

Multiplex PCR and Oligonucleotide Microarray for Detection of Single-Nucleotide Polymorphisms Associated with *Plasmodium falciparum* Drug Resistance^{∇†}

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Drug resistance in *Plasmodium falciparum* is a serious public health threat in the countries where this organism is endemic. Since resistance has been associated with specific single-nucleotide polymorphisms (SNPs) in parasite genes, molecular markers are becoming useful surrogates for monitoring the emergence and dispersion of drug resistance. In this study, a multiplex PCR (mPCR) and oligonucleotide microarray method was developed for the detection of these SNPs in genes encoding chloroquine resistance transporter (*Pfcr*t), multidrug resistance 1 (*Pfmdr*1), dihydrofolate reductase (*Pfdhfr*), dihydropteroate synthetase (*Pfdhps*), and ATPase 6 (*PfATPase*6) of *P. falciparum*. The results show that DNA microarray technology, combined with mPCR, is a promising and time-saving tool that supports conventional detection methods, allowing sensitive, accurate, simultaneous analysis of the SNPs associated with drug resistance in *P. falciparum*.

Malaria is still one of the most serious public health problems in the world; more than one million people worldwide die from it every year (35). This trouble is largely caused by the resistance of *Plasmodium falciparum* to most antimalarial drugs currently used, including chloroquine (CQ), sulfadoxine-pyrimethamine (SP), mefloquine (MQ), amodiaquine (AQ), and lumefantrine (LUM); furthermore, reduced susceptibility of *P. falciparum* to artemisinin and its derivatives has been also reported (15, 16, 34).

Antimalarial drug resistance has been associated with single-nucleotide polymorphisms (SNPs), in particular *P. falciparum* genes. The *P. falciparum* CQ resistance transporter (*Pfcr*t) gene K76T mutation has been linked to CQ and possibly AQ resistance (6, 17, 20); the *P. falciparum* multidrug resistance 1 (*Pfmdr*1) gene N86Y mutation has been associated with the response of the parasite to amino quinolines such as MQ (9, 25, 27) and LUM (26); mutations N51I, C59R, S108N/T, and I164L in the *P. falciparum* dihydrofolate reductase (*Pfdhfr*) gene have been associated with resistance to pyrimethamine (13, 14, 19, 21, 22); mutations S436A, A437G, K540E, A581G, and A613 S/T in the *P. falciparum* dihydropteroate synthetase (*Pfdhps*) gene have been shown to confer resistance to sulfadoxine (13, 14, 21, 22); and *P. falciparum* ATPase-6 (*PfATPase*6) S769N mutation has been associated with increased 50% inhibitory concentrations of artemether (11, 16, 18). These SNPs are believed to represent molecular

epidemiology surveillance tools of antimalarial drug resistance, which can complement the more conventional and logistically complex in vitro or in vivo test (7, 24, 33, 34).

Existing molecular methods for analysis of these SNPs include allele-specific PCR, PCR-restriction fragment length polymorphism (RFLP) analysis, multiplex PCR (mPCR)-RFLP, DNA sequencing, dot blot hybridization techniques, the molecular beacon method, real-time PCR, PCR-enzyme-linked immunosorbent assay, and pyrosequencing (1, 2, 6, 8, 10, 28, 31, 37, 38). Each of these techniques offers its own advantages and limitations. Among the techniques, PCR-RFLP protocols constitute the most common methodological approach for the analyses of these SNPs, but this approach is laborious and time-consuming. A rapid and high-throughput genotyping method would be ideal for large-scale population-based studies.

The oligonucleotide microarray technology, also known as DNA chip, has provided an incredible technical development in rapid, large-scale, and automatic analysis of SNPs (5, 36). Briefly, the principle of oligonucleotide microarray is based on reverse Southern hybridization on a glass or silicon substrate. The fluorescently labeled products hybridized to the immobilized probes on the substrate can be easily detected by using a fluorescence-detecting scanner, and the readable fluorescent signals obtained are combined to determine the genotype of genes (12). In recent years, a microarray technique has been successfully applied to the detection of resistance genes or resistance-associated SNPs in various pathogens (3, 4, 23, 29).

Here, we introduce a one-step mPCR in combination with an oligonucleotide microarray method for rapid, high-throughput detection of the antimalarial resistance-associated SNPs in *Pfcr*t, *Pfmdr*1, *Pfdhps*, *Pfdhfr*, and *PfATPase*6.

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TABLE 1. Primers used in the mPCR

Gene	Primer	Sequence (5'-3')	Amplicon size (bp)	Source or reference
<i>Pfprt</i>	P1F	GGAGGTTCTTGTC TTGGTAAAT	315	This study
	P1R	ATATTGGTAGGTG GAATAGATTCT		
<i>Pfmdr1</i>	P2F	TGTTGAAAGATG GGTAAAGAG CAGA	514	This study
	P2R	TCGTACCAATTCC TGAACACTCACTT		
<i>Pfdhps</i>	P3F	GATTCTTTTTCAG ATGGAGG	770	22
	P3R	TTCCTCATGTAAT TCATCTGA		
<i>Pfdhfr</i>	P4F	TGATGGAACAAG TCTGCGACGTT	594	22
	P4R	CTGGAAAAAATA CATCACATTCA TATG		
<i>PfATPase6</i>	P5F	AAAAATAAATACC ACATCAAC ACAT	437	This study
	P5R	TCAATAATACCTA ATCCACCTAAA		

MATERIALS AND METHODS

Samples. Three *P. falciparum* laboratory strains (3D7, HB3, and DD2) (30) and 92 field samples (positive for *P. falciparum* by microscopy) collected on 3MM filter paper (Whatman, Maidstone, United Kingdom) from China's Yunnan province (40 samples), Hainan province (27 samples), and Myanmar's Shan state (25 samples) were used in the present study. The genomic DNA of *P. berghei* (CS strain), *P. cynomolgi* (B strain) and *P. vivax* (collected from Anhui province, China) were used as controls. The research protocol was approved by the Chinese National Institute of Parasitic Diseases ethics committee.

DNA extraction. Genomic DNAs of the *P. falciparum* laboratory strains 3D7, HB3, and Dd2 were provided by the Malaria Research and Reference Reagent Resource Center (<http://www.mr4.org/>). Extraction of DNA from bloodspots on filter paper was carried out by the Chelex-100 (Bio-Rad Laboratories, Hercules, CA) method described by Wooden et al. (32) with some modifications described by Pearce et al. (22). The quality of the DNA samples was tested by determining the optical density at 260 and 280 nm.

SNPs studied. The following 21 SNPs were analyzed: *Pfprt* 391T/A, 392G/C, 399G/T, 400A/G, 402T/A, and 404A/C (corresponding to codons C72S, M74I, N75E, and K76T); *Pfmdr1* 256A/T and 257A/T (N86Y/F); *Pfdhps* 1482 T/G, 1483C/T/G, 1486 C/G, 1794 A/G, 1918 C/G, and 2013G/T/A (S436A/F/C, A437G, K540E, A581G, and A613S/T); *Pfdhfr* 148T/C, 152A/T, 153T/C, 175T/C, 323G/A/C, and 490A/T (C50R, N51I, C59R, S108N/T, and I164L); and *PfATPase6* 2306G/A (S769N). These SNPs are located at 11 nucleotide positions since some positions include two or more SNPs.

mPCR. To reduce the time and cost of DNA amplification, an mPCR was developed to amplify the DNA fragments containing all of the SNPs studied in one reaction. Five pairs of primers were designed by using Primer 5.0 software and checked for specificity in a BLAST search available through the National Center for Biotechnology Information (NCBI; <http://www.ncbi.nlm.nih.gov/BLAST/>). The primers used in the mPCR are shown in Table 1. mPCR was performed with the PCR amplification kit of Genscript (Piscataway, NJ) according to the manufacturer's instructions. Briefly, the mPCR was performed by using the PTC 200 cyclotherm (MJ Research, Inc., Waltham, MA) in a total volume of 50 μ l of reaction mix containing 2.5 μ l of template DNA, 25 μ l of 2 \times Genscript master mix, and 1.0 μ l of primer mixture (consisting of 3.2 μ l of 12.5 μ M P1F, 3.2 μ l of 12.5 μ M P1R, 2.0 μ l of 12.5 μ M P2F, 2.0 μ l of 12.5 μ M P2R, 2.0 μ l of 12.5 μ M P3F, 2.0 μ l of 12.5 μ M P3R, 2.0 μ l of 12.5 μ M P4F, 2.0 μ l of 12.5 μ M P4R, 2.0 μ l of 12.5 μ M P5F, and 2.0 μ l of 12.5 μ M P5R). The mPCR cycling conditions were 94°C for 15 min for 1 cycle; followed by 30 cycles of denaturation at 94°C for 40 s, annealing at 50°C for 2 min, and extension (ramping from 50 to 72°C by 0.1°C/s) for about 3 min 40 s; followed in turn by 72°C for 3 min for 1 cycle and holding at 4°C. The resulting amplicon covers all

of the 21 observed SNPs. A total of 3 μ l of PCR product was identified by electrophoresis on a 20-g/liter agarose gel to confirm the successful amplification.

Fragmentation and labeling of mPCR product. mPCR products were purified with a QIAquick PCR purification kit (Qiagen, Inc.) and treated with DNase I (Fermentas) at 0.001 U of DNA/ μ g to generate fragments of 50 to 200 bp. Reaction mixtures contained 1 mM Tris-HCl, 0.25 mM MgCl₂, and 0.05 mM CaCl₂. The fragmentation reaction was performed at 37°C for 18 min and then at 95°C for 10 min. Labeling of DNA was performed using terminal deoxynucleotidyltransferase, and reaction mixtures (25 μ l) contained 200 mM potassium cacodylate, 25 mM Tris-HCl (pH 7.2), 1 mM CoCl₂, 0.01% Triton X-100, 20 μ M Cy3-dCTP (GE Healthcare), and 20 U of deoxynucleotidyltransferase (Fermentas). The mixtures were incubated for 1 h at 37°C, followed by denaturation at 95°C for 10 min.

Oligonucleotide probe design and microarray manufacture. For each of the polymorphic sites associated with antimalarial drug resistance, a group of probes was designed, differentiated by the interrogated base(s) positioned in the central region of the probes. To reduce the number of potential ambiguous hybridization results, some oligonucleotide probes were designed to represent combinations of SNPs rather than one SNP. In all, 11 groups of probes were designed. The specificity of the oligonucleotide probes was verified through a BLAST search. Based on initial hybridization experiments, some probes were optimized by modifying their length and sequence to achieve more uniform fluorescence intensities across the microarray. Probes were commercially synthesized and modified (Table 2). Each probe contained a 5'-amino group for immobilization chemistry and a 15-mer poly(dT) spacer, followed by the nucleotide hybridization sequence.

The probes were suspended in 100 mM phosphate-buffered solution containing 0.05% sodium dodecyl sulfate (SDS) at a concentration of 25 μ M and printed on silylated slides (OPAldehydeSlide; CapitalBio Corp., Beijing, China) by an OmniGrid 100 Microarrayer (GeneMachine, San Carlos, CA). Each slide was spotted with 10 sets of three identical subarrays consisting of a 5 \times 10 matrix; a matrix includes 45 specific probes and 2 Cy3-labeled spotting control probes (sequence, ATTGCTTGCGGCGTAACG-Cy3), a positive hybridization control probe (TCTGCTTCTGCTTCTGCTT), a negative hybridization control probe (TCTGCTTCTCTTCTGCTT), and a blank control probe (100 mM phosphate-buffered solution containing 0.05% SDS). A schematic diagram of the probe positions on the microarray is shown in Fig. 1.

Hybridization and washing. Hybridization of fluorescently labeled single-stranded DNA samples to the oligonucleotide microarray was performed in 1 \times hybridization buffer (6 \times sodium chloride-sodium phosphate-EDTA buffer, 5% dimethyl sulfoxide, 0.1% Triton X-100) at 52°C for 1 h. Just before hybridization, 10 μ l of the Cy3-labeled single-stranded DNA sample was mixed with an equal volume of 2 \times hybridization buffer containing 1 nM Cy3-labeled control oligonucleotide (sequence, AAGCAGAAGCAGAAGCAGA), followed by denaturation at 95°C for 10 min and chilling on ice. After hybridization, the slides were washed at 42°C for 6 min with 2 \times saline sodium citrate (SSC) buffer containing 1% SDS, for 6 min with 1 \times SSC buffer containing 0.2% SDS, and for 3 min with 0.6 \times SSC buffer. Traces of buffer were removed by air stream.

Microarray scanning and analysis. Slides were scanned by using a scanner GenePix 4000B (Axon Instruments), and data acquisition and processing were performed by using GenePix Pro 3.0 software. The median of local background-corrected feature intensities (henceforth referred to as net intensities) and their signal-to-noise ratios (net intensities/standard deviation of the local background) were used for further analyses. Each signal was ranked from highest to lowest in each probe group according to their net intensities. The highest ranked signal in each group was regarded as a positive signal, and a signal with an intensity more than one-third of the strongest signal in that group was also considered a positive signal, whereas signals with intensities less than one-third of the strongest signal were considered negative. When hybridized to single-infection samples, only one probe in each group produced a positive signal as the perfect match. In multiple infections, two or more probes with the highest signal intensities in a group might be selected as positive.

Microarray analyses of synthesized oligonucleotides. Because genotype variations of the five genes from the samples studied here were limited, we also used a set of 37 synthesized 50-bp oligonucleotides (Table 3) harboring all known genotypes of the loci studied to evaluate the microarray. Twenty combinations (Table 4) of the synthesized oligonucleotides were detected by the microarray to test whether the method can correctly identify all possible genotypes in single or mixed infection. The labeling and hybridization conditions were as described above.

Traditional nested PCR-sequencing assay. To validate the results of microarray analysis, nested PCR-sequencing assays were performed in parallel. Nested PCRs described by Djimde et al. (6) were used to amplify fragments of the *Pfprt*

TABLE 2. Oligonucleotide probes used in the microarray

Gene and oligonucleotide	Corresponding genotype	Sequence (5'-3')
<i>Pfdhfr</i>		
A1	50CN	NH ₂ -(T)15-CCATGGAAATGTAATTCCTAGATAT
A2	50CI	NH ₂ -(T)15-CCATGGAAATGTATTTCCCTAGATAT
A3	50CN2	NH ₂ -(T)15-CATGGAAATGTAACCCCTAGATATG
A4	50RN	NH ₂ -(T)15-CCATGGAAACGTAATTCCTAGATAT
A5	50RN2	NH ₂ -(T)15-CATGGAAACGTAACCCCTAGATAT
A6	50RI	NH ₂ -(T)15-CCATGGAAACGTAATTTCCCTAGATAT
B1	59C	NH ₂ -(T)15-ATATGAAATATTTTGTGCAGTTACAACAT
B2	59R	NH ₂ -(T)15-ATGAAATATTTTCGTGCAGTTACAAC
B3	59 control	NH ₂ -(T)15-ATGAAATATTTTGGTGCAGTTACAAC
C1	108S	NH ₂ -(T)15-GAAGAACAAGCTGGGAAAGC
C2	108N	NH ₂ -(T)15-GAAGAACAACCTGGGAAAGC
C3	108T	NH ₂ -(T)15-GAAGAACAACCTGGGAAAGC
C4	108 control	NH ₂ -(T)15-GAAGAACAATCTGGGAAAGC
D1	164I	NH ₂ -(T)15-AAATGTTTTATTATAGGAGGTTCCGT
D2	164L	NH ₂ -(T)15-AAATGTTTTATTATAGGAGGTTCCGT
D3	164 control	NH ₂ -(T)15-ATGTTTTATTCTAGGAGGTTCCG
<i>Pfmdr1</i>		
E1	86N	NH ₂ -(T)15-ATTAAGAACATGAATTTAGGTGATGAT
E2	86Y	NH ₂ -(T)15-ATTAAGAACATGTATTTAGGTGATGAT
E3	86F	NH ₂ -(T)15-TTAAAGAACATGTTTTTAGGTGATGATA
E4	86 control	NH ₂ -(T)15-TTAAAGAACATGCATTTAGGTGATGA
<i>PfATPase6</i>		
F1	769S	NH ₂ -(T)15-TTTGCTTATAAAAAATTAAGTAGTAAAGATTTAAATAT
F2	769N	NH ₂ -(T)15-CTTTGCTTATAAAAAATTAAGTAGTAAAGATTTAAATAT
F3	769 control	NH ₂ -(T)15-TTTGCTTATAAAAAATTAAGTAGTAAAGATTTAAATAT
<i>Pfdhps</i>		
G1	436AA	NH ₂ -(T)15-AGAATCCGCTGCTCCTTTTGT
G2	436AG	NH ₂ -(T)15-AGAATCCGCTGGTCCTTTTGT
G3	436SA	NH ₂ -(T)15-GAGAATCCTCTGCTCCTTTTGT
G4	436SG	NH ₂ -(T)15-GAGAATCCTCTGGTCCTTTTGT
G5	436FA	NH ₂ -(T)15-GAGAATCCTTTGCTCCTTTTGT
G6	436FG	NH ₂ -(T)15-GAGAATCCTTTGGTCCTTTTGT
G7	436CA	NH ₂ -(T)15-GAGAATCCTGTGCTCCTTTTGT
H1	540K	NH ₂ -(T)15-CACATACAATGGATAAACTAACAAATTA
H2	540E	NH ₂ -(T)15-CACATACAATGGATGAACAACTAACAAATTA
H3	540 control	NH ₂ -(T)15-CACATACAATGGATCACTAACAAATTA
I1	581A	NH ₂ -(T)15-GATTAGGATTTGCGAAGAAACATG
I2	581G	NH ₂ -(T)15-GATTAGGATTTGGGAAGAAACATGA
I3	581 control	NH ₂ -(T)15-GATTAGGATTTGAGAAGAAACATG
J1	613A	NH ₂ -(T)15-AAAAAGATTTATTGCCCATTCATGA
J2	613S	NH ₂ -(T)15-AAAAAGATTTATTGCCCATTCATGAAT
J3	613T	NH ₂ -(T)15-AAAAAGATTTATTGCCCATTCATGAAT
J4	613 control	NH ₂ -(T)15-AAAAAGATTTATCCCATTCATGA
<i>Pfcr1</i>		
K1	72CVMNK	NH ₂ -(T)15-GTGTATGTGTAATGAATAAAAATTTTGCTA
K2	72CVIET	NH ₂ -(T)15-TGTATGTGTAATGAAACAATTTTGCTAA
K3	72SVMNT	NH ₂ -(T)15-TATTTATTTAAGTGAAGTGAATGAATACAATT
K4	72CVIEK	NH ₂ -(T)15-GTGTATGTGTAATGAAAAAATTTTGCTA
K5	72S ₂ VMNT	NH ₂ -(T)15-TATTTATTTAAGTGTATCTGTAATGAATACAATTTTG

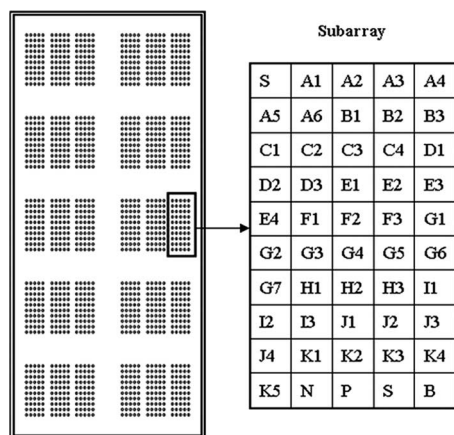


FIG. 1. Schematic diagram of the probe positions on the microarray. A1 to K5, specific probes for detection of the SNPs; S, spotting control probe; N, negative control probe; P, positive control probe; B, blank control probe. Each probe was spotted three times.

and *Pfmdr1* genes from genomic DNA. Nested PCRs as described by Pearce et al. (22) were used to amplify fragments of the *Pfdhfr* and *Pfdhps* genes. A nested PCR used to amplify fragments of the *PfATPase6* gene will be described elsewhere (L. H. Tang et al., unpublished data). Sequencing reactions were carried out by using an ABI Prism BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems) as specified by the manufacturer's protocol. The sequences of the amplicons were compared to the published data of the NCBI database by BLAST analysis.

RESULTS

mPCR. We began our analysis with mPCR amplification of the five antimalarial resistance related genes from laboratory strains and field samples. Gel electrophoresis of the resulting mPCR products indicated that they were of the expected sizes (Fig. 2). Under these amplification conditions, no nonspecific bands were observed for most samples. Moreover, the genomic DNAs of *P. vivax*, *P. berghei*, and *P. cynomolgi* did not yield any PCR products of the expected length.

TABLE 3. Thirty-seven synthesized oligonucleotides

Gene and oligonucleotide	Harboring genotype	Sequence (5'-3')
<i>Pf</i> <i>dhfr</i>		
A1	50CN	TGTAAGTGCACGAAAAATATTTTCATATCTAGGGAAATTACATTTCCATGGTA
A2	50CI	TGTAAGTGCACGAAAAATATTTTCATATCTAGGGAAATACATTTCCATGGTA
A3	50CN2	TGTAAGTGCACGAAAAATATTTTCATATCTAGGGAGTTACATTTCCATGGTA
A4	50RN	TGTAAGTGCACGAAAAATATTTTCATATCTAGGGAAATTACGTTTCCATGGTA
A5	50RN2	TGTAAGTGCACGAAAAATATTTTCATATCTAGGGAGTTACGTTTCCATGGTA
A6	50RI	TGTAAGTGCACGAAAAATATTTTCATATCTAGGGAAATACGTTTCCATGGTA
B1	59C	TGATTCATTCACATATGTTGTAAGTGCACAAAAATATTTTCATATCTAGGG
B2	59R	TGATTCATTCACATATGTTGTAAGTGCACGAAAAATATTTTCATATCTAGGG
C1	108S	AAATTTTTTGGAAATGCTTTCCAGCTTGTCTTCCATAACTACAACAT
C2	108N	AAATTTTTTGGAAATGCTTTCCAGCTTGTCTTCCATAACTACAACAT
C3	108T	AAATTTTTTGGAAATGCTTTCCAGCTTGTCTTCCATAACTACAACAT
D1	164I	TCTTGATAAACAACGGAACCTCCTATAATAAAACATTTATAGTAATTTAA
D2	164L	TCTTGATAAACAACGGAACCTCCTAAAATAAAACATTTATAGTAATTTAA
<i>Pf</i> <i>mdr1</i>		
E1	86N	TAGGATTAATATCATCACCTAAATTCATGTTCTTTAATATTACACCAAAC
E2	86Y	TAGGATTAATATCATCACCTAAATACATGTTCTTTAATATTACACCAAAC
E3	86F	TAGGATTAATATCATCACCTAAAAACATGTTCTTTAATATTACACCAAAC
<i>Pf</i> <i>ATPase6</i>		
F1	769S	TTCTTAATATTTAAATCTTTACTACTTAATTTTTTATAAGCAAAGCTAAG
F2	769N	TTCTTAATATTTAAATCTTTACTATTTAATTTTTTATAAGCAAAGCTAAG
<i>Pf</i> <i>dhps</i>		
G1	436AA	TTGGATTAGGTATAACAAAAGGAGCAGCGGATTCTCCACCTATATCTATA
G2	436AG	TTGGATTAGGTATAACAAAAGGACCAGCGGATTCTCCACCTATATCTATA
G3	436SA	TTGGATTAGGTATAACAAAAGGAGCAGAGGATTCTCCACCTATATCTATA
G4	436SG	TTGGATTAGGTATAACAAAAGGACCAGAGGATTCTCCACCTATATCTATA
G5	436FA	TTGGATTAGGTATAACAAAAGGAGCAAAGGATTCTCCACCTATATCTATA
G6	436FG	TTGGATTAGGTATAACAAAAGGACCAAAGGATTCTCCACCTATATCTATA
G7	436CA	TTGGATTAGGTATAACAAAAGGAGCAGGATTCTCCACCTATATCTATA
H1	540K	ACTAGATTATCATAATTTGTTAGTTTATCCATTGTATGTGGATTTCTCT
H2	540E	ACTAGATTATCATAATTTGTTAGTTTATCCATTGTATGTGGATTTCTCT
I1	581A	TTTAATAGATTGATCATGTTTCTTCGCAAATCCTAATCCAATATCAAATA
I2	581G	TTTAATAGATTGATCATGTTTCTTCGCAAATCCTAATCCAATATCAAATA
J1	613A	ACATTTTGATCATTATGCAATGGGCAATAAATCTTTTCTTGAATATCC
J2	613S	ACATTTTGATCATTATGCAATGGGAAATAAATCTTTTCTTGAATATCC
J3	613T	ACATTTTGATCATTATGCAATGGGTAATAAATCTTTTCTTGAATATCC
<i>Pf</i> <i>cr1</i>		
K1	72CVMNK	GTTCTTTTAGCAAAAATTTTATTCATTACACATACACTTAAATAAATAAT
K2	72CVIET	GTTCTTTTAGCAAAAATTTGTTTCAATTACACATACACTTAAATAAATAAT
K3	72SVMNT	GTTCTTTTAGCAAAAATTTGTTTCAATTACACATACACTTAAATAAATAAT
K4	72CVIEK	GTTCTTTTAGCAAAAATTTTCAATTACACATACACTTAAATAAATAAT
K5	72S2VMNT	GTTCTTTTAGCAAAAATTTGTTTCAATTACAGATACACTTAAATAAATAAT

Microarray analyses of laboratory strains and field samples. The net intensities achieved at the different oligonucleotide probes varied substantially, which is reflected by the wide range of signal-to-noise ratios from 5.6 ± 0.9 to 40.3 ± 6.4 . However, upon hybridization to perfectly matching target DNA, the signal-to-noise ratios at all probes were larger than three (Fig. 3A). This threshold is considered indicative of sufficient signal in fluorescence-based microarray hybridization (3). Also, the net intensities at matching probes were always at least five times as stronger than those at probes with mismatch(es) to the respective targets (Fig. 3B), which will suffice for robust genotyping.

All of the SNPs in the three *P. falciparum* laboratory strains could be correctly analyzed by the mPCR-microarray system. In 8 of 92 field samples, results could not be obtained due to failure of DNA extraction or mPCR amplification. Among 957

genotyped positions of the 87 successful hybridizations, 943 (98.5%) yielded usable data, and 12 (1.3%) had inconsistent genotyping results between the nested PCR-sequencing assay and the microarray assay (see Table S1 in the supplemental material). Four discrepancies were due to errors in the sequencing assay, three discrepancies were due to insufficient probe-spotting quality, three discrepancies were due to a non-specific microarray signal, and two discrepancies were due to false determination of mixed infection by microarray. Scanning images and genotyping results of *P. falciparum* 3D7 and Dd2 are shown in Fig. 4.

Serial 10-fold dilutions of genomic DNA ranging from 60 ng to 0.6 pg were used to test the sensitivity of the mPCR-microarray system. The results demonstrated that when the DNA was not less than 0.06 ng, all of the SNPs in the five genes could be identified. When the DNA was diluted to 6 pg, SNPs at

TABLE 4. Twenty combinations of the synthesized oligonucleotides

Combination no.	Oligonucleotide combination ^a
1	A1, B1, C1, D1, E1, F1, G1, H1, I1, J1, K1
2	A2, B2, C2, D2, E2, F2, G2, H2, I2, J2, K2
3	A3, B1, C3, D1, E3, F1, G3, H1, I1, J3, K3
4	A4, B2, C1, D2, E1, F2, G4, H2, I2, J1, K4
5	A5, B1, C2, D1, E2, F1, G5, H1, I1, J2, K5
6	A6, B2, C3, D2, E3, F2, G6, H2, I2, J3, K1
7	A1, B1, C1, D1, E1, F1, G7, H1, I1, J1, K2
8	A2, B2, C2, D2, E2, F2, G1, H2, I2, J2, K3
9	A3, B1, C3, D1, E3, F1, G2, H1, I1, J3, K4
10	A4, B2, C1, D2, E1, F2, G3, H2, I2, J1, K5
11	A1, B1, C1, D1, E1, F1, G4, H1, I1, J1, K1, A2
12	A1, B1, C1, D1, E1, F1, G4, H1, I1, J1, K1, B2
13	A1, B1, C1, D1, E1, F1, G4, H1, I1, J1, K1, C2
14	A1, B1, C1, D1, E1, F1, G4, H1, I1, J1, K1, D2
15	A1, B1, C1, D1, E1, F1, G4, H1, I1, J1, K1, E2
16	A1, B1, C1, D1, E1, F1, G4, H1, I1, J1, K1, F2
17	A1, B1, C1, D1, E1, F1, G4, H1, I1, J1, K1, C2, G3
18	A1, B1, C1, D1, E1, F1, G4, H1, I1, J1, K1, A2, B2, C2, E3, G6, J2, K2
19	A1, B1, C1, D1, E1, F1, G4, H1, I1, J1, K1, B2, C2, E2, G5, K2
20	A1, B1, C1, D1, E1, F1, G4, H1, I1, J1, K1, D2, K5

^a Combinations 1 to 10 simulate single infections with various genotypes; combinations 11 to 20 simulate mixed infections.

codons 50, 51, 59, 108, and 164 of gene *Pfdhfr* could not be identified, but other SNPs were identified successfully. When the DNA was diluted to 0.6 pg, only the SNPs at codons 436, 437, 540, and 613 of *Pfdhps* and the SNP at codon 86 of *Pfmdr1* could be detected.

The specificity of microarray assay was tested by using mPCR products from *P. berghei*, *P. cynomolgi*, and *P. vivax*. For these three parasites no positive signal was obtained with any specific probe except the spotting control probe and the positive hybridization control probe.

To determine at what level minor SNPs in mixed infections can be detected, we mixed the genomic DNA of the 3D7 and Dd2 strains. These two strains have different genotypes at *Pfdhfr* codons 51, 59, and 108, *Pfmdr1* codon 86, *Pfdhps* codons 436 and 613, and *Pfprt* codons 74, 75, and 76. Although the DNA concentration of one parasite strain was fixed at 3 ng/μl,

the DNA concentration of the other strain was serially 10-fold diluted from 30 ng/μl to 0.3 pg/μl. When the DNA concentration of 3D7 was fixed, Dd2 could be satisfactorily analyzed at 30 pg/μl. When the DNA concentration of Dd2 was fixed, 3D7 could also be satisfactorily analyzed at 30 pg/μl.

There was 98.2% (926/943) agreement among the genotypes obtained from three identical subarrays in the same slide for 87 samples. Thirty samples were investigated in two separate tests by the microarray method and interpreted blindly, and the reproducibility between the repeated typing was 96.1% (317/330).

Microarray analyses of the synthesized oligonucleotides. All SNPs in the 20 combinations of the synthesized oligonucleotides were identified without ambiguity by the microarray except for two genotyped positions of *Pfdhps* 540 that could not give usable signals due to spotting failure. The net intensity ratios of matching probes to the mismatch ones were more than five times for 83.9% of the total and between 2.7 and 5.0 times for the others. Figure 5 shows a scanning image of the combination of 18 of the synthesized oligonucleotides (simulation of mixed infection of *P. falciparum* 3D7 and *P. falciparum* Dd2).

DISCUSSION

In this study, we describe the development of an mPCR-based oligonucleotide microarray method that can simultaneously detect a set of SNPs, covering the genetic markers associated with the resistance of *P. falciparum* to the most common used antimalarial drugs, such as CQ, AQ, MQ, LUM, pyrimethamine, sulfadoxine, and artemether.

A high degree of concordance was observed when we compared the new microarray assay with the existing nested PCR-sequencing method, probably the most credible method currently used for detection of these SNPs. This indicates that the microarray technique for drug resistance-associated genes genotyping is reliable and accurate.

Traditional nested PCR method requires 10 PCRs for application of the five genes, which may take five workdays to accomplish. Here, the use of mPCR to amplify the five genes in one reaction leads to a significant decrease in time, cost, and

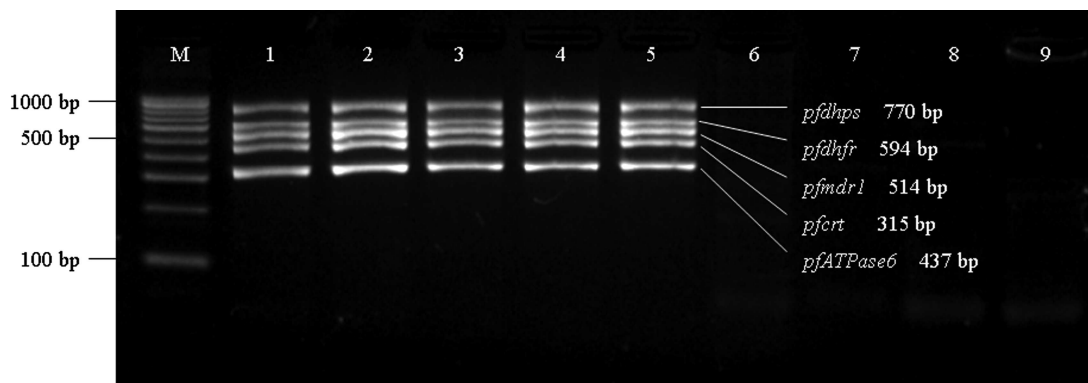


FIG. 2. Agarose gel electrophoresis of mPCR products. M, 100-bp DNA ladder. Lane 1, *P. falciparum* 3D7; lane 2, *P. falciparum* Dd2; lane 3, *P. falciparum* HB3; lanes 4 to 5, *P. falciparum* samples collected from field; lane 6, *P. vivax*; lane 7, *P. berghei*; lane 8, *P. cynomolgi*; lane 9, blank control (H₂O).

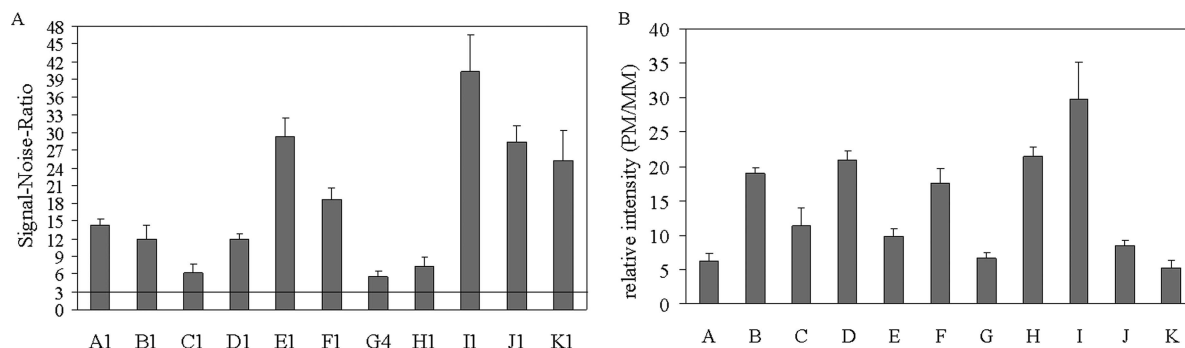


FIG. 3. Signal-to-noise ratios and mismatch discrimination. The data are based on three independent hybridizations of *P. falciparum* 3D7. (A) Signal-to-noise ratios (means and standard errors) of perfectly matching probes; (B) relative intensities of mismatched probes (means and standard errors).

number of manipulations. It takes a total of about 8 h to perform the entire detection procedure, from mPCR reaction (4.0 h), DNA labeling (2.0 h), hybridization (1.5 h), and microarray washing (0.25 h) to microarray scanning and analysis (0.25 h). There are 30 subarrays in a slide, allowing the genotyping of 10 different samples thrice by each assay, so approximately 100 samples could be analyzed for the 21 resistance-associated SNPs in one workday by two technicians. Therefore, this method is especially suitable for large-scale surveillance of the molecular markers. Moreover, the method requires less genomic DNA, which is propitious for analyzing samples with low parasite density.

The high throughput of the microarray method substantially reduces the cost. In estimating the cost, we included expenses associated with DNA isolation, multiplex-PCR, PCR product labeling, microarray production, microarray hybridization, and microarray washing. The equipment and labor costs were not included. We calculated a price of 2.9 Chinese yuan (\$0.41 [United States]) per SNP for the microarray method; this cost was lower than that for pyrosequencing (\$2.28 per SNP), conventional sequencing (\$3.66 per SNP), and RFLP (\$6.58 per SNP) (37).

Filter paper samples used in the present study offered con-

siderable advantages for field collection, transportation, and storage over frozen liquid samples, but eight samples collected from Hainan province in 2001 failed to be analyzed by microarray. We found that these samples were not stored in a dry environment and became moldy before tests were conducted. Thus, it is probable that the genomic DNA of the parasite in these samples was destroyed or degraded. Therefore, it is important to store samples properly to ensure high-quality DNA. In addition, since false microarray determinations were mainly caused by nonspecific signals and poor probe spotting, the microarray silylated slides should be carefully kept from dust contamination and more efforts should be made to further improve the spotting quality.

The testing results of the 20 combinations of the synthesized oligonucleotides show that the microarray is able to identify all of the possible genotypes at the loci studied. Furthermore, it indicates that the technique can also differentiate the SNPs of mixed infections with *P. falciparum* strains of different genotypes, which allows the application of microarrays in various regions for different genetic background of *P. falciparum*.

As a limitation, the microarray could only be used to analyze the known SNPs in the resistance-related genes. Further studies are needed to develop a microarray assay for detection of

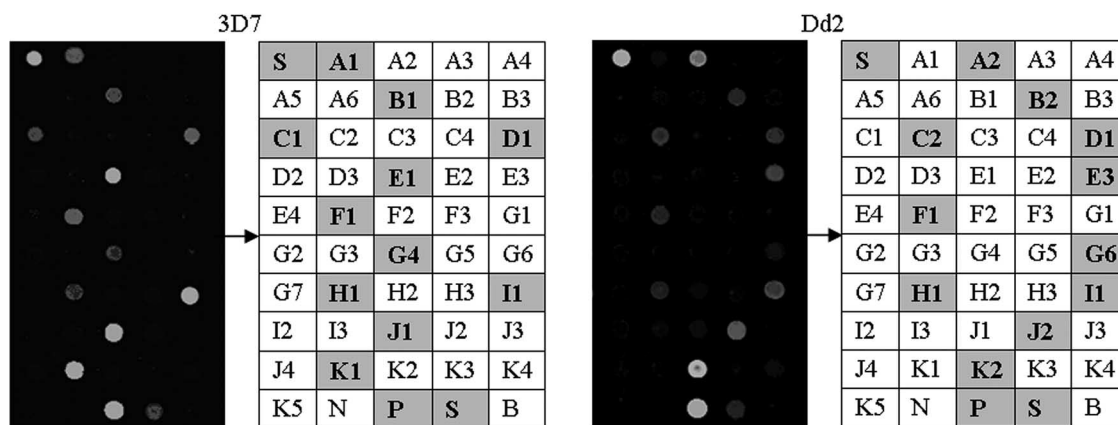


FIG. 4. Scanning images of *P. falciparum* 3D7 and Dd2. For 3D7, the positive probes were A1, B1, C1, D1, E1, F1, G4, H1, I1, J1, K1, P, and S (genotypes: *Pfdhfr*, 50/51CN, 59C, 108S, and 164I; *Pfmdr1*, 86N; *PfATPase6*, 769S; *Pfdhps*, 436/437 SG, 540K, 581A, and 613A; *Pfcrt*, 72-76CVMNK). For Dd2, the positive probes were A2, B2, C2, D1, E3, F1, G6, H1, I1, J2, K2, P, and S (genotypes: *Pfdhfr*, 50/51CI, 59R, 108N, and 164I; *Pfmdr1*, 86F; *PfATPase6*, 769S; *Pfdhps*, 436/437 FG, 540K, 581A, and 613S; *Pfcrt*, 72-76CVIET).

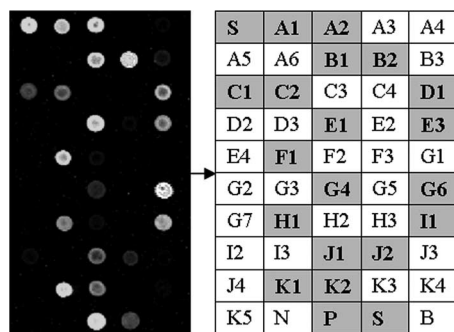


FIG. 5. Scanning image of simulated mixed infection of *P. falciparum* 3D7 and *P. falciparum* Dd2 (combination of 18 synthesized oligonucleotides). The positive probes were A1, B1, C1, D1, E1, F1, G4, H1, I1, J1, K1, A2, B2, C2, E3, G6, J2, K2, P, and S.

the *Pfmdr1* copy number, which has been associated with resistance against chloroquine, mefloquine, and probably artemisinins. Since new SNPs associated with antimalarial drug resistance continue to be discovered, the present microarray should be regularly updated by adding new probes.

It should be noted that microarray technology requires a microarray spotter and a microarray scanner, which may not be available in ordinary laboratories. We hereby suggest that a microarray laboratory be established in one or several countries where *P. falciparum* is endemic so that every site within the area can send filter paper samples to that laboratory for testing. Moreover, in the next phase, we will try to adopt chemiluminescence in the microarray technology so that the microarray assay can be performed in more laboratories.

In conclusion, mPCR-based microarray provides a promising tool for resistance-associated SNP detection in *P. falciparum*. This approach not only saves time and cost but also permits high-throughput detection of the SNPs. However, we consider that molecular techniques, including microarray, will complement rather than replace conventional in vivo or in vitro testing. Conventional resistance testing will remain indispensable for the detection of resistance because the molecular results do not always correlate with the clinical response associated with *P. falciparum*. Therefore, microarray-based analysis will be most useful in combination with conventional methods. We expect that the method developed here will considerably facilitate the molecular surveillance of antimalarial resistance in countries and regions where malaria is endemic.

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