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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 employed to detect mixed infections; infections with single genotype or having a minor allele smaller than a subjectively selected cut-off value are often considered single infection. We investigate 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.¹ 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;² measuring [³H] hypoxanthine incorporation,³ parasite lactate dehydrogenase (LDH) activity⁴ or signals from antibodies against histidine-rich protein II (HRP II)^{5,6} or DLH;⁷ and staining parasite DNA with SYBR green or DAPI dyes.⁸⁻¹² These assays have been widely used to study drug application efficacy, to monitor drug resistance, and to map drug resistant genes.¹³⁻¹⁷

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Many factors can affect drug test results. As 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, as bloods are usually drawn once and patients are treated with antimalarial agents thereafter. Repeated tests under similar conditions are critical for malaria drug assays, as 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.¹⁸⁻²¹ 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 parasite²¹.

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.^{20,22,23} 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 (MS) were used, which may provide more realistic estimates of parasite populations, although MS generally vary at higher rates than single nucleotide polymorphisms (SNP).²⁴⁻²⁶ 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?

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 investigate 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 investigate 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

P. falciparum parasite isolates HB3, Dd2, FCB, and Dd2¹⁹² used in this study have been described.^{13,27} The parasites were cultured *in vitro* according to the methods of Trager and Jensen.²⁸ Briefly, parasites were maintained in RPMI-1640 medium containing 25 mM HEPES, 5% human O⁺ erythrocytes (5% hematocrit), 0.5% Albumax (GIBCO, Life Technologies, Grand Island, NY), 24 mM sodium bicarbonate, and 10 µg/mL gentamycin at 37°C with 5% CO₂, 5% O₂, and 90% N₂.

Drug assays

Drug assays were performed using a SYBR green staining method modified from a procedure described previously.⁹ Briefly, parasites were diluted to 1% parasitemia with 1% hematocrit shortly after thawing; and the diluted parasites (150 µL) were added to wells in a 96-well plate containing 50 µL properly diluted CQ. The drug was two-fold diluted in triplicate with concentration ranging from 10,000 nM to ~10 nM. The parasites were incubated with the drug at 37°C and measured at 72 hr. After incubation, DNA were released from cultured parasite and stained with SYBR green dye as described.⁹ The plate was kept in the dark for 30 min, and signals were read in a FLUOstar OPTIMA reader (BMG Labtech, Germany). Data from the microplate reader were analyzed using Prism 4.0 software (GraphPad Software, Inc., 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 Biosystems, 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,²⁹ except individually labeled primers were used. Briefly, each 14 µL polymerase chain reaction (PCR) mix contained 7.5 µL H₂O, 1.4 µL 10× buffer [200 mM Tris-HCl (pH8.4), 500 mM KCl and 50 mM MgCl₂], 0.3 µL 10 mM dNTP, 0.4 µL each of the two primers (50 pmol/µL), 0.1 unit Taq polymerase, and 4 µL diluted DNA (~4 ng). The reactions were denatured at 94°C for 2 min and cycled at 94°C for 20 sec, 45°C and 40°C for 10 sec each, and 60°C for 30 sec for 40 cycles. Fluorescently labeled PCR products (2.5 µL) 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 (qPCR)

Real-time 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'-TTAGATCAAATGAGACATCTT-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 min at 94°C for initial denaturation, 95°C for 30 sec, 50°C for 30 sec, 60°C for 30 sec for 45 cycles, and a final extension at 60°C for 2 min. The quantity of DNA was calibrated using a serial-diluted plasmid DNA (PVLH1X) with primer sequences: R-LucF3, 5'-TTGTGTTTGTGGACGAAGTAC-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, Dd2¹⁹²:HB3, FCB:HB3) were sent to EpigenDX (Worcester, MA; <http://www.epigenDX.com>) for pyrosequencing. Two single-nucleotide polymorphisms (A/T at nucleotide position 3100 and A/G at position 3125) in the putative *P. falciparum* multiple-drug-resistant gene 1 (*Pfmdr1*) were amplified and sequenced. Primers for PCR amplification and pyrosequencing were: 5'-TTATTGTAAATGCAGCTTTATGGG-3', 5'-GGGACACCGCTGATCGTTTAGATCCAAACCAATAGGCAAAAC-3', and 5'-GCAGCTTTATGGGGATT-3'.

RESULTS

Measurement of parasite IC₅₀

We used a SYBR green drug assay modified from a protocol described previously⁹ to measure parasite IC₅₀. Parasite responses to CQ were tested at least three times to obtain reproducible IC₅₀ for each sample. The average IC₅₀ values for the parasites used in this study were 13.6 ±2.2 (standard deviation) nM for HB3 (21 repeats); 237±40.6 nM for Dd2 (11 repeats); 391.6 ±33.3nM for Dd2¹⁹² (11 repeats), and 196.6±23.0nM for FCB (11 repeats) (Table 2). These results were comparable with those obtained using the [³H] hypoxanthine assay (Dd2, 404.1 nM; HB3, 33.9 nM; FCB, 492.2 nM), although the absolute values were different.³⁰

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 hr 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 IC₅₀ (shifting the curve left or approximately double the IC₅₀), 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 Dd2¹⁹²/HB3 and FCB/HB3, respectively (Figure 1C-F).

Effect of a highly resistant parasite on drug IC₅₀ measurement

Because patients can carry parasites that are resistant to a drug at different levels, we similarly tested a parasite (Dd2¹⁹²) with higher IC₅₀ than that of Dd2 and investigated how the parasite affected drug tests in mixtures with different ratios of resistant and sensitive parasites (Figure 1C and D). Parasite Dd2¹⁹² was derived from Dd2 under continuous CQ pressure *in vitro*²⁷ and has an IC₅₀ higher than Dd2 (391 nM vs. 237 nM; Table 2). Drug test results from mixtures of various Dd2¹⁹² and HB3 ratios showed that Dd2¹⁹² indeed had a more profound effect on IC₅₀ measurements than Dd2 at the same parasite ratios, producing higher IC₅₀ at mixtures with 10% or higher resistant parasites (Table 2). Similarly, FCB parasite had an IC₅₀ (191 nM) slightly lower than that of Dd2; and mixtures with 30%-50% FCB parasite also had lower IC₅₀ than those of Dd2. The results showed that parasites with different resistance levels (IC₅₀) could also have different impacts on IC₅₀ measurements of mixed parasite populations.

Correlation of drug test IC₅₀ and genotype ratios using MS

Genotyping using genetic markers such as MS is frequently employed to detect mixed parasite populations in patient blood samples; however, it can be difficult to detect or to rule out a mixed infection. To investigate how well genotyping reflects the actual parasite (DNA) proportions in a mixture, we genotyped DNA mixtures with various ratios of CQR and CQS 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, as the standard deviations for the markers (except C1M4) were generally small, and the plots from DNA samples of Dd2:HB3 and Dd2¹⁹²: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, Dd2¹⁹², 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, Dd2¹⁹², and FCB were 4 to 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 and 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.³¹ 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 appeared 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 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 investigations. In this study, we show 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_{50} 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_{50} . 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 re-infection.³²⁻³⁸ Polymorphic genetic markers such as highly polymorphic genes or MS are often employed to screen mixed infections;^{25,39} 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 tests (Figure 1 and 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, or peaks from DNA sequencing signals were linearly related to the proportions of parasite DNA in a genetically mixed sample,^{31,40,41} 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 appeared 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 appeared 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 recommended 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 marker or method in correctly reflecting the true proportion of different alleles in the samples should be evaluated.

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Figure 1.

Growth inhibition drug assays of parasite mixtures. Chloroquine-resistant parasites (Dd2, Dd2¹⁹², and FCB) were mixed with chloroquine-sensitive parasite HB3 at various ratios. Inhibition data of Dd2:HB3 ratios from 100:0 to 50:50 were plotted in (A); from ratios of 1:99 to 7.5:92.5 in (B); Dd2¹⁹²(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 standard error bars indicate variations between tests performed at 48, 96, and 144 hr. Negative values were generated after subtraction from controls (no drug). A mixture of 10% of resistant parasites can greatly affect IC₅₀ of a sensitive parasite, whereas a mixture of 5% or less has minimum affects.

Figure 2.

Typing mixed DNA samples using microsatellite (MS) markers. DNA samples from mixed parasite populations of Dd2/HB3, Dd2¹⁹²/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, Dd2¹⁹² or FCB (minor alleles) over HB3 were plotted against ratios of mixed parasite populations (Dd2/Dd2¹⁹²/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 MS produced different estimates of DNA ratios.

Figure 3.

Estimating mixed DNA samples using real-time quantitative polymerase chain reaction (PCR) 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).

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 ¹⁹²	FCB		
C1M4	1	G38014	212	202	202	190	atctctacacagggaagca	ggcacataataatacatatc
BM17	6	G44440	142	140	140	138	aacgataatggtgatgataaa	gcacacatcgattatcatat
B5M5	3	G42731	207	213	213	225	taataataacaactactaatga	atcatgctctcagcattc
B5M124	11	G44571	226	194	194	176	taataagtgtaagaaatatgga	aaacagagcacaataaagcagt
C13M13	13	G44636	142	134	134	132	gtataaggtatagcagacga	taaatataatatacatattacgg
C14M17	14	G41045	186	190	190	216	acacaaagagaataggatata	ggcaatctatggcttaac

^{*} Product sizes were estimated using labeled primers and might not match the sizes in the genome or reported previously.²⁷

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

Table 2
IC₅₀ 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 ₅₀ (D42: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 ₅₀ (D42 ¹⁹² :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 ₅₀ (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 nM) were averages and standard deviations from 11 or more repeats. The rest of the measurements were from 4-6 repeats.