RAPID DETECTION OF MALARIA INFECTION IN VIVO BY LASER DESORPTION MASS SPECTROMETRY

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Abstract. Rapid diagnosis leading to effective treatment is essential to control escalating infectious diseases such as malaria. Malaria pigment (hemozoin) detection by laser desorption mass spectrometry (LDMS) was recently shown to be a sensitive (<10 parasites/µL) technique for detecting Plasmodium falciparum parasites cultured in human blood. To examine the use of LDMS in a rapid new malaria screening assay, we followed the time course of P. yoelii infections in mice in parallel with light microscopy and a colorimetric hemozoin assay. Hemozoin was detected by LDMS in 0.3 µL of blood within two days of infection independently of the inoculating dose of 10², 10⁴, or 10⁶ parasite-infected erythrocytes. Microscopy and colorimetric hemozoin determinations lagged the LDMS detection of infections by 2−4 and 3−5 days, respectively, except at the highest inoculation dose. The LDMS detection of hemozoin is a potentially more rapid screen than light microscopy for detecting malaria infection in this mouse model at parasitemias <0.1%.

INTRODUCTION

Despite development of immunochromatographic, polymerase chain reaction, flow cytometric and other detection methods, the microscopic examination of Giemsa-stained blood smears has endured for the past century as the benchmark method for malaria diagnosis with a working sensitivity of approximately 100 parasites/µL.1−7 Non-infected and low parasitemia samples limit the rate at which blood films can be scored. More than 30 minutes is required to examine the 200 fields needed to rule out an infection. Recently, the ultraviolet laser desorption mass spectrometry (LDMS) detection of hemozoin was demonstrated to be a novel and rapid method for the detection of Plasmodium falciparum parasites grown in vitro.8 This procedure is capable of achieving a sensitivity of <10 parasites/µL of blood. In contrast to microscopic analysis of blood smears, a prepared sample can be analyzed and scored by LDMS in less than one minute. The throughput of samples for microscopic examination could be increased by using LDMS as a malaria infection screening assay to triage samples and thereby minimize the number of sub-threshold parasitemias or non-infected samples submitted for analysis. Laser desorption mass spectrometry exploits the fact that intra-erythrocytic Plasmodium parasites catabolize most of the 5 mM host cell hemoglobin and sequester the released toxic heme as crystals called hemozoin (malaria pigment).9−15 Hemozoin strongly absorbs ultraviolet light resulting in vaporization and ionization of individual heme molecules. The laser desorption mass spectrum of hemozoin exhibits a heme cation radical (m/z 616) and more than five structurally characteristic heme fragment ions.8,16 This method does not enable malaria speciation. Heme-specific mass spectra have been previously observed during the fast atom bombardment mass spectrometric analysis of hemozoin isolated from the spleens of mice infected with P. yoelii.17

We followed the time course of non-lethal P. yoelii infections in mice to examine the utility of LDMS, combined with an abbreviated sample processing technique, for detecting infections in vivo. Groups of BALB/c mice were inoculated with high (10⁶), medium (10⁴), and low (10²) parasite-infected erythrocytes to compare LDMS with Giemsa-stained blood films and colorimetric hemozoin measurements to detect infection. Here we demonstrate that the LDMS detection of hemozoin in diluted whole blood samples provides a more rapid means of screening for infection than light microscopy at parasitemias <0.1%.

MATERIALS AND METHODS

Parasite growth. Plasmodium yoelii (17XNL) was propagated by serial passage in female BALB/c mice. This strain produces a non-lethal infection in these mice. It preferentially infects reticuloeytes and infections are asynchronous.

Animals. BALB/c mice (females, six weeks old) were obtained from the Charles River Laboratories, Inc−National Cancer Institute (Frederick, MD). Blood (100 µL) was collected from tail veins into heparinized phosphate-buffered saline (PBS). After blood collection prior to infection on day 0, groups of three mice were infected by intraperitoneal injection of 10⁶, 10⁴, or 10² parasite-infected erythrocytes in 100 µL of PBS. This protocol was approved by the Johns Hopkins University Committee overseeing the care and use of experimental animals.

Light microscopy. Parasitemia was determined via the light microscopic examination of Giemsa-stained thin blood smears after counting 10⁶ erythrocytes.

Hemozoin assay. Fifty microliters of frozen blood from individual timepoints was mixed with 50 µL of saponin (2 mg/mL), centrifuged at 13,000 × g for 10 minutes, sonicated in 150 µL of sodium docetyl sulfate (SDS)/bicarbonate buffer, pH 9, centrifuged, washed with 1.0 mL of 2% SDS, and quantitated at 400 nm after decrystalization in 10 mM NaOH, 2% SDS.14,18

Mass spectrometry. Blood (100 µL) was collected approximately every 48 hours during the 28 days following the infection of parasites. Blood samples (30 µL) were diluted 10-fold (v/v) upon collection in PBS containing heparin. Diluted samples (0.3 µL) were applied in triplicate to 20-well non-textured metal slides, allowed to air-dry, and 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. The laser flux (∼100 μJ/cm²) was selected to cause extensive, but generally incomplete, scission of the propionic acid side chains in the heme cation radical M⁺ (m/z 616) to yield a characteristic series of heme fragment ions of which m/z 498 is the most prominent. The simultaneous detection of the heme molecular ion and the more abundant heme-specific fragments enhances the specificity and sensitivity of hemozoin detection over alternative fluxes that exclusively yield either MH⁺ or fragment ions. One hundred individual laser shot spectra were acquired while the laser beam was rastered across each sample well (2 mm length).

**Mass spectral data processing.** Individual spectra were processed by in-house written software to detect heme using a matched-filter algorithm. The matched filter calculates a score \( h \), 0.0 ≤ \( h \) ≤ 1.0, representing the similarity of the test spectrum to that of heme, with \( h = 1.0 \) corresponding to an exact match and \( h << 1.0 \) corresponding to a highly non-conforming spectrum. The score \( h = 0 \) is obtained for spectra with all null data. *Plasmodium yoelii*-infected blood (2% parasitemia) was used as the template LDMS spectrum. The template spectrum was formed by averaging the ion intensities in each m/z bin for 100 consecutive laser shots for the range of m/z bins containing the heme molecular ion and its major fragment ions (m/z 450–620). The filter selectively passes spectra containing M⁺ (m/z 616) and fragment ions (m/z 571, 557, 526, 512, and 498). The matched filter was defined by treating the sample and template spectra as vectors, \( s \) and \( t \), respectively, with the intensity in each m/z bin representing one vector component. The two vectors were normalized so that the scalar products \( s \cdot s \) and \( t \cdot t \) = 1.0, then \( h \) was computed by forming the scalar product of \( s \) with \( t \); \( h = s \cdot t \). Heme was detected if two successively applied criteria were met: 1) the matched filter score \( h \) exceeded a threshold value (\( h > 0.5 \)) and 2), the signal-to-noise ratio (SNR) for the signal in the heme derived ion m/z range was ≥10.0. Background spectral noise was estimated by the signal intensity over the range (m/z ± 170) bracketing the heme ion range (m/z 450–620). The SNR value was calculated as the ratio of the mean ion intensity per m/z bin in the heme ion bands to that of the noise bands. Heme signal intensity was calculated by summing the signal intensities in the heme-ion specific m/z bands. For each data point, the mean and standard deviation were determined from the analysis of three sample wells.

**RESULTS**

**Laser desorption mass spectrometry spectra from infected and non-infected blood.** Representative heme spectra detected in diluted whole blood from a single mouse during the early phases of infection are shown in Figure 1. Hemin, synthetic β-hematin, and parasite-derived hemozoin crystals yielded mass spectra virtually identical to that of heme (Figure 1f). Heme complexed with hemoglobin in non-infected blood was not detected (Figure 1a and Supplementary Figure 1). Denaturation of proteins by the direct addition of 0.5 μL of 12 M formic acid to dry non-infected blood samples resulted in the LDMS detection of heme spectra, possibly due to its release after protein denaturation.

Heme spectra were not detected in blood collected from non-infected mice (Figure 1a and Supplemental Figure 1) and were absent from spectra obtained on the first day of infection (Figure 1b) in this mouse. Heme spectra were first detected by LDMS on day 2 (Figure 1c) and the intensity of spectra increased through day 6 (Figure 1d and e). Heme signal intensities achieved a plateau for the following 16 consecutive days and returned to non-detectable levels by day 28 in this mouse.

**Laser desorption mass spectrometry heme signal intensity, parasitemia, and hemozoin concentration during the infection time course.** The average LDMS heme signal, parasitemia, and colorimetrically determined hemozoin concentrations from representative mice at three parasite dose levels are shown in Figure 2. Data for the remaining mice are presented in Supplemental Figure 1. Micrographs of Giemsa-stained blood smears prepared at different time points are shown in Figure 3 for comparison with LDMS hemozoin data. Laser desorption mass spectrometry detected heme by day 1 in all samples from the medium dose group (Figure 2b and Supplemental Figure 1), by day 2 in all samples from the high dose group (Figure 2a), and by day 2 in two mice in the low dose group (Figure 2c). One mouse in the 10⁶ dose group did not sustain infection as determined by microscopic, colorimetric, and LDMS measurements. Parasites were detected by light microscopy at the same time as LDMS only for the high inoculum dose (Figure 3A). Microscopy lagged LDMS by 3 and 4 days, respectively, for the medium and low groups. Colorimetric hemozoin detection lagged LDMS even further by 2, 7, and 4–8 days respectively at the high, medium, and low parasite dose levels. Heme ion signal intensities rapidly increased after infection for all mice and reached a plateau after approximately six days. Over the next 14 days, parasitemia varied in a complex manner relative to heme ion intensity. The LDMS heme signal finally decayed over a 4–6-day period and disappeared entirely in more than half of the mice by day
24. Although no parasites were detected by microscopy by day 24 in these blood samples, leukocytes filled with hemozoin crystals were present (Figure 3F).

DISCUSSION

Automated miniature mass spectrometric instrumentation is being developed for the rapid detection of biologic warfare agents. The rugged design, ease of operation, and sensitivity of this technology could be translated to the more rapid and possibly automated detection and diagnosis of a wider range of diseases such as malaria. Promising initial LDMS studies detected *P. falciparum* parasites cultured in human erythrocytes at concentrations as low as 10 parasites/μL of whole blood. However, an extensive sample preparation protocol was used in these studies. To examine the utility of LDMS for rapidly detecting parasites in a more complex physiologic model of malaria infection and to increase assay throughput, the mouse time course measurements reported here were performed using a highly abbreviated sample dilution technique.

The high abundance of heme (~154 fg of heme/erythrocyte) in non-infected erythrocytes was originally expected to cause false-positive malaria detections. This suggested a need for the extensive removal of extra-parasitic haemoglobin in the previously reported sample preparation method. However, heme spectra are not detected in non-infected whole blood.

**Figure 2.** Time course of *Plasmodium yoelii* infection in three representative mice infected on day 0. 
A. High (10^6) parasite-infected erythrocyte inoculum dosed mouse (from Figure 1). B. Medium (10^5) dose. C. Low (10^4) dose. Parasites are detected via the laser desorption mass spectrometry (LDMS) detection of the heme cation radical and its corresponding fragment ions, light microscopy, and the colorimetric measurement of malaria pigment (hemozoin) concentration in blood. The LDMS average integrated heme ion intensity ± σ_n (△), parasitemia (■, %), and normalized hemozoin concentration (○, 100% = 297.4 μM). Supporting data are provided in Supplementary Figure 1.

**Figure 3.** Light microscopy of Giemsa-stained thin blood smears from the mouse in Figure 1 and Figure 2a during the time course of the asynchronous *Plasmodium yoelii* infection. The day 24 sample (F) was microscopically scored as parasite negative but the laser desorption mass spectrometry heme signal persisted due to the presence of hemozoin crystal-filled leukocytes. Arrows indicate hemozoin crystals. A. Day 2. An erythrocyte bearing a single-ring stage parasite is visible in the upper left quadrant. B. Day 4. C. Day 10. D. Day 18. E. Day 20. F. Day 24. Mouse erythrocytes are 3–5 μm in diameter.
samples that are diluted (10-fold) in water or neutral pH buffer. Therefore, it is not necessary to remove hemoglobin or other normally present heme sources to prevent false-positive LDMS results. Non-covalently bound heme can be detected by LDMS in non-infected whole blood samples only after heme-binding proteins are denatured (e.g., by the application of formic acid). For this reason, standard matrix-assisted laser desorption/ionization sample preparation methods using acidified organic solvents and organic acid matrices are not suitable for the detection of hemozoin in unprocessed blood samples. The tight binding of heme to hemoglobin and other proteins (e.g., hemopexin, albumin) renders heme LDMS silent in non-infected blood samples. For that reason, the centrifugation and washing steps previously used to prepare whole blood samples for malaria detection can be eliminated without incurring false-positive detections. Mass spectrometric methods generally require sample preparation steps to purify and concentrate target analytes. However, in the case of malaria, the parasite itself concentrates and purifies heme in the form of hemozoin crystals. These crystals present localized high heme concentration regions uniquely suited for its sensitive and specific detection by LDMS. Heme detection in hemozoin is an unusual and counterintuitive example in which parasites mitigate the sample processing problems typically encountered in the mass spectrometric analysis of biological specimens. Thus, the high capacity of blood proteins to tightly bind heme can be exploited to eliminate the centrifuge and reagents previously used for LDMS sample preparation, thereby increasing assay throughput and making this assay easier to perform under field conditions.

The LDMS detection of *P. yoelii* infections at the middle and lowest dose of infected erythrocytes by as much as four days earlier than light microscopy (≥0.1% parasitemia threshold) demonstrates that LDMS can be used as a rapid screening tool to detect malaria infections in this mouse model. Dose-dependent differences in the time to initial parasite detection by LDMS compared with microscopy and the colorimetric assay suggests that LDMS will be even more efficient at lower parasite doses.

Rapid saturation of the LDMS response early in infection at parasitemia levels approximately ≥0.1% prevented correlation of the LDMS heme signal intensity with parasitemia and indicates that the more extensive dilution of blood is required to achieve a larger dynamic range. Lack of the expected good correlation between the LDMS signal intensity and parasitemia in mice, although previously observed with *P. falciparum in vitro*, is presumably due to several factors: 1) non-linear signal detector response at high hemozoin densities; 2) fluctuations induced by different admixtures of parasite growth stages (stage-specific hemozoin content); 3) interrelated changes in the concentrations of parasites and erythrocytes caused by malaria-induced anemia; 4) post-infection persistence of hemozoin in leukocytes; and 5) complex biologic responses to infection such as fever, compensatory hematopoetic responses, immunologic reactions, and parasite death. Persistence of the LDMS heme signal after infections were microscopically observed to have been cleared (at the >0.1% parasitemia limit) is attributed to hemozoin present in circulating leukocytes as shown in Figure 3F. Similar to the clearance kinetics of pigment containing leukocytes in severe malaria, the LDMS heme signal disappeared as the population of hemozoin crystal-filled leukocytes decreased. Al-}

though the persistence of circulating hemozoin complicates its use as a biomarker of infection, it does not invalidate LDMS as a screening tool to more efficiently identify patient samples for further characterization by microscopy. It will be of interest to examine samples by LDMS and light microscopy in malaria-endemic regions where asymptomatic but persistent malaria infections have been identified.

A single erythrocyte harboring late stage trophozoites can produce more than 1 fmol of heme as hemozoin crystals. Ignoring sample surface area and deposited volume fluctuations, the amount of heme deposited for LDMS analysis can be estimated from the corresponding colorimetrically determined hemozoin concentration. On day 6 (Figure 2a), an estimated 1.3 pmole of heme (as hemozoin pigment) is present in the 30 nL of whole blood deposited for LDMS analysis. The LDMS ion intensities of the parasite infected (5.8% parasitemia) and 1.3 pmole heme standard samples are similar (Figure 1e and f), although the in vivo spectrum (Figure 1e) may be saturated. However, consistent measurement of hemozoin concentration in blood by LDMS during infection will require further evaluation. The physicochemical properties of blood are changed by malaria-induced anemia and dehydration. Dried samples obtained at different times during infection are expected to differ in thickness and protein composition. Variations in sample composition and morphology can produce variable rates of sample ionization that may further complicate the use of LDMS for more rigorously quantitative purposes. For instance, the intensity of the LDMS signal following exposure to successive laser shots decreases (Supplemental Figure 2) and could conceivably depend on sample thickness. More accurate measurement of parasite-derived heme crystal concentrations by LDMS and the relationship of this measurement to the circulating parasite burden may be achieved by several means. An isotopically labeled hemozoin internal standard may be used, together with the simultaneous measurement of hematocrit, hemoglobin concentration, and circulating parasite concentration.

The studies reported here demonstrate the successful use of a simple blood sample preparation method for the high throughput LDMS detection of hemozoin as a biomarker of malaria infection *in vivo*. The LDMS detection of hemozoin crystals *in vitro* and *in vivo* as a pan-malarial biomarker shows promise for rapidly differentiating infected from non-infected blood samples. However, this technique requires much further evaluation in human studies because the biology of malaria exhibits differences between animal models and the different species responsible for human infections, most notably *P. falciparum*. The presence and persistence of hemozoin in circulating leukocytes, hemozoin present as a residual body from the release of parasites by sequestered erythrocytes, and the relatively small circulating population of multiple hemozoin-bearing parasite stages are expected to enable the LDMS detection of *P. falciparum* parasites, even though the majority of mature *P. falciparum*-infected erythrocytes are sequestered in capillary beds. Therefore, the sequestration of *P. falciparum*-infected erythrocytes from peripheral circulation in humans is expected to only slightly diminish the effectiveness of the LDMS assay relative to the detection of *P. vivax*, *P. ovale*, and *P. malariae*. Preliminary data demonstrate that *P. falciparum* and *P. vivax* infections were detected by LDMS at <100 parasites/μL in human samples from Thailand and
samples from an ongoing large scale clinical study will be used to further characterize this assay (Feldman AB and others, unpublished data and http://www.jhuapl.edu/programs/rtdc/Pathogens/Malaria.html). Examination of the potentially confounding effects of other parasites (such as Schistosoma), bacterial, or viral infections, or genetic diseases such as glucose-6-phosphate dehydrogenase deficiency, sickle cell anemia, and the thalassemias will be important in determining the specificity of malaria detection by LDMS in future studies. The LDMS detection of parasites may additionally find application in the high throughput screening of drugs for anti-malaria activity. As rapid sample preparation techniques emerge for the mass spectrometric detection of malaria proteins, it may be possible to use the malaria proteome to practically diagnose infections at the Plasmodium species level. It may even become possible, in conjunction with robust miniature mass spectrometers, to perform malaria speciation and identify the drug resistance status of infections in the field.

Supplemental Figures 1 and 2 are available online at www.ajtmh.org.
**Supplemental Figure 2.** Examination of laser desorption mass spectrometry signal persistence by measuring the heme ion abundance in successive sets of 100 laser shots irradiating the same sample well. Mass spectra were acquired from one mouse in each dose group on day 14 and from blood drawn prior to infection on day 0. (–, low; –, medium; –, high parasite inoculum dose group). Raw spectra were averaged without applying the heme spectral matching filter. Heme was not detected in the preinfection sample and a stable heme-free background spectrum was obtained for all successive shot sets. Although the heme ion intensity in infected samples exponentially decreased as the number of laser shots increased, the accumulated heme signal magnitude increased.

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**REFERENCES**


