Unified Parasite Lactate Dehydrogenase and Histidine-Rich Protein ELISA for Quantification of Plasmodium falciparum

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Abstract. There is a need for more objective and quantitative tools to replace microscopy in malaria diagnosis. Emphasis has recently been placed on alternative methods such as immunochromatography-based rapid tests. However, these tests provide only qualitative results. Two bio-molecules, parasite lactate dehydrogenase (pLDH) and histidine-rich proteins (HRPs), that are released by the intra-erythrocytic stages of the parasite offer certain specific characteristics that could potentially improve malaria diagnosis. In this paper, we describe a protocol for a unified sandwich ELISA that allows for the separate but concurrent measurement of pLDH and HRP biomolecules in aliquots taken from the same samples. Freshly drawn blood from a healthy unexposed adult male was used to serially dilute in vitro cultivated and synchronized ring stage Plasmodium falciparum parasites. Commercially available ELISA formats were modified to allow for the measurement of pLDH and HRP from aliquots of the same samples. The pLDH and HRP levels in the samples spiked with known numbers of infected red blood cells (iRBCs) were measured, and the values were used to generate standard graphs. The standard graphs were used to estimate the numbers of iRBCs in test samples. Serially diluted recombinant proteins were similarly used to generate a calibration curve, allowing for the expression of test results in nanograms of their respective recombinant protein. Levels of pLDH and HRPs were determined by using 1) P. falciparum culture material (cells and medium) 2) P. falciparum infected human blood (N = 6) samples, and 3) plasma from P. falciparum–infected patient (N = 22) samples. The parasite density of all culture and infected patient samples was also estimated by microscopy. Both pLDH and HRP levels correlated positively with the parasite density assessed by microscopy: Pearson correlation coefficient pLDH (r = 0.754, P < 0.0001, 95% CI: 0.47–0.89); HRP (r = 0.552, P < 0.007, 95% CI: 0.16–0.79). The HRPs seem to be released in larger quantities than pLDH (in a ratio of ~1 pLDH:~6 HRP), making the detection of HRP in culture material, blood, and plasma easier. The modified ELISA assay with quantitative measurement of pLDH and HRPs may provide a valuable tool for malaria research and patient management.

INTRODUCTION

Reliable detection and quantification of Plasmodium falciparum parasite density is important for research and patient management.1 The most widely used technique, microscopy, is unable to consistently provide reliable results in conditions such as uncomplicated malaria, certain cerebral malaria presentations, and malaria during pregnancy.2,3 Research studies have established that both parasite lactate dehydrogenase (pLDH) and histidine-rich proteins (e.g., HRP-2) are released into plasma by the intra-erythrocytic stages and during schizogony.4 However, a role for these extracellular products in the development of sensitive diagnostic and quantitative immunoaassays for the disease has not been widely accepted for routine use. Even though these molecules are released into plasma, their binding characteristics and clearance rates vary. Recent studies have separately shown a direct correlation between plasma levels of HRPs and the severity of the infection5 on the one hand and blood levels of pLDH and response to therapy on the other.6 To date, the levels of pLDH and HRPs in infected blood have not to our knowledge been concurrently measured with a protocol standardized between the two assays for purposes of direct comparison or complementation.2,4,2 Such a practice, if routinely undertaken, may provide the clinician or researcher with a more robust and real-time data set to potentially improve diagnosis and focus treatment management. We have developed a unified ELISA assay for the measurement of pLDH and HRP under identical immunocapture condition and assay timings. Our unified ELISA is sensitive at levels equal to or better than those presently set for microscopy and rapid diagnostic tests (RDTs).

MATERIALS AND METHODS

Plasmodium falciparum culture and patient samples. The Dd2 strain of P. falciparum was cultured according to the method of Trager and Jensen.7 Cultures were synchronized to > 95% ring stages using 5% sorbitol.8 Molecular studies with this strain of P. falciparum have shown the absence of the HRP-2 subtype gene.9 It, however, secretes other HRPs such as HRP-1 and -3 (D Sullivan, personal communication, 2008). The P. falciparum cultures were set at 5% hematocrit (500 × 10⁶ red blood cells [RBCs]/mL) in 6 mL of RPMI 1640 medium supplemented with 10% human albumin in 25-cm² tissue culture flasks and followed until a working parasite density of up to a maximum of 3% was attained. The number of RBCs in the working culture was obtained by counting an aliquot of well-mixed culture material in a Coulter counter. The infected RBC (iRBC) in the working culture was serially diluted using whole blood from a healthy donor as the diluent. Giemsa-stained thin smears were prepared, and the parasitemia was estimated by experienced microscopists. Human blood samples, devoid of subject identifiers, were obtained from patients admitted to the Kisumu District Hospital through an Institutional Review Board–approved study protocol. Scientific and ethical approvals for use of these samples were obtained from the Ethical Review Committees of the Kenya Medical Research Institute, Nairobi, and the Human Use Research Committee of the Walter Reed Army Institute of Research, Silver Spring, Maryland. The microscopy results were expressed as the number of iRBCs per microliter of blood.
**Unified HRPs and pLDH capture ELISA protocol.** Two commercially available ELISA test kits manufactured under good manufacturing practice (GMP) conditions were used in these experiments: The Malaria Ag CELISA, from Cellabs Pty (Sydney, Australia) is for detection of *P. falciparum*-specific HRPs in human whole blood, serum, and plasma. The ELISA plates are coated with HRP-specific capture antibodies and enzyme-labeled HRP-specific detector antibodies are supplied along with the kit. The Malaria Antigen ELISA from Standard Diagnostics (Suwon, South Korea) is designed for the detection of pLDH in human whole blood. The ELISA plates coated with pLDH capture antibodies, and the enzyme-labeled pLDH detector antibodies were supplied in the kit along with the control reagents. Both of these kits work on the principle of sandwiching the specific target antigen between the capture and the detector antibodies. The assay protocols suggested in the respective package inserts differed in sample dilution, testing methods, and incubation timings. Use of the individual kit protocols made comparison of the results difficult, and therefore, a uniform assay protocol was developed for the detection of both pLDH and the HRPs present in the test sample using the respective kit reagents. Both HRP and pLDH malaria biomarkers were assayed under a single unified method designated “unified protocol.” Culture material (cells and medium), human blood or plasma samples of known parasite density were initially frozen overnight, subsequently thawed, vortexed and designated “test sample”. Next, using a clean 96-well microtiter tray, 50 µL of each test sample was mixed with 50 µL of PBS/T (phosphate-buffered saline supplemented with 0.05% Tween 20) and allowed to react for 15 minutes with 100 µL of the respective (pLDH or HRP) detector antibody solution. The reaction mixture was transferred into the ELISA test plate (pLDH and HRP plate) pre-coated with the capture antibody provided in the kits. Care was taken to completely transfer all of the reaction mixture. The plates were further incubated for 60 minutes at 37°C to allow effective binding of the analyte sandwiched between the corresponding capture and detector antibodies. After a washing step, each well received 100 µL of chromogen (TMB) substrate supplied in the kit, and the relative absorbance values were read in a VmaxKinetic Microplate Reader (Molecular Devices, Sunnyvale, CA) equipped with a dual wavelength (450/650 nm) filter. The amount of bound sandwiched target analyte was measured as optical density (OD). This unified protocol was used to measure the levels of pLDH and HRPs in culture material, whole blood (N = 6), and plasma (N = 22) from malaria-infected patients. The cut-off levels were calculated and set at the mean ± 3 SD values of parasite-free control material or uninfected human blood samples.

**Quantification of pLDH and HRP.** A known amount of the homogenous and purified recombinant protein preparation (pLDH or HRP) was tested in accordance with the unified protocol described above. The recombinant pLDH antigen was supplied in the pLDH kit as a positive control at 6.6 ng/µL concentration. This was titrated from 132 to 0.0423 ng/well, and the quantity was supplemented with the sample buffer to a total volume of 100 µL in each well. The recombinant pLDH provided a smooth graph on 5-fold dilution. In the case of the HRPs, a homogeneous recombinant (HB-3) HRP-2 protein (donated by Dr. David Sullivan, Johns Hopkins University) was titrated in the range of 1–0.0016 ng to generate a standard graph. The standard graph was used to convert the OD values of a test sample into nanogram of HRPs. A wide range of concentrations was tested (5–0.00032 ng), and based on the resultant graph, its inflection point was determined. Assays were performed in triplicate, and the resultant absorbance values were used to generate a standard graph. The standard graph was used to convert the absorbance values (ODs) of a test sample into nanograms of recombinant antigen. Considering the pLDH and HRP values at the intercept midpoint and the corresponding absorbance values, an inflection point was determined according to Sasaki and Mitchell as detailed in Figure 2. For example, at absorbance 1.1, the equivalent binding is 1.01 ng of recombinant pLDH protein, and an absorbance of 2.25 was equivalent to 0.2 ng of rHPs.

**Clinical samples.** The ELISA results of plasma samples were expressed in either number of iRBCs per microliter or picogram of pLDH and HRPs. The clinical samples were also tested with an HRP-based RDT (Cellabs Pty). Whole blood samples were used to assess the sensitivity of the unified ELISA on clinical samples from a population with hyperendemic malaria at the Kisumu District Hospital.

**Statistical method.** The pLDH and HRP ELISA test results were analyzed (F-test), and a comparison between the ELISAs and microscopy-based parasite density determination was done by Pearson correlation statistics.

**RESULTS**

**Range of detection of pLDH and HRP in in vitro–cultivated *P. falciparum* material.** Stock cultures of 1%, 0.1%, and 0.01% parasitemia were set and serially diluted to obtain a wide range of iRBC per milliliter samples. When diluted, the number of iRBCs in the 1% series ranged from 2,500 to 39.1 iRBC/µL; the 0.1% series ranged from 250 to 3.9 iRBC/µL; and the 0.01% series ranged from 25 to 0.4 iRBC/µL parasite densities. A 50-µL aliquot of in vitro culture material was freeze-thawed and well mixed with an equal volume (50 µL) of PBS/T in a fresh 96-well microtiter plate. An equal volume (100 µL) of detector antibody was added, and the plate was incubated for 15 minutes. The entire reaction mixture was transferred into assay wells pre-coated with capture antibody and provided in the test kits. ELISA assays were done on the three sets of cultures, and the pLDH and corresponding HRP levels were determined. Uninfected culture material was also tested in parallel as the negative control. The data (Figure 1) show that pLDH (cut-off value of OD = 0.12) was barely detectable in the 0.01% and 0.1% series but had a strong signal in the 1% series. The lowest detectable level of pLDH was measured at 156 iRBC/µL (OD = 0.234) in the 1% series. In contrast, HRP (cut-off value OD = 0.09) was detectable in all the dilution series tested. In particular, it was measurable at 1.6 iRBC/µL in the 0.01% series (OD = 0.133; Figure 1).

**Construction of standard graphs for pLDH and HRP-2.** A 5-fold and 2-fold dilution series was prepared for the recombinant pLDH and recombinant HRP2, respectively, and analyzed to obtain their linear ranges. Figure 2 shows standard graphs for pLDH (Figure 2A) and HRP-2 (Figure 2B) comprising both high and low reactivity values ($R^2 = 0.99$). Based on Sasaki and Mitchell and considering the linearity of the graph between 5.28 and 0.2116 ng pLDH, an inflection point (OD 1.1) was determined that is equivalent to 1.05 ng pLDH. Recombinant HRP values > 1 ng showed the characteristic.
Figure 1. Aliquots (50 µL) from serially diluted (0.3, 3.0, and 30 × 10⁶ iRBCs in 6 mL) stock cultures were tested using the unified ELISA. The levels of HRP were consistently higher than pLDH at all iRBC dilutions tested.

Saturation level seen in conditions of antigen excess. The linear portion of the graph was identified between 0.5 and 0.0625 ng, with an inflection point at OD 2.25, which is equivalent to 0.2 ng of rHRP-2.

Comparison of pLDH and HRP levels in graded whole culture material. Graded cultures were set up by inoculating *P. falciparum* to a final density of 0.01%, 0.1%, and 1% iRBCs. Cultures were prepared in 25-cm² tissue culture flasks at 5% hematocrit (500 × 10⁶ RBCs/mL) in 6 mL of RPMI 1640 medium supplemented with 10% human albumin and followed for 3 days. Samples containing iRBCs and the medium containing released biomarkers were used for testing. Parallel

Figure 2. Measured amounts of recombinant protein was diluted with quantity sufficient sample buffer to a total volume of (100 µL) per well. The levels of pLDH and HRP were determined using the unified ELISA protocol and the OD plotted against protein concentration.
cultural methods without the parasites were also set up as controls. This asynchronous culture material containing both iRBCs and the medium with released biomarkers was tested in the unified ELISA assay for the presence of pLDH and HRPs. Table 1 shows the overall comparison of the quantities of pLDH and HRPs present in the culture material (total of 6 mL) at the various densities (0.01%, 0.1%, and 1.0%) of iRBCs. The proportional differences between pLDH and HRPs were present at all densities tested with pLDH uniformly detected at lower levels than the HRP (Table 1). At 0.01% density, there was at least as much HRPs than pLDH, and at 0.1% density, the ratio was about seven times more HRPs (Table 1). This difference in the quantity of antigen produced may make it far easier to detect HRPs compared with pLDH when antigen detection assays (ELISA and RDTs), particularly at low parasite densities. These values show the relative amounts of pLDH and HRPs present in cultured material and should not be construed as a measure of the relative sensitivity of the ELISA test kits.

Comparison of microscopy and ELISA (pLDH and HRPs) from the same patient whole blood sample. Thick smear microscopy and immunochromatography-based RDTs require ~10 µL blood or test sample. A batch (N = 6) of microscopy-positive blood samples were tested by ELISA using 10 µL (equivalent to the volume used for thick smear microscopy and RDTs) and 40-µL volume. To each sample of whole blood (10- or 40-µL volume), sample buffer (PBS supplemented with Tween 20-PBS/T) was added to bring the volume in each ELISA microtiter plate test well to 100 µL. The use of PBS/T released the pLDH and HRPs present in the intact iRBCs. The levels of pLDH and HRPs were measured by the unified ELISA assay. Figure 3 shows that pLDH levels were low when 10-µL blood aliquots were used. However, higher levels of pLDH were detected when the sample test volume was increased 4-fold (to 40 µL blood). In comparison, HRP levels were detected with the low (10 µL) and high (40 µL) sample volume. These data corroborate the in vitro culture results and further suggest that the observed performance of pLDH versus HRP–based ELISAs and RDTs under field and clinical conditions may simply reflect the difference in the amounts of antigen present in the test sample rather than a difference in the sensitivity of the test. It is important to note that as much as 50 µL of blood sample can be used for either test in the unified ELISA assay protocol.

Measurement of pLDH and HRP in malaria-infected human plasma samples. A pilot study was undertaken to verify the performance of the unified ELISA assay for malaria diagnosis in patients presenting with a presumptive diagnosis of malaria at a district hospital in a malaria-endemic area in Kenya, East Africa. Twenty-two subjects participating in a hospital-based severe malaria study had their parasite density assessed by conventional microscopy and expressed in iRBCs per microliter of whole blood. Additionally, the unified ELISA assay for pLDH and HRP levels was performed by using one part (50 µL) of plasma sample mixed with an equal volume of sample buffer (PBS/T). An overall cut-off value (mean ± 3 SD) for pLDH (OD = 0.290) and HRP-2 (OD = 0.115) was calculated by using uninfected human plasma samples as previously described. Any plasma value read off the linear portion of their respective standard curves (Figures 2A and B) with an OD value greater than the cut-off value was considered to be positive. Figure 4 shows that pLDH and HRP can be measured from plasma samples, and some samples showed higher values (as high as 10 pg of pLDH and > 10 pg of HRP per microliter of plasma). There was a trend toward the detection of higher pLDH levels in plasma samples obtained from plasma samples showing > 15 parasites/µL. Although higher HRP levels were recorded in plasma samples derived from blood samples showing > 31 parasites/µL, some of the samples with 3, 5, and 15 parasites/µL also showed elevated levels. The correlation between the parasite density (parasite/µL of whole blood) and the plasma levels of pLDH and HRP (pg/µL of plasma) was highly significant as assessed by Pearson correlation statistics: pLDH (r = 0.753, P < 0.0001, 95% CI: 0.39–0.86) and HRPs (r = 0.552, P < 0.007, 95% CI: 0.16–0.79), with a higher significance for pLDH compared with the HRPs. Additionally, the pLDH and HRP ELISA assay results were significantly different from each other (F-test, P = 0.00032). The picogram units of pLDH and HRPs seen in 22 infected plasma samples were graded, and their values were compared with respective levels of HRPs (versus unit grades of pLDH) and pLDH (versus unit grades of HRPs). This overall analysis showed significant difference between the pLDH and HRPs (graded data analysis not shown). For every unit of pLDH detected, there were ~7 units of HRPs (P < 0.001) present in the infected human plasma samples. Even though a difference in the levels of pLDH versus HRPs was also observed in clinical samples, care must be taken in the interpretation and extrapolation of in vitro results to clinical cases given the complexity of parasite maturation and sequestration. Additional studies with synchronized parasites and measurements in the various blood compartments are needed before correlations to parasite burden or the clinical severity of the disease can be attempted. Microscopy detects iRBCs in circulating RBCs only, whereas the targeted biomarkers in both plasma and iRBCs are measured by the unified ELISA protocol. Before such studies can be reliably and reproducibly undertaken, technical difficulties must be overcome in the separation and washing of microliter amounts of plasma samples without the loss of cells and targeted biomarkers.

**DISCUSSION**

The family of HRPs was first identified as dense cytoplasmic inclusions granules associated with the rough endoplasmic reticulum of the asexual stages of the parasite *P. lophure*. Later, Kilejian and Jensen described a histidine-rich counterpart protein in *P. falciparum* with a relative molecular mass

<table>
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<tr>
<th>No of iRBCs in 6 mL culture material</th>
<th>pLDH (ng/mL)</th>
<th>HRPs (ng/mL)</th>
<th>Relative</th>
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<tr>
<td>Control 0 iRBCs</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>0.01% parasite density</td>
<td>1.87</td>
<td>3.78</td>
<td>2.02</td>
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<tr>
<td>0.3 x 10^4 iRBCs</td>
<td>3.13</td>
<td>22.5</td>
<td>7.19</td>
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<tr>
<td>0.1% parasite density</td>
<td>28.9</td>
<td>164.5</td>
<td>5.69</td>
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<tr>
<td>3.0 x 10^4 iRBCs</td>
<td>11.3</td>
<td>63.59</td>
<td>5.62</td>
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* Aliquot were taken from cultures with known parasite densities and the relative amounts of pLDH and HRPs measured. using the unified ELISA protocol. The HRP levels were found to be consistently higher than pLDH levels at all iRBC densities tested and a mean ratio of 5.62.
Subsequent work showed three types of HRPs (HRP-1, HRP-2, and HRP-3) in *P. falciparum*. The HRP-2 gene is absent from the Dd2 strain of *P. falciparum*, which nonetheless secretes the other HRPs. In contrast to *P. falciparum*, the HRP gene family is non-functional in other human malaria parasites. The HRPs are released in large quantities by the asexual (ring and trophozoites) and early gametocytes stages of *P. falciparum* and can be detected at parasite densities as high as ~55 kd.

**Figure 3.** Either (10 or 40 µL) aliquots of whole blood were taken from each of six patients with known *P. falciparum* parasite densities and the relative amounts of pLDH and HRPs measured, using the unified ELISA protocol. Increasing the sample volume from 10 µL to 40 µL greatly improved the relative absorbance for the pLDH versus the HRPs ELISA assay result.

**Figure 4.** Patients presenting to the Kisumu District Hospital with a presumptive diagnosis of malaria had their parasite density estimated by microscopy. Their plasma pLDH and HRP levels were measured by the unified ELISA protocol and the resulting values compared.
Hinrichs showed that pLDH uniquely depended on APAD in its ATP requirements. Furthermore, work by Makler and Druilhe and others showed direct plasmodial participation in the provision of lactate to pyruvate and uncovered its role in the design of genus- and species-specific immunodiagnostic tools for malaria diagnosis. Druilhe and others using the same set of monoclonal antibodies as those used by Piper and others showed much higher sensitivity for pLDH in a double site immunoenzymatic assay (88% sensitivity compared with 84% optimal RDTs). Unfortunately none of these pLDH assays, manufactured under GMP conditions, are available commercially and were therefore not used in this study. The clearance of pLDH from the plasma has been shown by previous studies to be rapid (3–5 days) compared with HRP (10–14 days). Consequently, pLDH levels more closely reflect the presence of metabolically viable parasites and can be used to monitor patient response to antimalarial therapy.

We evaluated three different types of analytes to understand the different levels and inter-relationship between pLDH and HRPs: 1) whole culture (cells + culture fluid) material (Figure 1; Table 1), 2) malaria-infected human whole blood (N = 6; Figure 3), and 3) plasma samples derived from infected human blood (N = 22; Figure 4). Our data support the notion that HRPs are released in higher amounts and can be detected with smaller blood volumes than pLDH. HRPs are not well suited for monitoring the antimalarial therapy in populations with hyperendemic malaria because of their slow clearance rates from plasma. Sensitivity of pLDH-based rapid assays remained relatively low for P. falciparum, detecting a lower limit of ~200 parasites/µL of blood compared with HRP based rapid tests (up to 99% sensitive), which detect ~50 parasites per microliter. This may be because of the fact that parasites release smaller regulated quantities of pLDH, a metabolically active enzyme, compared with the HRPs. The ELISA format offers reproducibility and high throughput of samples. All of these attributes are not shared to the same degree by tests based on immunochromatography technology. Furthermore, our data provides a standardized protocol for the measurement of both pLDH and HRP using separate kits but under similar conditions that allow for joint and complementary analysis of the test results from the same patient sample. The unified sandwich assay technique was tested not only with the spiked blood samples but also using clinical blood samples. The commercial HRP ELISA kit used in this study is a unified ELISA protocol described in this manuscript that has been used to determine the HRP-2 in blood, plasma, and serum in a recent publication by Kifude and others (Kifude CM, Rajasekariah GH, Sullivan DJ, Stewart VA, Angov E, Martin SK, Diggis CL, Watumbi JN, 2008 Enzyme-linked immunosorbent assay for detection of Plasmodium falciparum histidine rich proteins 2 in blood, plasma and serum. Clin Vaccine Immunol 15: 1012–1018).

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