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DETECTION OF *PLASMODIUM FALCIPARUM* IN PREGNANCY BY LASER DESORPTION MASS SPECTROMETRY

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Abstract. Detection of *Plasmodium falciparum* malaria during pregnancy is complicated by sequestration of parasites in the placenta, which reduces peripheral blood microscopic detection. Laser desorption mass spectrometry (LDMS) has previously demonstrated sensitive detection of hemozoin from *P. falciparum* blood cultures and the ability to track parasitemia in a *Plasmodium yoelii* malaria mouse model. Here we use a simple, dilution in water, blood sample preparation protocol for LDMS detection of malaria in 45 asymptomatic, pregnant Zambian women. We compare LDMS to microscopy and polymerase chain reaction (PCR) analysis. All women were microscopy negative. LDMS detected *P. falciparum* hemozoin in 15 out of 45 women, while PCR results were positive in 25 women. Compared with PCR, which analyzed 20–30 μL of blood, the sensitivity of LDMS, which analyzed < 1 μL of blood, was 52%, with a specificity of 92%. LDMS is a potentially rapid and more sensitive alternate diagnostic method than microscopy.

INTRODUCTION

Worldwide, 50 million pregnant women are exposed to malaria infection annually. In Africa, 42% of women have malaria at some point during their pregnancy.[1] Although frequently asymptomatic in hyperendemic areas, malaria infection during pregnancy is associated with maternal anemia, premature delivery, intrauterine growth retardation, and low birth weight, which is the greatest indicator of neonatal mortality.[2] These complications are associated with the infected erythrocyte sequestration in the placenta, which is thought to interfere with placental nutrient transfer.[3] Management of malaria in pregnancy is increasingly difficult due to a number of factors, among them the rapid rise of chloroquine-resistant *Plasmodium falciparum* in the past decade, a severely limited choice of alternative safe drugs, and limitations in diagnosis.[4–6]

Optical microscopic examination of peripheral-blood smears for parasitemia during pregnancy is known to miss a significant portion of placental infections.[7] Theoretically, thick film microscopy can detect approximately 50–100 parasites per microliter, while a practical working number is often 500–1,000 per microliter.[8] Immunochromatographic assays, based on detection of histidine-rich protein II (HRP II) or glycolytic enzymes, are designed with a working threshold of approximately 100 parasites per microliter.[9] PCR, a research "gold standard," has been estimated to detect as little as 5 parasites or less per microliter.[8] Many of these sensitivities will vary based on total volume of blood processed for analysis. A large study conducted in Cameroon showed that more than 20% of placental infections were missed by microscopic examination of peripheral blood and that the detection was improved to about 90% by additional use of the immunochromatographic test for HRP 2.[7]

The biomarker for detecting malaria infection by laser desorption mass spectrometry (LDMS) is heme from hemozoin.

An intraerythrocytic *Plasmodium* parasite digests up to ~0.4 femtomoles of hemoglobin, crystallizing the released toxic heme into hemozoin in the acidic, oxygen-rich food vacuole.[10] While trophozoites are named for the presence of heme crystals visible by light microscopy, ring-stage parasites also contain submicroscopic hemozoin.[11] During LDMS, hemozoin crystals efficiently absorb UV photons from a laser pulse, which liberates intact iron protoporphyrin IX (heme) molecules and heme ions from the deposited blood sample. Molecular-structure-specific fragmentation of heme during this desorption process produces a characteristic spectral signature based on the masses and relative abundances of the molecular ion and fragment ions.[12–14] Importantly, heme bound to hemoglobin in uninfected RBCs under normal physiologic conditions is not detected by LDMS. In saponin and acetic acid purifications of *in vitro* cultured parasites, LDMS has been shown to be highly sensitive with a detection limit of less than a femtomole of hemozoin heme, specific, and semiquantitative for *P. falciparum* hemozoin.[14] In an *in vivo* nonlethal *Plasmodium yoelii* mouse malaria model, LDMS accurately followed the time course of infection as monitored by thin film optical microscopic examination and a colorimetric hemozoin assay and did so with greater sensitivity in both cases.[12]

This human study examined the efficacy of LDMS for detecting *P. falciparum* malaria in a group of asymptomatic pregnant Zambian women who presented for routine antenatal follow-up and presumptive treatment of malaria infection with sulfadoxine-pyrimethamine. The LDMS results are compared with optical microscopy and PCR analysis. Prior to the initiation of this study, the efficiency of LDMS for detecting early-stage *P. falciparum*–infected erythrocytes using our simple blood dilution sample prepation protocol (*vide infra*) was not known. For this reason, we did not know *a priori* the blood volume that needed to be examined by LDMS for the direct comparative analysis of sensitivity and specificity with the other diagnostic methods. In this initial study, less than 1 μL of whole blood per patient was scanned by LDMS for the presence of hemozoin, while the sample preparation for PCR analysis processed about 20–30 μL of whole blood. Microscopy results were based on examination 100 thick-film fields

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of a Giemsa-stained blood smear, or roughly 0.1–0.25 μL of whole blood. LDMS of *P. falciparum* culture parasites diluted in whole blood was also performed to help interpret the human subject data, to estimate detection efficiency for infected erythrocytes, and also to guide the development of an assay protocol suitable for future diagnostic use. This study was conducted as part of a larger study, also reported here, aimed at examining the frequency of drug-resistant genotypes as predictors of parasite resistance to chloroquine or sulfadoxine-pyrimethamine in Zambia.

MATERIALS AND METHODS

Study site and subjects. The study to examine drug-resistant molecular markers to sulfonamide and pyrimethamine during pregnancy was conducted during March–June 2003 at the outpatient maternal-child health service in Macha Mission Hospital, Choma district, southern Zambia. *P. falciparum* infection is the most common diagnosis and one of the top 10 causes of child mortality and maternal morbidity in the Macha area. The peak of malaria transmission, associated with an entomological inoculation rate of 2–20 per week, occurs during the rainy season from December to June. During the other months, many residents have low-level parasitemias without symptoms and are considered semi-immune to malaria. About 20% of live births have a low birth weight and 25% of pregnancies result in stillbirths (Macha Hospital Annual Report, 2000).

Fifty-two women were recruited and screened from the Macha antenatal clinic and also five mobile clinics surrounding the hospital. The nature and detailed procedures of the study were described both verbally and in writing in Chitonka, the native language. Exclusion criteria included women whose gestational duration was less than 12 weeks, whose age was less than 21 years, who had a history of allergy to sulfa drugs or pyrimethamine, who were unavailable for a follow-up, who lived outside of a radius of approximately 20 km, or who have participated in other drug studies in the past 90 days. After written consents were obtained from the women eligible for the study, a complete history was taken, physical and obstetric examinations were performed, fingerprick blood collected, and sulfadoxine and pyrimethamine was given. Thick blood smears were air-dried and stained with Giemsa stain. The smears were read independently by two microscopists. A third reader was available if there was a disagreement between the two. Blood samples were also deposited onto filter papers for subsequent PCR analysis. Three additional positive controls from children presenting to the clinic for malaria were processed in a similar fashion. Finally, 25 negative nonendemic control patient samples were obtained from unlinked, unidentifiable leftover blood collections from the hematology laboratory at Johns Hopkins Hospital. The antenatal study and other blood collections were approved by the local hospital management board, the Zambian UNZA Research Ethics Committee, as well as the Committee on Human Research of the Johns Hopkins University (JHU) Bloomberg School of Public Health.

Parasite culture. *P. falciparum*, strain 3D7, was maintained in culture plates at 37°C and 5% parasitemia in RPMI 1640 supplemented with 10% (v/v) heat inactivated human serum under a $O_2/CO_2/N_2$ atmosphere as described by Trager and Jensen.[15] Cultures were synchronized by sorbitol lysis,[16]

washed in PBS to minimize extracellular hemozoin, and diluted into whole blood at 50% hematocrit to 100, 1,000, 10,000, and 100,000 infected erythrocytes per microliter. Samples were prepared using the early ring forms and trophozoite stage.

LDMS. For LDMS metal slide preparation, 2 μL of human whole blood was diluted with addition of 18 μL of distilled water. A 0.2-μL aliquot of the mixture was pipetted onto each of 20 wells of a metal slide, air-dried, and stored in a 15-mL plastic conical tube at room temperature for up to a year. The metal slides in the conical tubes were shipped by air to the Johns Hopkins University. Only 45 patient samples out of 52 were available for LDMS analysis. Samples from *P. falciparum* culture were run in triplicate (3 slides, 20 wells each) at each parasitemia. Each metal slide was inserted into a commercial time-of-flight mass spectrometer (Kratos Discovery, Shimadzu Analytical, Chesnut Ridge, NY). Positive ionization laser desorption mass spectra were acquired in the linear mode with a 20-kV delayed extraction potential as previously described.[12,14] The laser fluence (~100 μJ/cm²) was optimized for the sensitivity and specificity of detection of both the heme molecular ion and heme-specific fragment ions. The laser beam was rastered linearly across each sample well, 2 mm in length, in 20 μm steps, and LDMS spectra were acquired from each location. One hundred individual laser shot spectra were acquired for each of 20 wells for a total of 2,000 spectra per patient sample. Previous investigation of this protocol using UV sensitive paper inserted into the mass spectrometer indicated that only ~25% of the 0.2 μL dried, diluted blood in each well was interrogated by the laser beam (beam width ~100 μm). Thus, for the 20 wells, the surface of ~1 μL of the dried, diluted whole blood was interrogated for the presence of parasite hemozoin, or ~0.1 μL of original whole blood.

Mass spectral data processing. Individual spectra were initially processed by in-house written software in which a heme spectral matching filter is applied.[12,14] The filter compares each individual spectrum to a template heme spectrum and yields a score, h ($0 \leq h \leq 1.0$), representing the degree of correlation between the observed spectrum and the template. A low score h ~0.0 corresponds to a poorly conforming spectrum, while high scores, h ~1.0, indicate a highly conforming spectrum. The template for this study was derived from an average hemozoin spectrum from a *P. yoelii*–infected mouse. For this study, spectra with h $\geq$ 0.45, and signal-to-noise (SNR) ratio > 50 were designated as containing heme from hemozoin (Feldman AB and others, in preparation). The SNR value was computed by forming the ratio of the integrated ion signal intensity within the template mass/charge (*m/z*) range (450 to 616) to that in two *m/z* domains (each half the width of the template range) adjacent to the low-end and high-end of the template.

PCR analysis. Each filter paper ($1 \times 2 \, cm^2$) was air-dried and stored in an individual sealed plastic bag at room temperature for up to a year. Parasite DNA was extracted from the filter paper by a modified standard saponin extraction method.[17] The strips were placed in 1 mL of 0.5% saponin in phosphate-buffered saline (PBS), soaked for 15 minutes at 37°C, and incubated overnight at 4°C. The supernatant was aspirated out and replaced with 1 mL of PBS. The tubes were incubated at 4°C for 30 minutes. The PBS was aspirated out, and 200 μL of heated water treated with 20% chelex-100

(Bio-Rad, Richmond, CA). The tubes were vortexed at high speed for 20 seconds, placed in a cycler at 95°C for 10 minutes, vortexed briefly once again, and centrifuged. After centrifugation, the supernatant was collected and used for PCR or stored at 4°C. A nested PCR reaction was performed by established protocols and primers.[18–20] Positive PCR templates were restricted to detect the multiple single nucleotide polymorphisms for the genes for merozoite surface protein 2 (Pfmsp2), chloroquine-resistance (PfCRT), quinoline-resistance modulation (PfMDR1), pyrimethamine-resistance (PfDHFR), and sulfadoxine-resistance (PfDHPS).

RESULTS

Demographics. Fifty-two asymptomatic women with an average age of 26 years (range 21–39) were enrolled in the study. Only 8% of women were primigravida or secundagravida. Forty percent of women were in their second trimester and the rest were in their third trimester (average of 6.6 months gestation). None were microscopy positive for *P. falciparum* parasites upon presentation to the clinic. Twenty-nine of 52 samples had a positive amplification product on multiple nested PCR reactions.

LDMS. Seven metal slides from the group of 52 enrolled patients, including 4 that were PCR positive, were not available for LDMS examination. LDMS spectra were analyzed for the presence of hemozoin, and for each patient sample the number of spectra with detected heme out of the 2,000 acquired was recorded. Representative spectra from malaria-infected and noninfected subjects are averaged over the 2,000 total laser shot spectra (Figure 1). The heme signal from *P. falciparum* hemozoin is a cluster of peaks at *m/z* 616 for intact iron protoporphyrin IX, and at *m/z* 571, 557, 527, 511, 498, and 485 for the fragment ions derived from iron protoporphyrin IX. The spectrum from a Zambian, microscopy smear positive, *P. falciparum* control had 27 spectra that were positive for heme according to our detection criteria (Figure 1A). This spectrum is similar to a representative spectrum of a pregnant patient's blood that was microscopy negative, but PCR positive (Figure 1B). This sample had only three positive spectra with detected heme (out of 2,000 overall spectra). The LDMS spectrum from the negative control, a JHU patient, clearly has no characteristic pattern of heme ion peaks (Figure 1C). In total, LDMS detected *P. falciparum* infection in 15 patients, none of which were identified by microscopy (Table 1). While we have used a signal processing algorithm to determine the presence of hemozoin heme in each single laser shot spectrum, heme is readily identifiable visually in these individual spectra (Figure 2). Single-shot heme positive spectra from two PCR positive and one PCR negative antenatal sample all had hemozoin template matching filter scores h > 0.5. Both of the LDMS-positive/PCR-negative patient samples had only one positive heme signal spectrum out of the 2,000 as did 8 other LDMS positive samples that were also PCR positive. Comparison of the LDMS test performance versus PCR in a Fisher's exact test for significantly different proportions (Table 2) demonstrated a *P* value of 0.003, indicating the concordance of the LDMS and PCR results was unlikely to have occurred by chance. The sensitivity of the 0.1 μL LDMS assay is 52% and specificity of 92%, compared with the PCR standard.

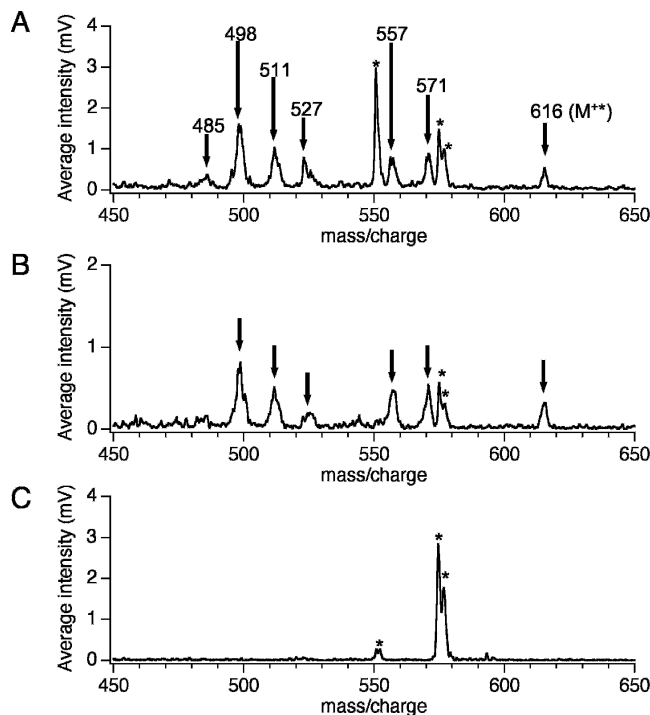An LDMS standard curve from laboratory culture of syn-

FIGURE 1. LDMS spectra: **A**, a positive endemic control, 27 individual shot spectra averaged; **B**, a microscopy negative, but PCR positive blood sample, 3 individual shot spectra averaged. **C**, The LDMS spectrum of a nonendemic negative control blood sample has background ions but clearly contains no heme. Heme characteristic ion peaks at mass/charge values of 616, 571, 557, 527, 511, 498, and 485 are indicated by the arrows. Background ions, unrelated to heme, are indicated by an asterisk.

chronized *P. falciparum* early rings and *P. falciparum* trophozoites was generated to compare the count of heme detections to sample parasitemia and to estimate the detection efficiency of infected erythrocytes (Figure 3). The error bars indicate 1 standard error of the mean based on the 3 replicate samples. Results for the endemic positive controls are shown for comparison. The positive non-pregnant Zambian controls had 6, 27 and 37 positive heme signal spectra corresponding to 11,799, 27,830 and 109,911 parasites per microliter, respectively.

Drug-resistance genotyping. Each of the PCR positive samples had different nested PCR-RFLP banding pattern for the *Pfmsp2* gene as expected in this region of Africa with high parasite diversity. The chloroquine-resistant genotype PfCRT K76T was present in all of the PCR positive samples with only one mixed sensitive and resistant genotype. The Pfmdr1 N86Y was present in 88% of the samples with 28% mixed sensitive and resistant genotype. The dihydrofolate reductase S108N allele was present in all of the samples indicating Resistance level 1 (Res1) to antifolate drugs (Figure 4).[4] The DHPS genotype resistance was present at less than 20% of samples. Only one sample had all five DHFR-DHPS mutations indicating high level drug resistance (Res2).[4] All the patients including the PCR positive patients were blood film and PCR negative on follow-up visit, indicating full parasitological response to antifolate medications.

DISCUSSION

Diagnosis of malaria during pregnancy in women who live in endemic areas is complicated by sequestration of parasites

TABLE 1

Comparison of parasitemia, PCR result, and number of LDMS heme shot detections out of 2,000 laser shots for human samples and trophozoite and ring-stage parasites

	Sample	Parasite/µL	PCR	Number of positive heme shots/2,000
	E005	neg	pos	1
	E006	neg	pos	1
	E007	neg	pos	1
	E017	neg	pos	1
	E027	neg	pos	1
	E028	neg	pos	1
Human pregnancy study	E032	neg	neg	1
	E040	neg	pos	1
	E049	neg	neg	1
	E009	neg	pos	2
	E019	neg	pos	2
	E035	neg	pos	2
	E016	neg	pos	3
	E044	neg	pos	3
	E031	neg	pos	4
Children positive controls	ma03081	11,799	pos	6
	ma04083	27,830	pos	27
	ma03082	109,911	pos	37
	Trophozoites	100	N.D.	0
	Trophozoites	100	N.D.	5
	Trophozoites	100	N.D.	6
	Trophozoites	1,000	N.D.	13
	Trophozoites	1,000	N.D.	19
	Trophozoites	1,000	N.D.	21
	Trophozoites	10,000	N.D.	88
	Trophozoites	10,000	N.D.	142
	Trophozoites	10,000	N.D.	157
In vitro cultured infected erythrocytes	Trophozoites	100,000	N.D.	50
	Trophozoites	100,000	N.D.	111
	Trophozoites	100,000	N.D.	151
	Rings	1,000	N.D.	0
	Rings	1,000	N.D.	2
	Rings	1,000	N.D.	3
	Rings	10,000	N.D.	11
	Rings	10,000	N.D.	12
	Rings	10,000	N.D.	15
	Rings	100,000	N.D.	35
	Rings	100,000	N.D.	59
	Rings	100,000	N.D.	60

N.D., not determined.

in the placenta, which reduces the number of circulating ring-stage parasites in peripheral blood able to be identified by traditional Giemsa stain–based microscopy. All patients in this study were enrolled during the peak malaria season and were blood film negative, but some were PCR positive and LDMS positive, suggesting low ring-stage parasitemias in these patients. Ginsburg and others have measured the hemozoin content of ring-, trophozoite-, and schizont-stage parasites to be approximately 1, 3, and 4–5 micromoles per 10^{10} parasites, respectively. These correspond to an average of 0.1, 0.3, 0.4–0.5 femtomoles per parasite, which suggests that LDMS, with subfemtomole sensitivity for heme, can detect hemozoin from a single individual infected erythrocyte. Even though hemozoin is not visible under light microscopy in ring-stage parasites, electron micrographs depict recognizable heme crystals during this stage.[11] The LDMS detection of heme in clinical *P. falciparum* samples is further evidence of the presence of submicroscopic hemozoin in ring-stage parasites.

The general agreement between the heme detection counts
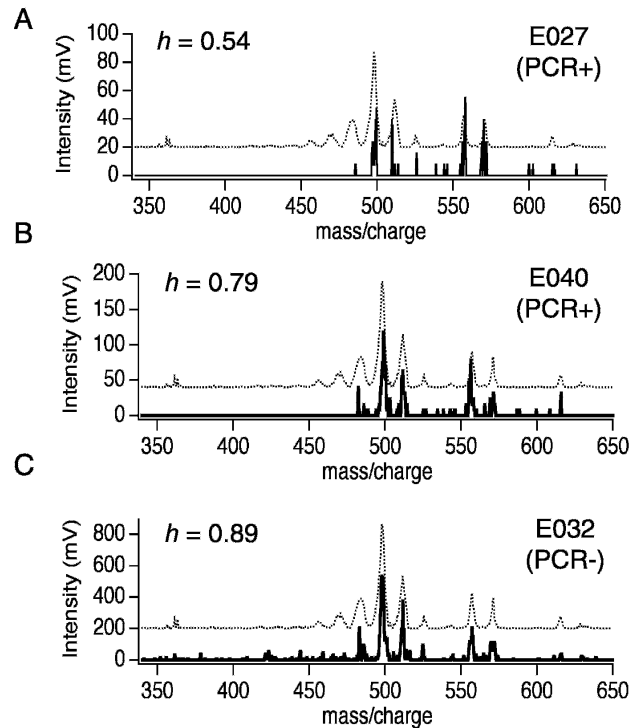
SINGLE SHOT SPECTRA OF DETECTED HEME

FIGURE 2. Single-shot LDMS spectra with detected heme from two PCR positive (**A**, **B**) and a PCR negative (**C**) patient. A value of h ≥ 0.45 was required for an individual laser shot spectrum to be classified as a heme detection.

for the endemic positive controls and those predicted by the ring-stage standard curve suggests that the population of ring-stage parasites harvested from the culture is reasonably representative of an *in vivo* population. Note also that the detection count tracks the parasitemia in the range of clinical interest from 0.1% parasitemia. The number of heme detections per 2,000 laser pulses is quite low, indicating a low efficiency of detection of infected erythrocytes. For example, at 10,000 parasites per microliter, the LDMS assay scanned the surface of a dried blood volume containing ~1000 ring-stage parasites and averaged ~10 heme positive spectra (~1% efficiency). This value is actually an upper bound to the true efficiency, as detections sometimes occurred in spatially contiguous clusters (consecutive pulses), which is expected when the beam width is much larger than the beam step used during sample scanning. "Shading" effects, that is, overlapping of

TABLE 2

Comparison of LDMS and PCR by Fisher exact test for significant proportions

	PCR		
	Positive	Negative	Total
LDMS			
Positive	13	2	15
Negative	12	18	30
Total	25	20	45

PCR analyzed 20–30 µL of blood, whereas LDMS analyzed approximately a tenth of a microliter of whole blood. Sensitivity is 52%, specificity is 92%, and *P* value is 0.003.
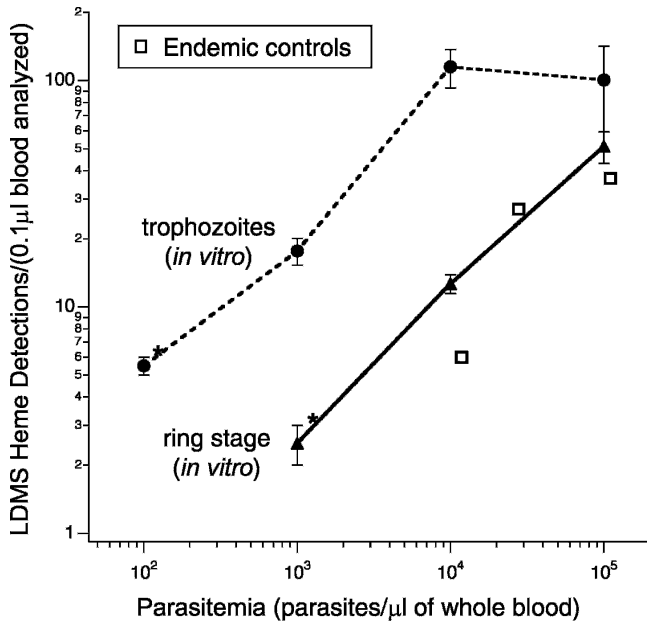
FIGURE 3. Comparison of parasitemia versus number of LDMS heme signal detections for *in vitro* cultured synchronized *P. falciparum* parasites. Trophozoites (circles), which by definition contain more hemozoin, scored a higher number of heme detection counts than rings (triangles) for a given parasite dilution. The three blood samples from *P. falciparum* smear positive patients are also indicated (empty squares). Values are means of triplicate measurements.

infected with uninfected erythrocytes when samples are prepared following the dilution protocol used here, likely contributes to the low detection efficiency we observed.

Another important feature of the *in vitro* data is that the number of heme detections is an order of magnitude higher for trophozoites than for ring-stage at comparable parasitemias. This indicates that the more mature parasite forms are detected more efficiently by the LDMS method, presumably due to the larger and more numerous hemozoin crystals. It also suggests that for a fixed blood volume analyzed, LDMS will on average be more sensitive for the three non-*falciparum* species of human *Plasmodium*, in which the later stage parasites circulate in the peripheral blood. This is not normally the case for *P. falciparum*, where later stage parasites sequester in internal organs. Previous pilot studies demonstrated detection of *Plasmodium vivax* infection at parasitemias < 100 parasites per microliter using the identical sample preparation protocol as in this study.[21] The data in Figure 2 and Table 1 show the detection of *P. falciparum* trophozoites at 100 parasites per μl, but in only 2 out of the 3 replicates tested. A similar detection probability was found for *P. falciparum* rings at 1,000 parasites per microliter. Generally, the probability of detecting infection by LDMS depends on the parasite stages, the detection efficiency for each stage, and the total blood volume scanned by the laser. When the expected detection count is low (e.g., extrapolating Figure 3 suggests that for ring stages, only 0.2 detections are expected per 2000 spectra at 100 parasites per microliter), it is possible to increase the detection probability by increasing the blood volume analyzed. The detection counts will scale linearly with the amount of blood scanned. As the limit of detection for field microscopy is ~100–1,000 per microliter, the LDMS detection of 13 microscopy negative, malaria positive cases out
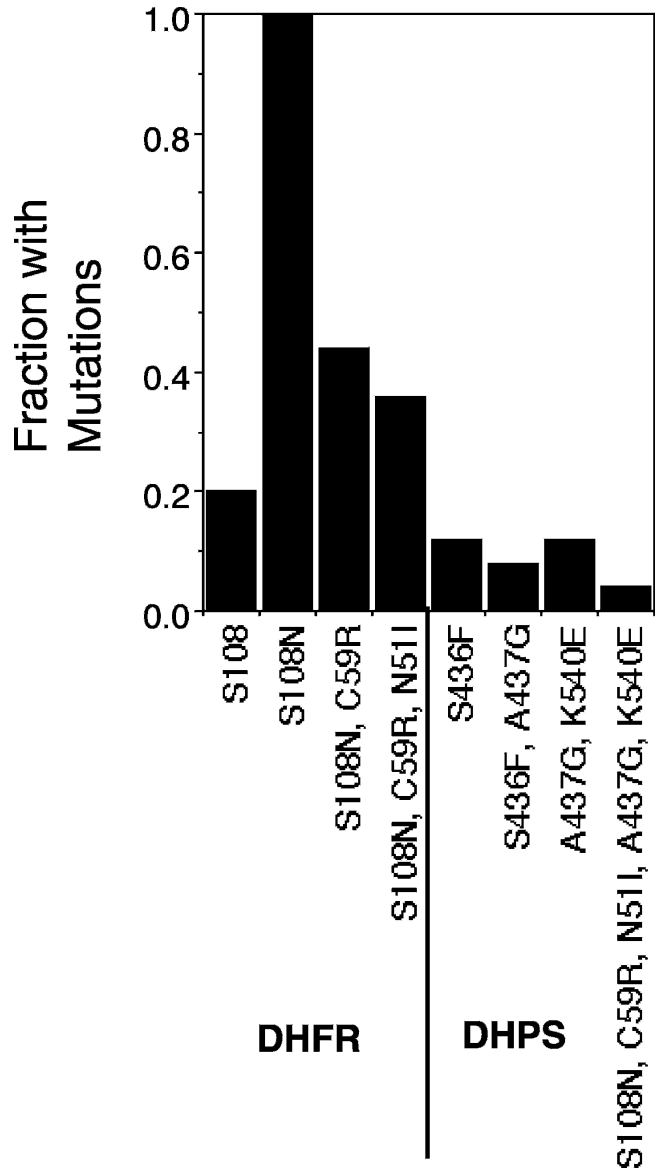

FIGURE 4. Fraction of DHFR and DHPS drug-resistant mutations out of 25 PCR positive patients.

of 25 positive PCR cases is evidence that the trial LDMS assay performed here was sensitive for *P. falciparum* parasitemias in the 100–1,000 per microliter range, but the probability of detection was only modest in this range. For the LDMS positive samples, four patients had 3 or more heme detections, three patients had 2 heme detections, and nine patients had only 1 heme detection per 2,000 spectra. Based on the ring-stage standard curve, we can infer that the parasitemias for the pregnant patients were in the 200–1,000 parasites per microliter range. A more quantitative analysis of the *in vitro* data based on Poisson counting statistics (Feldman AB and others, in preparation) predicts an average detection probability for this 0.1 μL–based LDMS assay to be ~50% over the 10–1,000 parasites per microliter parasitemia range. This is in good agreement with our clinical finding of 52% (13 LDMS positive out of 25 PCR-positive), assuming the parasitemias of these samples are distributed uniformly over this parasitemia range.

The two samples that were LDMS positive and PCR negative could have resulted from detection of leukocytes containing hemozoin, but not parasite DNA. When the ruptured infected erythrocyte releases merozoites, the residual body containing remnant parasite membranes surrounding hemozoin is released into the circulation. Hemozoin and/or the residual body containing hemozoin can be ingested by circulating neutrophils or monocytes. Immunochromatographic tests can detect sequestered parasites in pregnant patients through circulating antigens, as the schizont rupture of the infected erythrocytes in placental tissues releases *P. falciparum* aldolase, HRP II, or lactate dehydrogenase into the plasma in addition to that contained inside circulating erythrocytes. Therefore, like the immunochromatagraphic tests, LDMS has the capability to detect the molecular remnants of previous generations of *P. falciparum* parasites, in addition to the intact intraerythrocytic ring-stage parasites in the blood sample.

This work establishes the detection of the predominant ring-infected erythrocytes of *P. falciparum* by LDMS. The field and hospital use of LDMS will require further study in larger clinical trials using more carefully designed assay protocols that scan sufficient blood to meet a targeted sensitivity and probability of detection. A potential role may be as a screening tool of large populations before more time- and labor-intensive diagnostics are used. The current development of miniature, field-portable MS systems may make this LDMS method more widely available. Although the LDMS instrument costs are expensive, the protocol we have developed is simple-to-perform, rapid, and requires no consumables other than water and a lancet for obtaining the droplet of blood.

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