A Multiplex Ligase Detection Reaction-Fluorescent Microsphere Assay for Simultaneous Detection of Single Nucleotide Polymorphisms Associated with *Plasmodium falciparum* Drug Resistance[▽]

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Incomplete malaria control efforts have resulted in a worldwide increase in resistance to drugs used to treat the disease. A complex array of mutations underlying antimalarial drug resistance complicates efficient monitoring of parasite populations and limits the success of malaria control efforts in regions of endemicity. To improve the surveillance of *Plasmodium falciparum* drug resistance, we developed a multiplex ligase detection reaction–fluorescent-microsphere-based assay (LDR-FMA) that identifies single nucleotide polymorphisms (SNPs) in the *P. falciparum* *dhfr* (9 alleles), *dhps* (10 alleles), and *pfcr* (3 alleles) genes associated with resistance to Fansidar and chloroquine. We evaluated 1,121 blood samples from study participants in the Wosera region of Papua New Guinea, where malaria is endemic. Results showed that 468 samples were *P. falciparum* negative and 453 samples were *P. falciparum* positive by a *Plasmodium* species assay and all three gene assays (concordance, 82.2%). For *P. falciparum* infections where the assay for each gene was positive, 2 samples carried resistance alleles for all three genes, 299 carried resistance alleles for *dhfr* and *pfcr*, 131 carried resistance alleles for only one gene (*dhfr* [*n* = 40], *dhps* [*n* = 1], or *pfcr* [*n* = 90]), and 21 carried only sensitive alleles at all three genes. Mixed-strain infections characterized 100 samples. Overall, 95.4% (432/453) of *P. falciparum*-infected samples carried at least one allele associated with resistance to Fansidar or chloroquine. In view of the fact that 86.3% (391/453) of *P. falciparum*-infected samples carried *pfcr* mutations, chloroquine is largely ineffective against *P. falciparum* in Papua New Guinea. Surveillance of additional *dhfr* and *dhps* polymorphisms in order to monitor the continued effectiveness of Fansidar is recommended.

Plasmodium falciparum strains exhibit resistance to many antimalarial drugs in most regions of the world where malaria is endemic. In some regions, individual strains are resistant to more than one drug. With a very limited arsenal of safe and effective antimalarial drugs, complex genetic factors contributing to drug resistance pose a constant challenge to efforts to control this important human parasite. Moreover, it has been observed that as drug-resistant *P. falciparum* evolves and spreads within regions of endemicity and resistant strains become predominant, both transmission and the morbidity and mortality attributable to malaria increase (29). With increasing travel around the world, drug-resistant malaria parasites present further challenges in prescribing effective prophylactic treatment for tourists, military personnel, and humanitarian aid workers. Additionally, infected travelers are likely to increase the exchange of parasite strains between regions where different patterns of drug resistance are observed.

Molecular genetic studies of *P. falciparum* have enabled identification of a number of specific mutations in genes linked to resistance to specific antimalarial drugs (34, 35). These

include genes encoding the *P. falciparum* chloroquine resistance transporter (*pfcr*) (10), dihydrofolate reductase (*dhfr*) (7, 24), and dihydropterate synthetase (*dhps*) (30, 31), which confer resistance to chloroquine, pyrimethamine, and sulfadoxine, respectively. Mutations in the latter two genes confer resistance to the drug combinations Fansidar (pyrimethamine-sulfadoxine) and LAPDAP (chlorproguanil-dapsone) (22, 36). Our interest in monitoring these genes for single nucleotide polymorphisms (SNPs) associated with drug resistance was based on a number of technical and field surveillance objectives. Whereas numerous PCR-based approaches have been used to analyze polymorphisms in the *P. falciparum* *dhps*, *dhfr*, and *pfcr* genes, most strategies involve post-PCR restriction fragment length polymorphism or DNA probe hybridization methods that are cumbersome (2, 9, 18, 25, 26, 33). Here we describe a post-PCR approach for screening 22 different allelic variants of the *dhps*, *dhfr*, and *pfcr* genes associated with drug resistance in a single-tube multiplex assay by using a ligase detection reaction–fluorescent-microsphere assay (LDR-FMA) strategy (17). This approach avoids many limitations that decrease the efficiency of other post-PCR analyses. LDR-FMA analyses also enable semiquantitative comparisons between strain-specific polymorphisms. Finally, our studies have been motivated by the need to increase the capacity for evaluating these polymorphisms in Papua New Guinea, where chloroquine and Fansidar constitute the government's recommended first-line antimalarial treatment. Overall, our study

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TABLE 1. PCR primers and amplification conditions for *P. falciparum* *dhfr*, *dhps*, and *pfcr*

Reaction ^a	Primer sequence	Thermocycling conditions
<i>dhps</i>	5'-AATGATAAATGAAGGTGCTAGT-3' 5'-ATGTAATTTTGTGTGTATTTA-3'	35 cycles of 95°C for 30 s, 56°C for 30 s; 60°C for 60 s
<i>dhfr</i> N1	5'-TTTATATTTTCTCCTTTTAA-3' 5'-ACATTTTATTATTCGTTTTC-3'	15 cycles of 95°C for 30 s, 50°C for 30 s; 60°C for 60 s
<i>dhfr</i> N2	5'-ATGATGGAACAAGTCTGCGAC-3' 5'-CATTTTATTATTCGTTTTC-3'	35 cycles of 95°C for 30 s, 50°C for 30 s; 60°C for 60 s
<i>dhfr</i>	5'-TAACTACACATTTAGAGGTCTA-3' 5'-GTTGTATTGTTACTAGTATATAC-3'	35 cycles of 95°C for 30 s, 56°C for 30 s, 72°C for 60 s
<i>pfcr</i> N1	5'-CATTGTCTTCCACATATATGACATAAA-3' 5'-TTGGTAGGTGGAATAGATTCTCTT-3'	15 cycles of 95°C for 30 s, 56°C for 30 s; 60°C for 60 s
<i>pfcr</i> N2	5'-CCGTTAATAATAAATACACGCAG-3' 5'-CGGATGTTACAAACTATAGTTACC-3'	35 cycles of 95°C for 30 s, 56°C for 30 s; 60°C for 60 s

^a N1, nest 1; N2, nest 2.

demonstrates new potential for efficient large-scale evaluation of antimalarial drug resistance in drug efficacy studies and molecular epidemiologic surveillance of *P. falciparum* populations.

MATERIALS AND METHODS

Study population and blood sample collection. The study was part of an ongoing (1990 to the present) effort to monitor asymptomatic and clinical *Plasmodium* species infections in the Wosera, East Sepik Province, Papua New Guinea (11, 12). In this region of northern, lowland Papua New Guinea, all four

human malaria parasite species, *P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale*, are holoendemic. Finger prick blood samples evaluated in this study ($n = 1,121$; collected between August 2001 and June 2003) were used to produce thick or thin blood smears, determine hemoglobin concentration, and extract DNA (K^+ -EDTA Microtainers). Informed consent was obtained from all study participants; this study was approved by the Medical Research Advisory Committee of Papua New Guinea and by the Institutional Review Board for Human Investigation at University Hospitals of Cleveland, Cleveland, OH. We also analyzed samples from 95 American Red Cross blood donors (37). All donors (18 to 55 years of age) were self-identified as Caucasian-American and had no history of malaria exposure.

FIG. 1. Overview of post-PCR LDR-FMA diagnosis of drug resistance-associated SNPs. Allele-specific oligonucleotides include 5' TAG extensions of 24 nucleotides. TAG sequences ($n = 100$), containing no G residues, have been designed to reduce the potential for cross-hybridization with sequences amplified from naturally occurring target sequences. Conserved sequence oligonucleotides are 5' phosphorylated and 3' biotinylated. Fluorescent-microsphere sets ($n = 100$) are embedded with varying ratios of red and infrared fluorochromes to result in unique fluorescent “classification” signatures. Each microsphere set is precoupled to unique anti-TAG oligonucleotides specifically complementary to the TAG sequences. Following ligation detection and TAG-anti-TAG hybridization reactions, doubly labeled ligation products are detected, and the “reporter” signal is classified into allele-specific bins by the BioPlex array reader and BioPlex Manager (version 3.0) software. Data from these reactions are immediately available in Excel spreadsheets.

TABLE 2. LDR primers for *P. falciparum* *dhfr*, *dhps*, and *pfcr*

Gene	Primer	Sequence ^a	FlexMAP microsphere set ID no. ^b
<i>dhps</i>	436-7 SA	5'-ttacctttatctctttttttacTAGGTGGAGAATCCTCTGC-3'	30
	436-7 SG	5'-caattcaaatcacaataatcaatcTAGGTGGAGAATCCTCTGG-3'	5
	436-7 AG	5'-caattactacatacaatacatcacAGGTGGAGAATCCGCTGG-3'	77
	436-7 FG	5'-ctattacacttttaaacatcaatacATAGGTGGAGAATCCTTTGG-3'	92
	436-7 CM ^c	5'-phosphate-TCCCTTTTGTATACCTAATCCA-biotin-3'	
	540 K	5'-ctacaacaacaacattatcaaGGAATCCACATACAATGGATA-3'	28
	540 E	5'-ctttaactctttatcattatcaGAAATCCACATACAATGGATG-3'	17
	540 CM	5'-phosphate-AACTAACAAATTATGATAATCTAG-biotin-3'	
	581 A	5'-aatctacaacactcctcaataacTTGATATTGGATTAGGATTTGC-3'	76
	581 G	5'-ctttcaattacaataactcattacaTTGATATTGGATTAGGATTTGG-3'	43
	581 CM	5'-phosphate-GAAGAAACATGATCAATCTATTA-biotin-3'	
	613 A	5'-tacacttttaacttactacactaaGATATTCAAGAAAAAGATTTATTG-3'	95
	613 S	5'-aaacaacttcacatctcaataatGGATATTCAAGAAAAAGATTTATTT-3'	48
	613 CM	5'-phosphate-CCCATTGCATGAATGATCAAA-biotin-3'	
<i>dhfr</i>	51 I	5'-caatttcattcattcatttcaGAGTATTACCATGGAAATGTAT-3'	35
	51 N	5'-ctactatacatcttactatactttGAGTATTACCATGGAAATGTAA-3'	14
	51 CM	5'-phosphate-TTCCCTAGATATGAAATATTTT-biotin-3'	
	59 R	5'-cttttcattctttcatttcaatTCACATATGTTGTAAGTGCACG-3'	37
	59 C	5'-tacaactttttctttcttttTCACATATGTTGTAAGTGCACA-3'	12
	59 CM	5'-phosphate-AAAAATTTTCATATCTAGGGAAWTA-biotin-3'	
	108 T	5'-ctaactaacaataattaactaacTTGTAGTTATGGGAAGAACAAAC-3'	80
	108 S	5'-ctataacatattacattcacatcTTGTAGTTATGGGAAGAACAAAG-3'	69
	108 N	5'-tcataatcaactcttttcaactttTTGTAGTTATGGGAAGAACAAA-3'	59
	108 CM	5'-phosphate-CTGGGAAAGCATTCCAAAAAAA-biotin-3'	
	164 I	5'-ctttctatctttactcaataatGAAATTAATTAATAAATGTTTTATTA-3'	94
	164 L	5'-ctatctttaaactacaatactaacGAAATTAATTAATAAATGTTTTATTT-3'	100
	164 CM	5'-phosphate-TAGGAGGTTCCGTTGTTTATC-biotin-3'	
<i>pfcr</i>	CVMNK	5'-aatctacaatccaataatctcatATTTAAGTGTATGTGTAATGAATAA-3'	60
	CVIET	5'-tcataatctcaaatctttctttAATTAAGTGTATGTGTAATTGAAAC-3'	68
	SVMNT	5'-aatctcttttaactcaataatcaATTTAAGTGTGTAATGAATAC-3'	21
	72-76 CM	5'-phosphate-AATTTTTGCTAAAAGAAGCTTTAAAC-biotin-3'	

^a Lowercase nucleotides (24 bases) represent TAG sequences added to the 5' ends of each allele-specific LDR primer. W, T or A.

^b ID, identification. One hundred unique Luminex microsphere sets are synthesized to exhibit unique fluorescence. Each microsphere set is coupled to different anti-TAG sequences. Anti-TAG sequences are complementary to allele-specific TAG sequences.

^c CM, common sequence primer positioned immediately downstream from the allele-specific primer.

Laboratory-adapted *P. falciparum* strains. The following *P. falciparum* laboratory-adapted strains were obtained from the Malaria Research and Reference Reagent Resource (MR4; ATCC, Manassas, VA): HB3 (MR4-155), Dd2 (MR4-150), K1 (MR4-159), 3D7 (MR4-102), FCB (MR4-309), VS/1 (MR4-176), and 7G8 (MR4-154). The Papua New Guinean *P. falciparum* strain 1917 was kindly provided by Karen Day. *P. falciparum* was grown in vitro as described previously (17). Thin blood smears were fixed with 100% methanol for 30 s, stained with 4% Giemsa stain for 30 min, and examined by microscopy with an oil immersion objective (100×). Parasitemia was calculated as (number of infected erythrocytes)/(number of infected plus uninfected erythrocytes [$n = 1,000$]).

DNA template preparation. DNA was extracted from whole blood (200 μ l) using the QIAamp 96 DNA blood kit (QIAGEN, Valencia, CA). Genomic DNA was extracted from *P. falciparum* cultures (200 μ l) by using the QIAamp DNA blood minikit (QIAGEN, Valencia, CA).

PCR amplification of *Plasmodium* species rDNA and *P. falciparum* *dhps*, *dhfr*, and *pfcr* target sequences. All reactions (25 μ l) were performed in a buffer containing 3 pmol of appropriate upstream and downstream primers, 67 mM Tris-HCl (pH 8.8), 6.7 mM MgSO₄, 16.6 mM (NH₄)₂SO₄, 10 mM 2-mercaptoethanol, 100 μ M (each) dATP, dGTP, dCTP, and dTTP, and 2.5 U of thermostable DNA polymerase. Amplification reactions were performed in a Peltier thermal cycler (PTC-225; MJ Research, Watertown, MA). The specific primers and thermocycling conditions used to amplify small-subunit ribosomal DNA (rDNA) gene segments for *Plasmodium* species diagnosis have been described previously (14, 17, 19). The specific primers and thermocycling conditions used to amplify *P. falciparum* *dhps*, *dhfr*, and *pfcr* target sequences for evaluation of polymorphisms associated with antimalarial drug resistance are listed in Table 1. Following analysis of 630 samples, minor adjustments to the PCR protocol eliminated the necessity for performing nest 1 reactions to evaluate *dhfr* and *pfcr* (for comparisons, see Results). After PCR amplification, products were loaded

on 2% agarose I (Amresco, Solon, OH) gels, and electrophoresis was performed in 1× TBE buffer (8.9 mM Tris, 8.9 mM boric acid, 2.0 mM EDTA). The gels were first stained for 30 min with SYBR Gold (Molecular Probes, Eugene, OR) diluted 1:10,000 in 1× TBE buffer, and DNA products were visualized on a Storm 860 PhosphorImager using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

LDR-FMA evaluation of *Plasmodium* species and of *P. falciparum* *dhps*, *dhfr*, and *pfcr* sequence polymorphisms. We have recently provided a detailed description of all methods and strategies used to perform LDR-FMA diagnosis of *Plasmodium* species infections (17). In the current study, these methods have been adapted directly to perform analysis of SNPs associated with resistance to sulfadoxine in *dhps*, pyrimethamine in *dhfr*, and chloroquine in *pfcr*. The following brief description and the summary in Fig. 1 provide an overview of the three-step, post-PCR LDR-FMA procedure.

Following PCR amplification of the gene-specific target sequences carrying the drug resistance-associated SNPs, products are combined into a multiplex LDR (step 1) where allele-specific upstream primers ligate to conserved sequence downstream primers. Upstream, allele-specific primers include 5' extensions of unique TAG sequences (see Fig. 1 legend); downstream, conserved sequence primers are modified by 5' phosphorylation and 3' biotinylation. The 5' ends of the LDR products receive "classification" labeling in a second multiplex reaction (step 2), where hybridization occurs between the TAG sequences added to the allele-specific primers and anti-TAG (complementary-sequence) oligonucleotide probes bound to fluorescent microspheres. Following this hybridization reaction, products are incubated (step 3) in a solution containing streptavidin-R-phycoerythrin to allow "reporter" labeling through binding to the 3'-biotin on the conserved sequence primers. Detection of doubly labeled ligation products occurs through dual-fluorescence flow cytometry in the Bio-Plex array reader (Bio-Rad Laboratories, Hercules, CA) and leads to collection of "reporter"

FIG. 2. Detection density plot of LDR-FMA products. White areas represent expected result targets given the microspheres specified. Classification 1 and classification 2 each represent the signal intensity from one of two fluorochromes applied to Luminex FlexMAP polymer microspheres. Dots within white areas represent individual detection events as determined by the BioPlex suspension array system, where allele-specific LDR products are labeled with both “classification” (the fluorescent microsphere at the 5’ end) and “reporter” (R-phycoerythrin at the 3’ end) fluorescent signals. Individual *dhps* alleles are identified by Arabic numerals above the target area as follows: 1, 436S–437A; 2, 436S–437G; 3, 436A–437G; 4, 436F–437G; 5, 540K; 6, 540E; 7, 581A; 8, 581G; 9, 613A; 10, 613S. Individual *dhfr* alleles are identified by lowercase letters at the upper right edge of the target area as follows: a, 51N; b, 51I; c, 59C; d, 59R; e, 108S; f, 108N; g, 108T; h, 164I; i, 164L. Individual *pfert* alleles are identified by Roman numerals below the target area as follows: I, CVMNK; II, SVMNT; III, CVIET. Analysis was performed using BioPlex Manager (version 3.0) software.

signals in unique allele-specific bins. Anti-TAG oligonucleotide probes bound to fluorescent microspheres (2.5×10^5 beads/ml; \$25.00) are available from Luminex Corporation (Austin, TX); individual assays receive 1 μ l of required microsphere solutions (\$0.25/microsphere/reaction).

Specific LDR primers used in this assay are listed in Table 2. These LDRs were performed in a solution (15 μ l) containing 20 mM Tris-HCl buffer (pH 7.6), 25 mM potassium acetate, 10 mM magnesium acetate, 1 mM NAD⁺, 10 mM dithiothreitol, 0.1% Triton X-100, 10 nM (200 fmol) each LDR probe, 1 μ l of each PCR product, and 2 U of *Taq* DNA ligase (New England Biolabs, Beverly, MA). Reaction mixtures were initially heated at 95°C for 1 min, followed by 32 thermal cycles at 95°C for 15 s (denaturation) and 58.0°C for 2 min (annealing/ligation). The multiplex LDR product (5 μ l) was then added to 60 μ l of hybridization solution (3 M tetramethylammonium chloride [TMAC], 50 mM Tris-HCl [pH 8.0], 3 mM EDTA [pH 8.0], 0.10% sodium dodecyl sulfate) containing 250 Luminex FlexMAP microspheres from each allelic set (total number of alleles, 22). Mixtures were heated to 95°C for 90 s and incubated at 37°C for 40 min to allow hybridization between SNP-specific LDR products and bead-labeled anti-TAG probes. Following hybridization, 6 μ l of streptavidin–R-phycoerythrin (Molecular Probes, Eugene, OR) in TMAC hybridization solution (20 ng/ μ l) was added to the post-LDR mixture and incubated at 37°C for 40 min in Costar 6511 M polycarbonate 96-well V-bottom plates (Corning Inc., Corning, NY). Hybrid complexes consisting of SNP-specific LDR products and microsphere-labeled anti-TAG probes were detected using a Bio-Plex array reader (Bio-Rad Laboratories, Hercules, CA); the plate temperature was set to 37°C throughout

detection. All fluorescence data were collected using Bio-Rad (Hercules, CA) software, Bio-Plex Manager 3.0.

We used a heuristic method to differentiate positive from negative fluorescent signals in samples from their field study participants across the array of targeted SNPs associated with antimalarial drug resistance. Here we compared fluorescence intensities from the individual drug resistance SNP assays and diagnostic results for *P. falciparum*-positive and -negative signals detected by our recently described species-specific diagnostic assay (17). In the first step of this heuristic method, we partitioned all samples into one of two categories: *P. falciparum* infected or not *P. falciparum* infected. Next, we evaluated fluorescent signals in stepwise fashion for each drug resistance-associated SNP allele (1 U of fluorescence per step) among *P. falciparum*-infected and non-*P. falciparum*-infected samples to determine cutoff thresholds for each allele. These comparisons produced an array of outcomes representing different levels of concordance among the *Plasmodium* species assay and the *P. falciparum dhps*, *dhfr*, and *pfert* assays. The *Plasmodium* species diagnostic assay determined infection status (infected or not infected with *P. falciparum*) in step 1; *P. falciparum*-infected samples were distinguished from non-*P. falciparum*-infected samples as described by Kasehagen et al. (14). Species assay results were then compared with individual drug susceptibility markers by evaluating allele-specific median fluorescent intensity (MFI) units (1 to 25,000) for all samples to determine cutoff thresholds for each allele (see Table 4). Samples were then judged positive or negative for both species and drug susceptibility markers and were classified as concordant or discordant. For samples with incomplete drug-resistant haplotypes, the heuristic

FIG. 3. LDR-FMA analysis of a series of HB3 and Dd2 mixed genomic DNA preparations at two loci on the *dhps* gene, neighboring codons 436 (S or F) and 437 (A or G). The six genomic DNA preparations were arranged to carry HB3/Dd2 ratios of 12,500:12, 3,150:48, 781:195, 195:781, 48:3,150, and 12:12,500 parasitized erythrocytes per μ l, respectively.

FMA shows that allele-specific background signals ranged from 150 to 744 and positive allele-specific signals ranged from 8,779 to 15,142. Strain-specific LDR-FMA results were 100% concordant with those predicted (GenBank accession numbers: AF233068 for HB3, AF030694 for Dd2, and AF233067 for 7G8).

Semiquantitative detection of alleles associated with drug sensitivity and resistance. As indicated above, the fluorescence array reader accumulated reporter signals within SNP-specific bins in relation to the amount of LDR product generated for each individual allele. To determine whether these allele-specific signals reflected relative differences in strain-specific parasitemia, we show results from a representative mixing experiment focused on the *dhps*(A613S) SNP; this allele is known to differ between HB3 (613A) and Dd2 (613S). In this experiment, genomic DNAs for the two strains were mixed in varying ratios approximating those of HB3-Dd2 parasitemias: 10,000:10, 3,100:50, 800:200, 200:800, 50:3,100, and 10:10,000. The results from this semiquantitative mixing experiment (Fig. 3) show that the rise and fall of allele-specific fluorescent signals correspond to changes in estimated strain-specific parasitemias. Additionally, the results show that the major allele was identified by a stronger relative fluorescent signal in each mixture.

Evaluation of field samples from regions of Papua New Guinea where malaria is endemic. Given the specificity of the allele-specific LDR-FMA, we were interested in evaluating this multiplex assay for drug resistance polymorphisms in the context of 1,121 Papua New Guinea study participant samples collected from a health center and community surveys in the Wosera. First, we compared results between *P. falciparum* diagnosis using our recently described *Plasmodium* species LDR-FMA and each of the *dhps*, *dhfr*, and *pfprt* diagnostic assays individually. Comparisons made between *P. falciparum* positivity or negativity and signal intensities for each allele-specific assay were performed by a heuristic method to identify cutoff thresholds for each allele. Table 4 reports both median fluorescence intensity (MFI) cutoff thresholds and maximum MFI

values for each allele. We observed an interassay concordance of 90.4 to 93.1%. For discordant samples, the *dhps* and *dhfr* assays were more often negative, while the *pfprt* assay was more often positive, for *P. falciparum* than the *Plasmodium* species diagnostic assay. Overall, a concordance of $\geq 90\%$ was observed regardless of whether the drug resistance assays were performed one gene at a time (3 to 10 allele-specific probes) or as a multiplex comprising all three genes (22 allele-specific probes) (data not shown). In a further evaluation (Table 5) among the *Plasmodium* species assay and the *dhps*, *dhfr*, and *pfprt* diagnostic assays, we observed 82.2% (921/1,121) concordance for positive and negative detection of *P. falciparum* among all four assays, suggesting that these gene-specific assays detect *P. falciparum* infection comparably.

Further technical results in Fig. 4A and B show representative comparisons for *dhfr* and *pfprt* to illustrate the identification of samples showing the presence of single- and mixed-strain *P. falciparum* infections at the Papua New Guinea study site. Given the results shown in Fig. 3, suggesting that semi-quantitative comparisons between alleles within mixed-strain infections can be performed, we were interested in observing the relationships among signal intensities for infections in vivo. Recalling data from Table 5 indicating that 468 patient samples showed no fluorescence signal for any of the *dhps*, *dhfr*, or *pfprt* allele-specific probes, it is clear that a large number of data points converge at the origin in the graphs. The results for both polymorphisms in Fig. 4 (that at *dhfr* codon 59 and that for *pfprt*) show a broad range of fluorescence signals for both

TABLE 4. MFI detection thresholds and maxima for *P. falciparum* *dhfr*, *dhps*, and *pfprt* in field samples

Gene and allele	MFI	
	Threshold ^a	Maximum
<i>dhps</i>		
436S-437A	237	25,450
436S-437G	453	12,963
436A-437G ^b	569	
436F-437G ^b	961	
540K	537	23,623
540E ^b	762	
581A	489	22,004
581S ^b	2,145	
613A	610	21,778
613S ^b	1,850	
<i>dhfr</i>		
51I ^b	2,443	
51N	1,283	25,902
59R	660	13,790
59C	1,012	23,938
108T	859	10,234
108S	3,109	21,670
108N	2,544	24,452
164I	311	24,547
164L ^b	897	
<i>pfprt</i>		
CVIET ^b	1,960	
CVMNK	4,824	15,490
SVMNT	6,579	21,439

^a MFI above which samples are judged to be infected with a *P. falciparum* strain carrying the designated allele.

^b Allele not detected in the current population survey.

TABLE 5. Population survey data for fully concordant samples^a for *P. falciparum* infection among *Plasmodium* species and *dhps*, *dhfr*, and *pfcr* diagnostic assays

<i>dhps</i> haplotype	<i>pfcr</i> haplotype ^b	No. of samples with the indicated <i>dhps</i> and <i>pfcr</i> haplotypes and the following <i>dhfr</i> haplotype:						
		NCSI	NCNI	NCTI	NRNI	Mixed	None	Total
SAKAA	CVMNK	21	4		27	9		61
	SVMNT	88	6	1	205	69		369
	Mixed	2			7	11		20
	None							0
	Total	111	10	1	239	89	0	450
Mixed-haplotype infections ^c	CVMNK							0
	SVMNT				2			2
	Mixed							0
	None							0
	Total	0	0	0	2	0	0	2
None	CVMNK							0
	SVMNT							0
	Mixed							0
	None						468	468
	Total	0	0	0	0	0	468	468

^a Samples were judged fully concordant if the *Plasmodium* species assay and the *P. falciparum dhps*, *dhfr*, and *pfcr* assays were all positive or all negative. These include the 921 samples identified here (450 with the *dhps* SAKAA allele, 2 with *dhps* mixed-haplotype infections, 468 with no *dhps* allele, and 1 carrying *pfcr* CVMNK, *dhfr* NCSI, and *dhps* SGKAA).

^b The CVIET *pfcr* haplotype was not observed in the samples screened for this study.

^c Two *P. falciparum* infections were characterized by parasite strains carrying both *dhps* SAKAA and SGKAA alleles.

alleles, and it appears that when viewed from the perspective of a single polymorphism, the majority of infections were characterized by a single allele. At this time, differences in the maximum fluorescence detected (Table 3) are likely to be characteristics of specific microsphere sets and not differences in strain-specific parasitemia. (Further information related to this assessment is available from Luminex Corporation.) Results for *dhfr* appear to have detected more mixed-strain infections ($n = 89$) than were observed for *pfcr* ($n = 20$).

Results from Table 5 also illustrate the drug susceptibility characteristics of *P. falciparum* in infections surveyed at the Papua New Guinea study site. Our results show that 49.2% (453/921; 450 samples carrying *dhps* SAKAA, 2 samples carrying mixed *dhps* SAKAA-SGKAA, and 1 sample carrying *dhps* SGKAA) of the samples fully concordant for the four LDR-FMA assays were *P. falciparum* infected. These results show that 86.3% of samples (391/453) were characterized by parasites carrying chloroquine-resistant alleles (*pfcr* SVMNT only), 75.1% (341/453) were infected with parasites carrying *dhfr* mutations associated with pyrimethamine resistance, and 0.6% (3/453) were infected with parasites carrying *dhps* mutations associated with sulfadoxine resistance (*dhps* S₄₃₆G₄₃₇K₅₄₀A₅₈₁A₆₁₃ only). The major drug-resistant *dhfr* allele, observed in 327 infected samples, was N₅₁C₅₉N₁₀₈I₁₆₄, which differs from the drug-sensitive *dhfr* allele N₅₁C₅₉S₁₀₈I₁₆₄ at codons 59 and 108. The N₅₁C₅₉N₁₀₈I₁₆₄ and N₅₁C₅₉T₁₀₈I₁₆₄ *dhfr* alleles, characterized by single amino acid substitutions, were observed in samples from 13 individuals and 1 individual, respectively. Of the *P. falciparum*-infected samples, 95.4% (432/453) carried at least one chloroquine, pyrimethamine, or

sulfadoxine resistance allele, 66.0% (299/453) carried both chloroquine and pyrimethamine resistance alleles, and only 2 infected samples carried *pfcr*, *dhfr*, and *dhps* alleles associated with resistance to chloroquine and Fansidar.

DISCUSSION

Our study introduces a new approach for evaluating *P. falciparum* polymorphisms associated with resistance to multiple antimalarial drugs. Whereas recent discussion has strongly suggested that chloroquine and Fansidar are no longer useful for malaria treatment and control in many regions of endemicity (3), we have focused on developing an assay to evaluate mutations in the *dhps*, *dhfr*, and *pfcr* genes associated with resistance to these drugs for two practical reasons. Monitoring the status of more than 20 SNPs in these genes in a single-tube multiplex assay is well beyond the capabilities of existing restriction fragment length polymorphism and real-time PCR diagnostic methods. The LDR-FMA strategy described here, therefore, presents unique advantages in monitoring *P. falciparum* drug resistance and introduces a new approach for strain-specific surveillance of microbial pathogens more generally. Beyond the technical objectives of our study, we were interested in developing this multiplex diagnostic assay for monitoring *P. falciparum* resistance to chloroquine and Fansidar, because this has become the first-line antimalarial treatment recommended by the government of Papua New Guinea, where our malaria field studies are based.

Our results using genomic DNA samples of laboratory-adapted *P. falciparum* strains showed that all SNPs were de-

FIG. 4. LDR-FMA diagnosis of 921 patient samples at *dhfr* codon 59 (A) and *pfcr* codons 72 to 76 (B). Results from the multiplex LDR-FMA show fluorescence captured for two alleles simultaneously for each individual sample: 59C and 59R for *dhfr* and CVMNK and SVMNT for *pfcr*. Data points on the x or y axis suggest single infections with parasites carrying *dhfr* 59C or 59R, respectively (A), or *pfcr* CVMNK or SVMNT, respectively (B). Data points occurring off the x and y axes suggest that infections included a mixture of parasites carrying both alleles.

tected in complete concordance with *dhps*, *dhfr*, and *pfcr* polymorphisms as characterized previously by other groups. To distinguish between alleles, sequence-specific LDR probes relied on differences in the last 3' nucleotide position (position 1) for seven SNPs. LDR probes distinguishing among polymorphic codons 436 and 437 of the four *dhps* alleles relied on polymorphisms in position 1 as well as positions 4 and 5 (nucleotides upstream from the 3' end); probes specific for *pfcr* alleles differed at positions 1, 3, 5, 6, and 14. The results demonstrate that both the signal intensities at the lower limits of detection and the maximum signal intensities differ according to individual alleles (Table 4; Fig. 3 and 4). One factor that may influence both outcomes is differences in the overall number of oligonucleotides coupled to the individual microspheres chosen for this assay. Also, the overall array of polymorphism between alleles may influence potential background hybridization between different allelic sequences. Of note, the *dhps*

probes 436-7 SG and 436-7 FG differ from each other only at position 4; higher background signals between these probes in Table 3 may be expected, since there are no differences between the probes and allelic sequence targets at nucleotide positions 1 to 3.

Our results derived from Papua New Guinea field samples followed comparisons with the results of the same assays performed with North American controls who had no previous exposure to malaria parasites and comparisons among the *Plasmodium* species assay and the three gene-specific assays. The heuristic model described in Materials and Methods described all possible outcomes from comparisons among the *Plasmodium* species assay and the three drug resistance SNP diagnostic assays. Following evaluation of the data, we excluded all samples where discordance was observed among any of the assays. As a result, cutoff values identifying negative MFIs corresponded to the highest fluorescent signals for samples judged to be *P. falciparum* negative by the *Plasmodium* species diagnostic assay. The results from these comparisons are summarized in Table 4.

Efforts to define the cutoff between negative and positive results in the gene-specific assays presented an important challenge for the LDR-FMA methods described here. The MFI values observed following evaluation of 70 North American Caucasian samples were no higher than 425 for any of the allele-specific assays. Similar values were observed for Papua New Guinea samples where no PCR product was observed following agarose gel electrophoresis. In infected samples where we observed high MFI values for one allele, we observed what appeared to be elevated levels of background signal from the other allele. This can be observed in the scatter plots for *dhfr* SNP 59 and the *pfcr* CVMNK and SVMNT comparisons in Fig. 4A and B. Here, as MFI increases above 5,000 for *dhfr* 59C, data points representing infected individuals do not rest on the x axis. We observed a similar outcome in analyzing the data for individuals infected with parasites carrying the *pfcr* CVMNK allele. We attempted to determine if individual samples characterized by this outcome were carrying low levels of the *dhfr* 59R allele or the *pfcr* SVMNT allele by cloning PCR products and analyzing individual clones by LDR-FMA. In these assessments we observed no evidence of the anticipated minor allele. Future studies will examine the relationship between true-positive and negative MFI signals through development of statistical models.

Our study provided the opportunity to determine the frequency of drug resistance polymorphisms in samples from individuals suspected to be infected with *P. falciparum* because of their residence in a region where malaria is holoendemic and their presentation at a local health center with a history of febrile illness ($>37.5^{\circ}\text{C}$). Our finding that 468 samples were *P. falciparum* negative by the three gene-specific assays and the *Plasmodium* species diagnostic assay was not unexpected, because bacterial, viral, or filarial infections and/or other *Plasmodium* species commonly underlie febrile illness in this region of Papua New Guinea. Approximately 50% of the samples from our study participants were infected with *P. falciparum*; 65% of the samples were infected with either *P. falciparum* or some other human *Plasmodium* species parasite. Our results showing that 82% of *P. falciparum*-infected samples carried *pfcr* mutations associated with chloroquine resis-

tance suggest that Fansidar is acting alone against *P. falciparum* at the Papua New Guinea site studied. Further, we found that more than 70% of *P. falciparum*-infected samples carried a resistance-associated double-mutation pattern at *dhfr* positions 59 (C→R) and 108 (S→N), similar to patterns observed in India (1, 4, 5) and Sri Lanka (13) and in contrast with the East African pattern at positions 51 (N→I) and 108 (S→N) (8, 15, 21, 25, 28). Drug resistance-associated mutations were virtually absent in *dhps*. These findings were similar to those of Casey et al., who performed similar studies in Papua New Guinea communities in the Wosera and Madang (6). Interestingly, in vivo efficacy studies of chloroquine plus Fansidar conducted in the Wosera have shown reduced sensitivity of *P. falciparum* parasites to this combination treatment, with adequate clinical and parasitological responses for only 83.9% and 78.3% of patients tested in 2003 and 2004, respectively (16). Additionally, Mita et al. have recently reported the identification of *P. falciparum* strains carrying polymorphisms associated with in vivo resistance to Fansidar [*dhps*(A437G), *dhps*(K540E), *dhfr*(C59R), *dhfr*(S108N)] (20) near Wewak, East Sepik Province, Papua New Guinea. Therefore, it will be important to continue monitoring the status of polymorphisms in *P. falciparum dhps*, *dhfr*, and other genes involved in drug resistance. Finally, since the samples evaluated here were collected largely from a health center in the Wosera, it will be important to perform similar studies on community-based controls who did not visit the health center. A comparison of this nature will make it possible to assess the level at which *P. falciparum* strains carrying drug resistance-associated polymorphisms contribute to clinical malaria in this region of Papua New Guinea.

In conclusion, limited resources contribute in many ways to the failure of drugs against malaria parasites. The results from our study introduce an approach for screening larger survey populations for a wide range of mutations associated with antimalarial drug resistance. Application of high-throughput diagnostic methods for the evaluation of large numbers of individual samples would enable more-complete characterization of *P. falciparum* strain diversity in regions of endemicity. If coordinated with efforts to develop an open-access database (27) to monitor regions of malaria endemicity around the world, the application of high-throughput molecular epidemiologic tools would contribute to better surveillance of drug resistance-associated polymorphisms in parasite populations and more-timely predictions regarding antimalarial drug effectiveness.

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