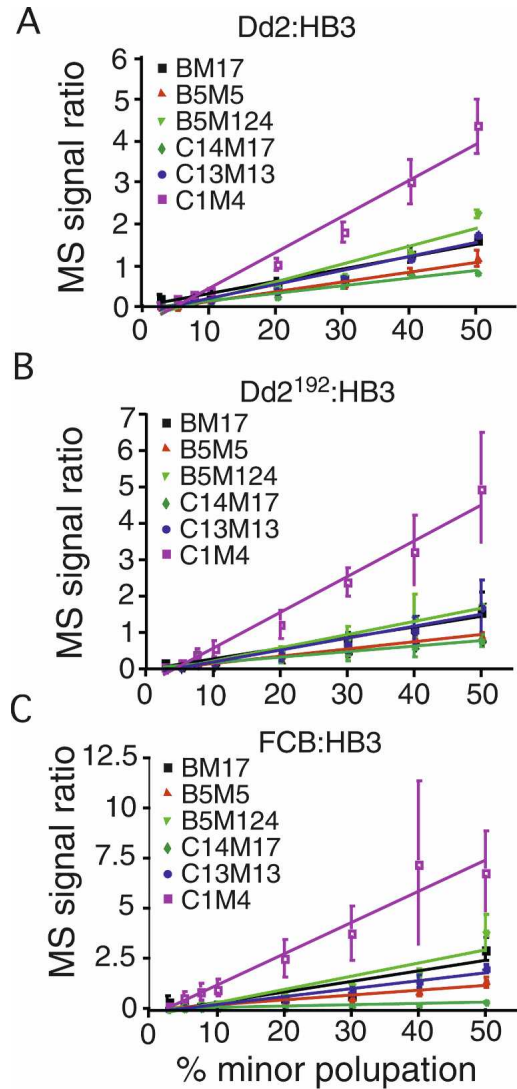


FIGURE 2. Typing mixed DNA samples using microsatellite (MS) markers. DNA samples from mixed parasite populations of Dd2/HB3, Dd2<sup>192</sup>/HB3, and FCB/HB3, respectively, were typed with six MS markers (BM17, B5M5, B5M124, C14M17, C13M13, and C1M4) three times each. Signal ratios of Dd2, Dd2<sup>192</sup>, or FCB (minor alleles) over HB3 were plotted against ratios of mixed parasite populations (Dd2/Dd2<sup>192</sup>/FCB:HB3; 1:99, 2.5:97.5, 5:95, 7.5:92.5, 10:90, 20:80, 30:70, 40:60, and 50:50). Different MSs produced different estimates of DNA ratios.

of the difficulty in adapting parasites from patient bloods to *in vitro* culture and the labor-intensive procedures needed to maintain parasite cultures, drug tests are often performed using parasites isolated freshly from patient bloods. In addition to various factors that can affect drug test results, the effect of mixed populations of drug-resistant and -sensitive parasites on a drug test is an important issue that requires additional study. In this study, we showed that mixed infections with a minor population as low as 10% could greatly influence the outcome of a drug test. Mixtures of parasite populations with resistant and sensitive parasites not only can affect the IC<sub>50</sub> of the parasites but also can change the dynamics of drug tests. In fact, a mixed infection of both CQR and CQS parasites can be predicted based on the shape of a parasite growth inhibition curve. A pure parasite population,

either resistant or sensitive to a drug, will produce a curve with a sharp inhibition of parasite growth at a relatively narrow range of drug concentration, whereas a mixed population with a minor resistant population of 10% or higher will produce a flattened curve or a double-hump curve, reflecting two parasite populations with different IC<sub>50</sub>. Additionally, resistant parasites having different levels of resistance to a drug will have different effects on the drug test outcomes.

Many methods have been developed to study mixed infections in patient bloods, parasite population dynamics, therapeutic efficacy, and to distinguish recrudescence and reinfection.<sup>32–38</sup> Polymorphic genetic markers such as highly polymorphic genes or MSs are often used to screen mixed infections<sup>25,39</sup>; and different cut-off values have been used to identify mixed infections. Our results suggest that samples with detectable minor alleles should be considered as mixed infections, particularly for studies that require accurate measurement of parasite drug responses. Although a 10% drug-resistant parasite in a mixture could greatly influence the outcome of a drug test (Figure 1; Table 2), it was difficult for the majority of the MS (or other methods) to reliably detect the signal in a mixture (Figure 2). If possible, a cut-off value for a minor allele should be established based on experimental data showing that the minor allele will have a minimum effect on phenotype measurement.

Many current techniques for typing mixed infections are PCR-based methods, which are generally sensitive; however, these methods may not faithfully reflect the real DNA ratios in a mixture because DNA present in different amounts may not be amplified proportionally. Previous studies have shown that relative band intensities of AFLP markers, signals from real time qPCR and pyrosequencing, or peaks from DNA sequencing signals were linearly related to the proportions of parasite DNA in a genetically mixed sample,<sup>31,40–42</sup> but conditions for these assays might have to be carefully controlled or raw data from some tests had to be normalized to obtain a linear relationship. Here we used different methods to estimate the proportions of different alleles in mixed parasite populations. In our hands, although signals from the majority of the MS markers we tested showed a linear relationship with the ratios of DNA mixtures, most of the MS markers could not accurately measure the true DNA amounts of the two mixed alleles. The amplification signals seemed to be biased toward smaller amplicons (MS). Similar results were obtained using pyrosequencing, with a tendency to overestimate minors at higher minor allele ratios. Real-time qPCR seemed to be a good method for estimating mixed DNA samples, but we only tested one polymorphism in PfAMA1.

Mixed infection is a relative term, depending on the nature of the genetic markers and the number of markers used. The more markers used, the more likely it is to detect different alleles between two parasites. Clearly, determining whether a sample is clonal or has mixed genotypes requires careful selection of markers because most randomly selected MS markers may not faithfully reflect the true proportion of different alleles in a DNA mixture. We recommend that a sample be considered a mixed infection as soon as a minor allele is detected if accurate measurement of drug response is an important issue for a study (such as genetic mapping). We also suggested that, before using a MS marker or a method to quantitate DNA in mixed infections, the performance of the

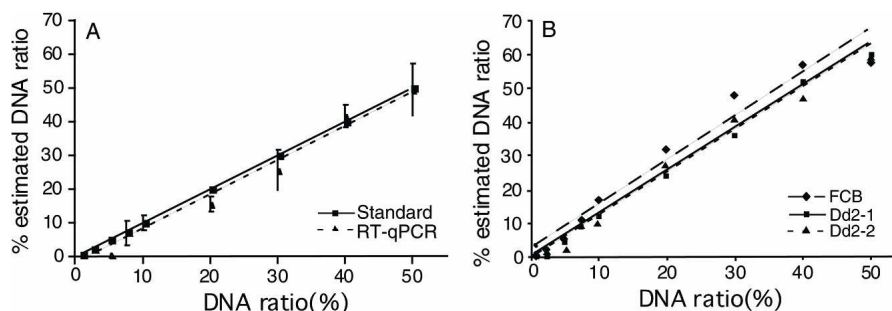


FIGURE 3. Estimating mixed DNA samples using real-time qPCR and pyrosequencing. Known DNA ratios from various parasite mixtures (Dd2 or FCB:HB3 at ratio: 1:99, 2.5:97.5, 5:95, 7.5:92.5, 10:90, 20:80, 30:70, 40:60, and 50:50) and the ratios estimated using real-time qPCR (A) and pyrosequencing (B) were plotted. “Standard” in A is the ratios of known parasite proportions; “RT-qPCR” indicates ratios estimated from real-time qPCR. The plots in B are from pyrosequencing of DNA mixtures of FCB:HB3 and Dd2:HB3, with ratios of Dd2:HB3 estimated twice (Dd2-1 and Dd2-2).

marker or method in correctly reflecting the true proportion of different alleles in the samples should be evaluated.

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## REFERENCES

- Yeung S, Pongtavornpinyo W, Hastings IM, Mills AJ, White NJ, 2004. Antimalarial drug resistance, artemisinin-based combination therapy, and the contribution of modeling to elucidating policy choices. *Am J Trop Med Hyg* 71: 179–186.
- Rieckmann KH, Campbell GH, Sax LJ, Mrema JE, 1978. Drug sensitivity of *Plasmodium falciparum*. An in-vitro microtechnique. *Lancet* 1: 22–23.
- Desjardins RE, Canfield CJ, Haynes JD, Chulay JD, 1979. Quantitative assessment of antimalarial activity in vitro by a semi-automated microdilution technique. *Antimicrob Agents Chemother* 16: 710–718.
- Makler MT, Ries JM, Williams JA, Bancroft JE, Piper RC, Gibbins BL, Hinrichs DJ, 1993. Parasite lactate dehydrogenase as an assay for *Plasmodium falciparum* drug sensitivity. *Am J Trop Med Hyg* 48: 739–741.
- Noedl H, Wernsdorfer WH, Miller RS, Wongsrichanalai C, 2002. Histidine-rich protein II: a novel approach to malaria drug sensitivity testing. *Antimicrob Agents Chemother* 46: 1658–1664.
- Noedl H, Bronnert J, Yingyuen K, Attlmayr B, Kollaritsch H, Fukuda M, 2005. Simple histidine-rich protein 2 double-site sandwich enzyme-linked immunosorbent assay for use in malaria drug sensitivity testing. *Antimicrob Agents Chemother* 49: 3575–3577.
- Druilhe P, Moreno A, Blanc C, Brasseur PH, Jacquier P, 2001. A colorimetric in vitro drug sensitivity assay for *Plasmodium falciparum* based on a highly sensitive double-site lactate dehydrogenase antigen-capture enzyme-linked immunosorbent assay. *Am J Trop Med Hyg* 64: 233–241.
- Bennett TN, Paguio M, Gligorijevic B, Seudieu C, Kosar AD, Davidson E, Roepe PD, 2004. Novel, rapid, and inexpensive cell-based quantification of antimalarial drug efficacy. *Antimicrob Agents Chemother* 48: 1807–1810.
- Smilkstein M, Sriwilaijaroen N, Kelly JX, Wilairat P, Riscoe M, 2004. Simple and inexpensive fluorescence-based technique for high-throughput antimalarial drug screening. *Antimicrob Agents Chemother* 48: 1803–1806.
- Bacon DJ, Latour C, Lucas C, Colina O, Ringwald P, Picot S, 2007. Comparison of a SYBR Green I based assay with an HRPII ELISA method for in vitro antimalarial drug efficacy testing and application to clinical isolates. *Antimicrob Agents Chemother* 51: 1172–1178.
- Baniecki ML, Wirth DF, Clardy J, 2007. High-throughput *Plasmodium falciparum* growth assay for malaria drug discovery. *Antimicrob Agents Chemother* 51: 716–723.
- Johnson JD, Dennull RA, Gerena L, Lopez-Sanchez M, Roncal NE, Waters NC, 2007. Assessment and continued validation of the malaria SYBR green I-based fluorescence assay for use in malaria drug screening. *Antimicrob Agents Chemother* 51: 1926–1933.
- Wellems TE, Panton LJ, Gluzman IY, do Rosario VE, Gwadz RW, Walker-Jonah A, Krogstad DJ, 1990. Chloroquine resistance not linked to mdr-like genes in a *Plasmodium falciparum* cross. *Nature* 345: 253–255.
- Wongsrichanalai C, Lin K, Pang LW, Faiz MA, Noedl H, Wimonwattawatee T, Laoboonchai A, Kawamoto F, 2001. In vitro susceptibility of *Plasmodium falciparum* isolates from Myanmar to antimalarial drugs. *Am J Trop Med Hyg* 65: 450–455.
- Noedl H, Wongsrichanalai C, Wernsdorfer WH, 2003. Malaria drug-sensitivity testing: new assays, new perspectives. *Trends Parasitol* 19: 175–181.
- Ferdig MT, Cooper RA, Mu J, Deng B, Joy DA, Su X-z, Wellems TE, 2004. Dissecting the loci of low-level quinine resistance in malaria parasites. *Mol Microbiol* 52: 985–997.
- Cooper RA, Lane KD, Deng B, Mu J, Patel JJ, Wellems TE, Su X-z, Ferdig MT, 2007. Mutations in transmembrane domains 1, 4 and 9 of the *Plasmodium falciparum* chloroquine resistance transporter alter susceptibility to chloroquine, quinine and quinidine. *Mol Microbiol* 63: 270–282.
- Anderson TJ, Haubold B, Williams JT, Estrada-Franco Section Sign JG, Richardson L, Mollinedo R, Bockarie M, Mokili J, Mharakurwa S, French N, Whitworth J, Velez ID, Brockman AH, Nosten F, Ferreira MU, Day KP, 2000. Microsatellite markers reveal a spectrum of population structures in the malaria parasite *Plasmodium falciparum*. *Mol Biol Evol* 17: 1467–1482.
- Bendixen M, Msangeni HA, Pedersen BV, Shayo D, Bodker R, 2001. Diversity of *Plasmodium falciparum* populations and

- complexity of infections in relation to transmission intensity and host age: a study from the Usambara Mountains, Tanzania. *Trans R Soc Trop Med Hyg* 95: 143–148.
20. Kobbe R, Neuhoﬀ R, Marks F, Adjei S, Langefeld I, von Reden C, Adjei O, Meyer CG, May J, 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.
  21. Lee SA, Yeka A, Nsohya SL, Dokomajilar C, Rosenthal PJ, Talisuna A, Dorsey G, 2006. Complexity of *Plasmodium falciparum* infections and antimalarial drug eﬃcacy at 7 sites in Uganda. *J Infect Dis* 193: 1160–1163.
  22. Viriyakosol S, Siripoon N, Petcharapirat C, Petcharapirat P, Jarra W, Thaithong S, Brown KN, Snounou G, 1995. Genotyping of *Plasmodium falciparum* isolates by the polymerase chain reaction and potential uses in epidemiological studies. *Bull World Health Organ* 73: 85–95.
  23. Babiker H, Ranford-Cartwright L, Sultan A, Satti G, Walliker D, 1994. Genetic evidence that RI chloroquine resistance of *Plasmodium falciparum* is caused by recrudescence of resistant parasites. *Trans R Soc Trop Med Hyg* 88: 328–331.
  24. Ferdig MT, Su X-z, 2000. Microsatellite markers and genetic mapping in *Plasmodium falciparum*. *Parasitol Today* 16: 307–312.
  25. Anderson TJ, Nair S, Qin H, Singlam S, Brockman A, Paiphun L, Nosten F, 2005. Are transporter genes other than the chloroquine resistance locus (pfcrt) and multidrug resistance gene (pfmdr) associated with antimalarial drug resistance? *Antimicrob Agents Chemother* 49: 2180–2188.
  26. Nair S, Nash D, Sudimack D, Jaidee A, Barends M, Uhlemann AC, Krishna S, Nosten F, Anderson TJ, 2007. Recurrent gene ampliﬁcation and soft selective sweeps during evolution of multidrug resistance in malaria parasites. *Mol Biol Evol* 24: 562–573.
  27. Dolan SA, Herrfeldt JA, Wellems TE, 1993. Restriction polymorphisms and ﬁngerprint patterns from an interspersed repetitive element of *Plasmodium falciparum* DNA. *Mol Biochem Parasitol* 61: 137–142.
  28. Trager W, Jensen JB, 1976. Human malaria parasites in continuous culture. *Science* 193: 673–675.
  29. Su X-z, Ferdig MT, Huang Y, Huynh CQ, Liu A, You J, Wootton JC, Wellems TE, 1999. A genetic map and recombination parameters of the human malaria parasite *Plasmodium falciparum*. *Science* 286: 1351–1353.
  30. Mu J, Ferdig MT, Feng X, Joy DA, Duan J, Furuya T, Subramanian G, Aravind L, Cooper RA, Wootton JC, Xiong M, Su X-z, 2003. Multiple transporters associated with malaria parasite responses to chloroquine and quinine. *Mol Microbiol* 49: 977–989.
  31. Cheesman SJ, de Roode JC, Read AF, Carter R, 2003. Real-time quantitative PCR for analysis of genetically mixed infections of malaria parasites: technique validation and applications. *Mol Biochem Parasitol* 131: 83–91.
  32. McNamara DT, Thomson JM, Kasehagen LJ, Zimmerman PA, 2004. Development of a multiplex PCR-ligase detection reaction assay for diagnosis of infection by the four parasite species causing malaria in humans. *J Clin Microbiol* 42: 2403–2410.
  33. Brockman A, Paul RE, Anderson TJ, Hackford I, Phaiphun L, Looareesuwan S, Nosten F, Day KP, 1999. Application of genetic markers to the identiﬁcation of recrudescence *Plasmodium falciparum* infections on the northwestern border of Thailand. *Am J Trop Med Hyg* 60: 14–21.
  34. Nyachieo A, VAN Omervier C, Laurent T, Dujardin JC, D'Alessandro U, 2005. *Plasmodium falciparum* genotyping by microsatellites as a method to distinguish between recrudescence and new infections. *Am J Trop Med Hyg* 73: 210–213.
  35. Colborn JM, Koita OA, Cisse O, Bagayoko MW, Guthrie EJ, Krogstad DJ, 2006. Identifying and quantifying genotypes in polyclonal infections due to single species. *Emerg Infect Dis* 12: 475–482.
  36. Greenhouse B, Myrick A, Dokomajilar C, Woo JM, Carlson EJ, Rosenthal PJ, Dorsey G, 2006. Validation of microsatellite markers for use in genotyping polyclonal *Plasmodium falciparum* infections. *Am J Trop Med Hyg* 75: 836–842.
  37. Certain LK, Sibley CH, 2007. *Plasmodium falciparum*: a novel method for analyzing haplotypes in mixed infections. *Exp Parasitol* 115: 233–241.
  38. Takala SL, Smith DL, Stine OC, Coulibaly D, Thera MA, Doumbo OK, Plowe CV, 2006. A high-throughput method for quantifying alleles and haplotypes of the malaria vaccine candidate *Plasmodium falciparum* merozoite surface protein-1 19 kDa. *Malar J* 5: 31.
  39. Cojean S, Noel A, Garnier D, Hubert V, Le Bras J, Durand R, 2006. Lack of association between putative transporter gene polymorphisms in *Plasmodium falciparum* and chloroquine resistance in imported malaria isolates from Africa. *Malar J* 5: 24.
  40. Martinelli A, Hunt P, Cheesman SJ, Carter R, 2004. Amplified fragment length polymorphism measures proportions of malaria parasites carrying specific alleles in complex genetic mixtures. *Mol Biochem Parasitol* 136: 117–122.
  41. Hunt P, Fawcett R, Carter R, Walliker D, 2005. Estimating SNP proportions in populations of malaria parasites by sequencing: validation and applications. *Mol Biochem Parasitol* 143: 173–182.
  42. Cheesman S, Creasey A, Degnan K, Kooij T, Afonso A, Cravo P, Carter R, Hunt P, 2007. Validation of pyrosequencing for accurate and high throughput estimation of allele frequencies in malaria parasites. *Mol Biochem Parasitol* 152: 213–219.