



Sensitive Immunoassay Detection of *Plasmodium* Lactate Dehydrogenase by Inductively Coupled Plasma Mass Spectrometry

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Specialty section:

This article was submitted to
Parasite and Host,
a section of the journal
Frontiers in Cellular and Infection
Microbiology

Received: 22 October 2020

Accepted: 24 November 2020

Published: 11 January 2021

Citation:

Mu J, Yu LL and Wellem's TE (2021)
Sensitive Immunoassay Detection of
Plasmodium Lactate Dehydrogenase
by Inductively Coupled
Plasma Mass Spectrometry.
Front. Cell. Infect. Microbiol. 10:620419.
doi: 10.3389/fcimb.2020.620419

Rapid, reliable, and sensitive detection of *Plasmodium* infection is central to malaria control and elimination. Many Malaria Rapid Diagnostic Tests (RDTs) developed for this purpose depend upon immunoassays that can be improved by advances in bound antibody sensor technology. In a previous study, immuno-polymerase chain reaction (PCR) was shown to provide highly sensitive detection of *Plasmodium falciparum* lactate dehydrogenase (PfLDH) in monoclonal antibody (mAb) sandwich assays. Here, we show comparably high immunoassay sensitivity by inductively coupled plasma mass spectrometry (ICP-MS) detection of gold nanoparticles (AuNPs). Following capture of PfLDH with the primary mAb and binding of the AuNP-labeled detection mAb, ICP-MS signals from the AuNPs provided quantitative measures of recombinant PfLDH test dilutions and *P. falciparum*-infected erythrocytes. A detection limit of 1.5 pg/mL was achieved with the PfLDH protein. Parasitemia in cultures of *P. falciparum*-infected erythrocytes could be detected to a lower limit of 1.6 parasite/ μ l (p/ μ l) for early ring-stage forms and 0.3 p/ μ l for mixed stages including mature trophozoites and schizont-stages. These results show that ICP-MS detection of AuNPs can support highly sensitive and accurate detection of *Plasmodium* infection.

Keywords: Gold nanoparticles, malaria, diagnostic tests, antigen-based detection, ICP-MS

INTRODUCTION

Asymptomatic and submicroscopic *Plasmodium* infections that go undetected in endemic populations and sustain transmission through mosquitoes are a continuing impediment to malaria elimination (Wu et al., 2015). Rapid and reliable diagnostic tests are needed that can detect these infections and be widely deployed for use at the primary healthcare level (Gitta and Kilian, 2020). Microscopy of Giemsa-stained blood smears falls short of these needs, not only because of the expertise and time it demands, but also by its detection threshold, estimated to be 50 to 500 parasites/ μ l (p/ μ l) under field conditions (Poschl et al., 2010). Highly sensitive molecular tests based on polymerase chain reaction (PCR) (Poschl et al., 2010), real-time quantitative PCR (qPCR) (Grignard et al., 2020), reverse transcription polymerase chain reaction (RT-PCR)

(Bourgeois et al., 2010), loop-mediated isothermal amplification (LAMP) (Han et al., 2007; Kongkasuriyachai et al., 2017; Mohon et al., 2019) and a clustered, regularly-interspaced palindromic repeat (CRISPR)-based assay (Cunningham et al., 2020) are much more sensitive, detecting parasitemia as low as 2 to 5 p/μl. However, the requirements of nucleic acid extraction, amplification, and detection often limit the use of these methods in resource-poor endemic settings.

Between the relatively low sensitivity of microscopy and the high sensitivity of nucleotide sequence detection methods, a mid-range of sensitivity is offered by Rapid Diagnostic Tests (RDTs), of which some can detect as few as 10 p/μl (Hemben et al., 2018). Overall, an estimated 1.92 billion RDTs were distributed between 2010 and 2017 (Slater et al., 2015; Wu et al., 2015). Antigens detected by these RDTs include the *P. falciparum* histidine-rich protein II and III (PfHRP-II, PfHRP-III), lactate dehydrogenase of *P. falciparum* (PfLDH) as well as other *Plasmodium* species, or a *Plasmodium* aldolase, all of which are abundantly produced by the parasites during the erythrocytic cycle. Although the tests can provide rapid results and require no specific training or equipment, the performances of RDTs call for improvements on their mid-range sensitivity (Das et al., 2018) and false-negative results, which can result from loss of PfHRP-II/III expression from some *P. falciparum* strains (Gatton et al., 2017; Verma et al., 2018; Bosco et al., 2020) or bloodstream parasitemias below the detection threshold (Bell et al., 2006).

To improve the performance of RDTs, research efforts in recent years have included searches for alternative parasite antigens (Mu et al., 2017), new detection methods for hemozoin from parasite digestion of hemoglobin (Peng et al., 2014; Rifaie-Graham et al., 2019), and improved biosensors and immunosensors (Krampa et al., 2020). Electrochemical techniques, with labeled amperometric as well as label-free impedimetric strategies, have been applied to malaria diagnostic studies and outperformed optical methods (Sharma et al., 2011; Low et al., 2019; Obisesan et al., 2019). A sensitive, magnetic nanoparticle (MNP) labeled immunosensor has been shown to detect PfHRP-II with a limit of detection (LOD) of 360 pg/mL (Castilho Mde et al., 2011). Aptamer-based sensors targeting PfLDH demonstrated high sensitivity and specificity with LOD measuring 120.1 fmol/L (4 pg/ml) (Lee et al., 2012). Dirkwager et al. (Dirkwager et al., 2016) used aptamer functionalized microbeads to measure the enzymatic activity of recombinant PfLDH and showed a LOD of 4.9 ng/ml. Further, a portable microfluidic biosensor, integrated with the aptamer-tethered enzyme capture (APTEC) assay, was developed for highly specific detection of *P. falciparum* in cultures and clinical samples (Fraser et al., 2018). Receptor-target binding can be detected in label-free formats by piezoelectric immunosensors (Sharma et al., 2011) or indicator displacement assays (Chakma et al., 2016). Although these methods are considered to have advantages for field applications, their sensitivity (12.0 ng/ml) needs to be improved further.

Gold nanoparticles (AuNPs) offer highly useful physicochemical properties including high density, good biocompatibility, stability, and catalysis activities, which have spurred their application in bioassays and diagnostics, particularly to meet the needs of points of

care (Cordeiro et al., 2016). Indeed, for malaria parasite detection, assays with AuNPs have been developed for rapid, simple, and cost-effective detection of *P. falciparum* infections, although the estimated LOD for these assays was poor (2.4 μg/ml for PfHsp70 and PfHSP90; ≈1,000 p/μl for PfLDH) (Castilho Mde et al., 2011; Guirgis et al., 2012; Jeon et al., 2013). To improve on the detection sensitivity of biosensors for AuNPs, non-optical bioassays have been developed with piezoelectric and electrochemical biosensors (Jiang et al., 2018). AuNP-labeled sandwich assay on the surface of a screen-printed gold electrode provided improved sensitivity of PfHRP2 detection to 36 pg/ml (Hemben et al., 2017) and of PfLDH to 19.0 pg/ml (Hemben et al., 2018). AuNPs have served for an electrochemical sensor in the detection of malaria parasites in clinical samples (Obisesan et al., 2019).

Inductively coupled plasma mass spectrometry (ICP-MS), is a highly sensitive technique for element and isotope analysis. The high-temperature ionization characteristics of the inductively coupled plasma (ICP), together with the sensitive and fast scanning modern mass analyzer, have boosted the capability of ICP-MS detection for biomolecules with metallic nanoparticle (NP) labels. In biomedical diagnostics, ICP-MS measurement of AuNP labels for HIV-1 p24 antigen achieved a detection limit of 1.49 pg/ml (He Q. et al., 2014). Detection of AuNPs has also served as a proxy for assays of anti-erythropoietin antibodies (Lu et al., 2009), virus-specific RNA (Hsu et al., 2011), human vascular endothelial growth factor (Thompson et al., 2010), and sialic acids on cancer cells (Zhang et al., 2016). Use of AuNPs for PfHRP-II biomolecular analysis has also been described (Wilschefski and Baxter, 2019). Because of its unmatched sensitivity for a wide range of metals and several non-metals, ICP-MS has increasingly been used for bioanalytic quantifications with element-tagged immunoassays in clinical diagnosis and single-cell analysis (Liu et al., 2014; Wilschefski and Baxter, 2019). Here, we describe the ICP-MS detection of AuNPs as a proxy for the sensitive immunoassay quantification of PfLDH, both as a purified recombinant protein and as antigen present in *P. falciparum*-infected erythrocytes.

METHODS

Plasmodium falciparum Parasite Cultures and Test Sample Preparations

P. falciparum parasites (3D7 line) were propagated *in vitro* in O⁺ human red blood cells at 2% hematocrit in RPMI1640 medium with L-Glutamine, 25 mmol/L Hepes, 50 μg/ml Hypoxanthine (KD Medical, Columbia, MD) supplemented with 10 μg/ml gentamycin (Gibco, ThermoFisher Scientific, Grand Island, NY) and 0.5% Albumax I (ThermoFisher Scientific, Grand Island, NY) (Cranmer et al., 1997). Synchronization of parasite cultures with 5% sorbitol (Sigma) was performed as previously described (Lambros and Vanderberg, 1979). Parasitemias were determined from Giemsa-stained thin blood films, and erythrocytes per unit volume were counted by hemocytometer. The total number of parasite-infected erythrocytes was calculated as: (% parasitemia/100) × (erythrocytes/ml) × (ml of culture).

For sample preparations, *P. falciparum* infected erythrocytes were washed three times with PBS (10 mmol/L PO_4^{3-} , 137 mmol/L NaCl, 2.7 mmol/L KCl) and lysed in a radioimmunoprecipitation assay buffer (RIPA buffer, Thermo Fisher Scientific, CA, USA) containing 1× protease inhibitor (Thermo Scientific Halt Protease Inhibitor Cocktail, Thermo Fisher Scientific, CA, USA). The samples were assayed immediately or aliquoted and stored at -20°C .

AuNP-Labeled PfLDH Antibodies and Sample Preparations

Malaria PfLDH mouse monoclonal antibodies (mAbs) MBS498007 and MBS498008 were purchased from MyBioSource, Inc. (San Diego, CA). Detection mAb MBS498008 was labeled with AuNPs using a 60 nm gold conjugation kit (ab188216, Abcam, CA), which covalently attached the ultra-stable AuNPs. Briefly, stock mAb MBS498008 (5 mg/ml) was diluted to 0.1 mg/ml with the gold 60 nm Antibody Diluent. For each reaction, 42 μl of the 60 nm AuNP reaction buffer was added to 12 μl of diluted antibody and mixed thoroughly by pipetting up and down at least 5 times. Forty-five μl of the mixture was then transferred to a vial of 60 nm AuNPs (provided in the Abcam kit) and mixed by gently pipetting up and down. Finally, 5 μl of quencher reagent was added and the AuNP-labeled mAb was stored at 4°C before use.

Immunoassay Detection of PfLDH Antigens

The PfLDH immunoassay was performed as previously described (Mu et al., 2017) with some modifications. Test tubes (TBS0211, Bio-Rad) were coated with anti-PfLDH mAb MBS498007 (6 $\mu\text{g}/\text{ml}$) overnight at 4°C . After three washes with PBS plus 0.05% Tween 20, the plates were blocked with tris-buffered saline, 4% bovine serum albumin (BSA), 0.05% Tween 20 (MilliporeSigma) for 2 h. Two-fold serial dilutions of recombinant PfLDH protein (MBS5308810, MyBioSource, Inc.) in 30 μl PBS buffer or 3D7 parasite lysate in RIPA buffer were added and incubated for 2 h at 37°C . After six additional washes, 30 μl of AuNP-labeled mAb MBS498008 (200 ng/ml) was incubated with the captured antigen for 1 h at 37°C . Unlabeled mAb was removed by five washes and samples were stored dry in the test tubes at 4°C before the ICP-MS was performed.

Inductively Coupled Plasma Mass Spectrometry

ICP-MS was performed as described previously (Assumpção et al., 2013). Briefly, a 200 μl aliquot of freshly prepared aqua regia, a mixture of one part nitric acid and three parts hydrochloric acid by volume, was deposited into the test tube. The tubes were capped, and the contents were allowed to react at room temperature of 21°C on the bench overnight. The contents in the tube were quantitatively transferred to a pre-weighed 15 ml Falcon centrifuge tube (Corning 352097, Thermo Fisher Scientific) and diluted to 5 ml with locally purified water distilled below the boiling point. The mass of the resulting sample was weighed on a Mettler Toledo (Columbus, OH) model AT261 Delta Range analytical balance by difference. Procedural blanks

were prepared similarly. Samples and the blanks were analyzed using the SemiQuant mode of Agilent Technologies (Santa Clara, CA) Model 7500CS inductively coupled plasma mass spectrometer. The sample introduction system of the mass spectrometer consisted of a perfluoroalkoxy alkanes (PFA) microflow (0.1 ml/min) concentric nebulizer, a PFA Scott-type double-pass spray chamber, and a sapphire injector. The SemiQuant mode is capable of quantifying elements of the entire periodic table by using the spectral information of the elements. The instrument was calibrated using a solution containing 20 $\mu\text{g}/\text{kg}$ each of 31 elements (lithium, beryllium, boron, sodium, magnesium, aluminum, calcium, scandium, vanadium, chromium, manganese, cobalt, nickel, copper, zinc, arsenic, selenium, strontium, molybdenum, silver, cadmium, antimony, barium, lanthanum, europium, holmium, ytterbium, thallium, lead, thorium, and uranium) prepared by diluting Catalog No. ICP-MS/SCS ICP-MS Calibration Standard (High Purity Standards, Charleston, SC) with 1.5% volume fraction nitric acid (Optima grade, Thermo Fisher Scientific) in water. National Institute of Standards and Technology Standard Reference Material (SRM) 1643f, Trace Elements in Water, was measured with the samples, serving as the quality assurance for the measurement.

Determinations of PfLDH-Based Immunoassay Sensitivity Limits

PfLDH determinations of LOD were calculated from serially-diluted samples of recombinant PfLDH protein (10 $\mu\text{g}/\text{ml}$ to 0 $\mu\text{g}/\text{ml}$) by the formula: $\text{LOD} = 3.3(\text{Sy}/\text{S})$, where Sy and S represent the standard deviation of the response (Sy), and slope of the calibration curve (S), respectively (Evard et al., 2016). S and Sy values were obtained using “SLOPE” and “STEYX” functions in MS Excel. *P. falciparum* erythrocytic stage detection sensitivities were determined by two-fold serial-dilutions of parasite-infected cultures from 40 p/ μl to 0.07 p/ μl . Synchronized ring-stage and mixed stage parasite cultures were evaluated separately. ICP-MS background signals from uninfected control erythrocyte dilutions (0 p/ μl) were subtracted against signals from the parasite-infected cell dilutions for analysis. One-way ANOVA and 95% confidence intervals were calculated in Prism 8 (GraphPad Software, La Jolla, CA, USA). All experiments were performed in duplicate.

RESULTS

Sensitive Immunoassay Detection of PfLDH by Inductively Coupled Plasma Mass Spectrometry of Bound AuNP-Labeled Antibody

For the immunoassay detection of PfLDH, capture of the protein by the primary capture mAb and binding of the detection mAb were as previously described for high sensitivity immuno-PCR assays (Mu et al., 2017). However, instead of the biotinylated DNA tag used for immuno-PCR, the detection mAb was labeled with AuNPs (Figure 1). Following disruption of the immune complex with aqua regia solution, then vaporization and

ionization in the nebulizer and spray chamber, gold atoms were separated according to their mass-charge ratio (m/z) and quantified by ICP-MS. The amount of PfLDH in the sample is calculated with the following equation:

$$C_{\text{PfLDH_sample}} = \frac{I_{\text{Au_sample}} \times C_{\text{PfLDH_standard}}}{I_{\text{Au_PfLDH_standard}}} \quad (1)$$

where $C_{\text{PfLDH_sample}}$ and $C_{\text{PfLDH_standard}}$ are the concentration of PfLDH in the sample and the standard, respectively; $I_{\text{Au_sample}}$ and $I_{\text{Au_PfLDH_standard}}$ are the instrument response to ^{197}Au in the PfLDH sample and the standard, respectively. To calibrate detection sensitivity, 30 μl volumes of a dilution series of recombinant PfLDH protein dilutions from 0.3 $\mu\text{g/ml}$ to 10 $\mu\text{g/ml}$ were tested by the assay protocol. ICP-MS quantification of the bound gold atoms (**Figure 2**) demonstrated sensitive detection of PfLDH protein in the $\mu\text{g/ml}$ range. Note that each point on the curve was obtained from a PfLDH sample subjected to the AuNP tagged immunoassay procedure. The results from the negative controls (**Table 1**) showed that the carry-over of AuNP or unbound AuNP reagents had been effectively minimized by the multiple washes with PBS. Linear fit of the data yielded a calibration curve expressed as $y = 2.370x - 0.772$, $R^2 = 0.9839$, with a PfLDH LOD of 1.5 $\mu\text{g/ml}$.

Sensitive Detection of *Plasmodium falciparum*-Infected Erythrocytes by the Inductively Coupled Plasma Mass Spectrometry PfLDH Immunoassay

We next evaluated the sensitivity of the ICP-MS PfLDH immunoassay for detection of *P. falciparum*-infected erythrocytes. Experiments were performed with mixed stage-infected erythrocytes (**Figure 3A**) or with synchronized ring stage-infected erythrocytes (**Figure 3B**). The Au signals at 0.57 to 0.60 ng from the lowest test parasitemia (0.07 $\text{p}/\mu\text{l}$) were more than 10 \times higher compared to that of the control sample (0.05 ng,

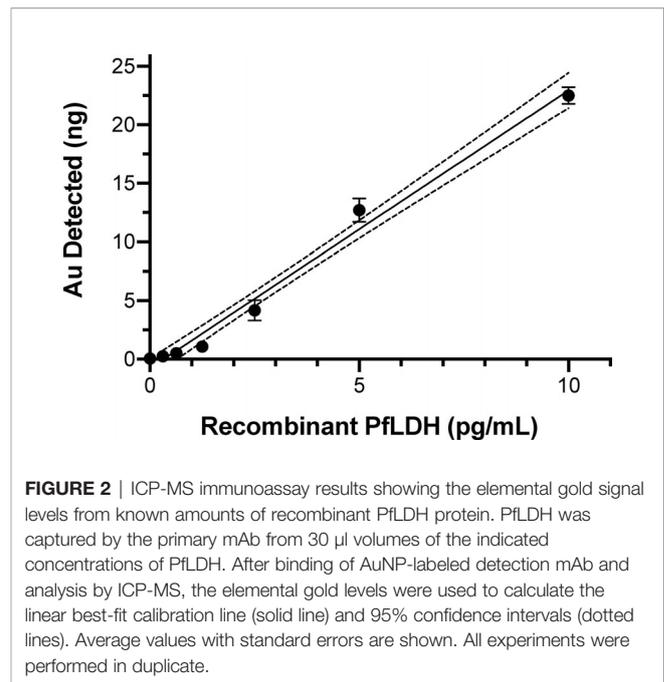


Table 1), testifying to the high sensitivity of ICP-MS based immunoassay in detection of *P. falciparum* infections. Statistical analysis with 95% confidence interval of difference (**Table 1**), showed that the significant difference between the test samples and control could be confidently detected down to a level of 0.32 $\text{p}/\mu\text{l}$ for mixed stage (**Figure 3C**) or 1.6 $\text{p}/\mu\text{l}$ for ring stage test samples (**Figure 3D**).

DISCUSSION

Recent years have seen remarkable advancement in the application of ICP-MS technology for molecular diagnostics,

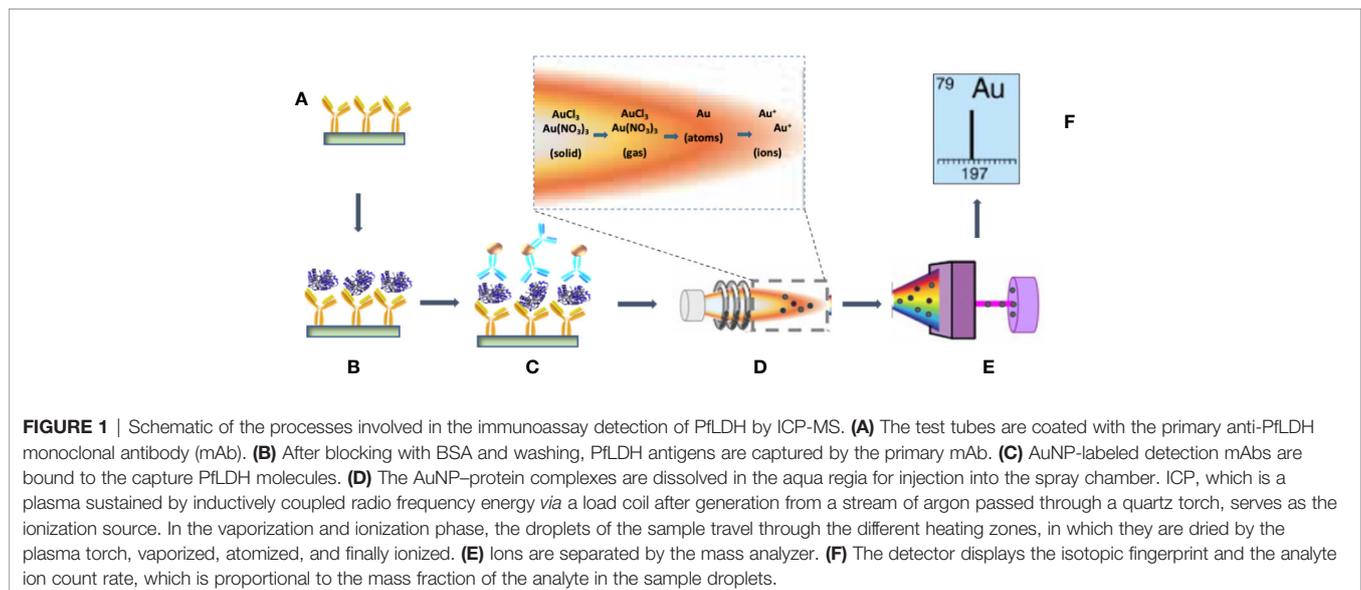


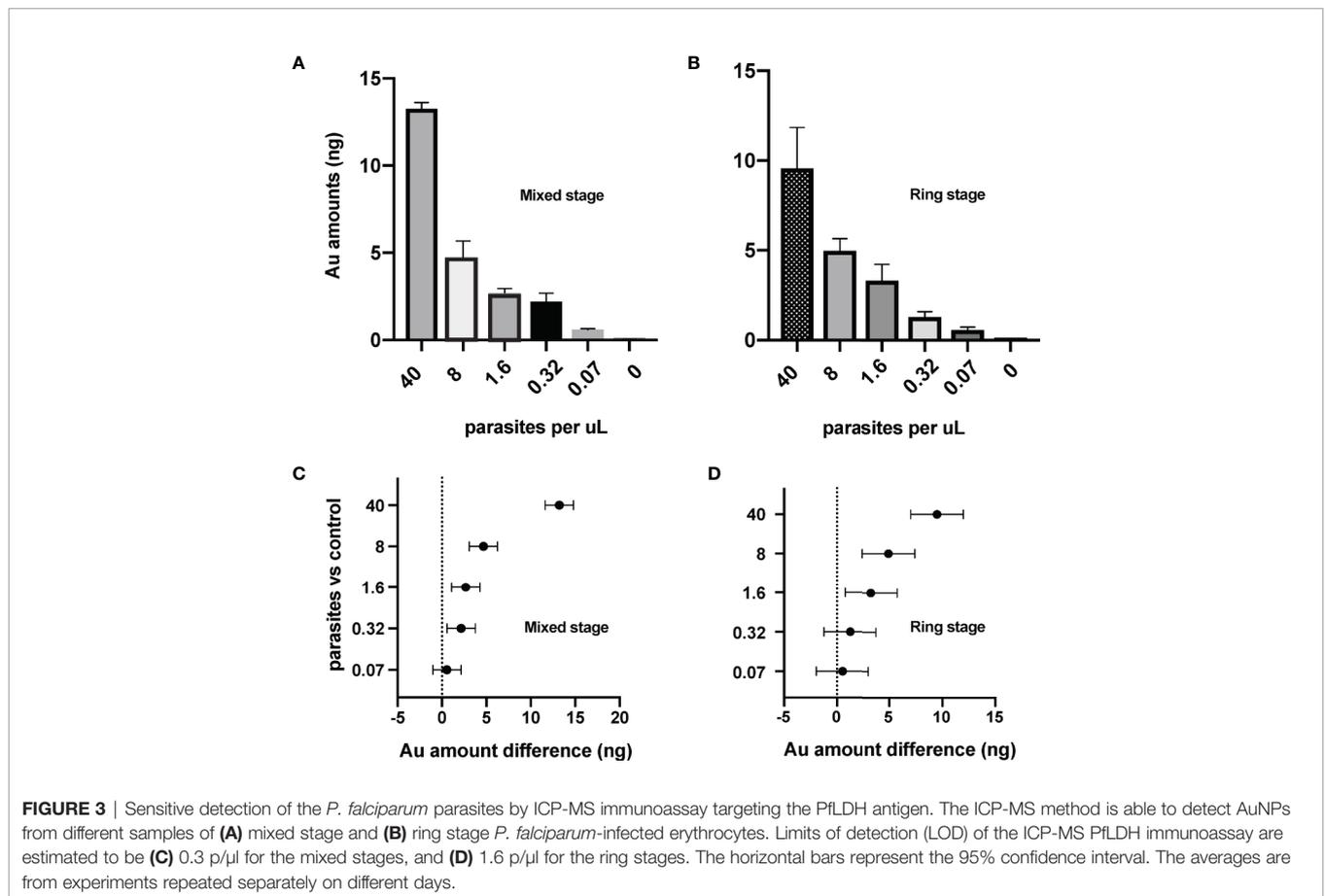
TABLE 1 | AuNP quantifications of parasite-infected relative to control erythrocyte samples by ICP-MS immunoassays.^a

	Sample (p/μl)	AuNP quantification (ng) ^c	PfLDH amount (pg/ml)	PfLDH Mean Difference - Parasites vs control (pg/ml)	95% CI ^b (pg/ml)	Adjusted P Value	Significance
Mixed stage parasitemia	0.07	0.60	0.58	0.56	-0.11 to 1.23	0.671	ns
	0.32	2.21	1.26	1.24	0.56 to 1.91	0.0133	*
	1.6	3.31	1.92	1.70	0.78 to 2.13	0.0046	**
	8	4.72	2.32	2.30	1.62 to 2.97	0.0002	***
	40	13.3	5.92	5.90	5.22 to 6.57	<0.0001	****
Control	0	0.05					
Ring stage parasitemia	0.07	0.57	0.57	0.55	-0.51 to 1.60	0.9562	ns
	0.32	1.30	0.87	0.85	-0.20 to 1.90	0.4836	ns
	1.6	2.73	1.48	1.46	0.65 to 2.75	0.0101	*
	8	4.97	2.42	2.40	1.36 to 3.45	0.0004	***
	40	9.56	4.36	4.34	3.29 to 5.39	<0.0001	****
Control	0	0.05					

^aOne-way ANOVA analysis was used for multiple comparisons test.

^b95% confidence interval of difference. Significance indications: ns, not significant; *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.

^cMean of two independent biological repeats.



particularly when it is applied to molecular targets with metal nanoparticle labels (Wilschefski and Baxter, 2019). Adding to the growing list, the ICP-MS PfLDH immunoassay described here detects *P. falciparum*-infected erythrocytes at the level of ≈1 p/μl,

comparable to the detection limit of immuno-PCR in sandwich assays with the same mAbs (Mu et al., 2017).

A mass spectrometer is an ion counter. By labeling PfLDH with AuNP, the analyte density as defined by the number of

PfLDH molecules is effectively multiplied by a factor which is the number of Au atoms in the average number of AuNPs attached to each PfLDH molecule. Note that the number of Au atoms, and hence the signal enhancement factor by ICP-MS detection, is proportional to the third power of the diameter of attached AuNPs. It may therefore be possible to improve detection sensitivity further by using AuNPs of larger diameter for labeling. With the 60 nm AuNP, ICP-MS is already capable of detecting the amount of Au at 10× below the amount associated with 0.07 p/μl, which is far below the detection limit of 0.32 p/μl to 1.6 p/μl discussed above. Therefore, the limiting factor to the LOD of the parasite is the reproducibility of the sandwich immunoassay procedure especially at low PfLDH concentration end rather than ICP-MS detection of Au. Strategies such as chemically modified nucleotide-based elemental tags (Hu et al., 2019), three-dimensional structure modifications of the AuNPs (Wang et al., 2018), rolling circle amplification of parasite DNA detected with AuNP-sensors (He Y. et al., 2014), and multiplex assays of biomarker proteins with AuNP-tagged antibodies (Terenghi et al., 2009) may also provide opportunities for higher sensitivity detection by ICP-MS. The ultimate goal will be to develop high-sensitivity detection of AuNPs with practical, field-robust devices at point of care. Design and development of the necessary equipment, the possibility of which brings to mind the successful production of a low-cost, portable, field-programmable nuclear magnetic resonance spectrometer (Peng et al., 2014), will support this goal.

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DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

JM and TW initiated the study and drafted the manuscript. JM and LY conducted the lab work and analyzed the data. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the Intramural Research Program of the Division of Intramural Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health.

ACKNOWLEDGMENTS

We express our thanks to Roy Faiman, Kristin D. Lane, T. Parks Remcho, and Juliana M. Sá for discussions and comments on the manuscript.

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