Towards the Use of a Smartphone Imaging-Based Tool for Point-of-Care Detection of Asymptomatic Low-Density Malaria Parasitemia

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Abstract

Background: Globally, there are over 200 million cases of malaria annually and over 400,000 deaths. Early and accurate detection of low density parasitemia and asymptomatic individuals is key to achieving the WHO's 2030 sustainable development goals of reducing malaria-related deaths by 90% and eradication in 35 countries. Current rapid diagnostic tests are neither sensitive nor specific enough to detect the low parasite concentrations in the blood of some asymptomatic individuals.

Methods: Here, we combine an imaging-based sensing technique, particle diffusometry (PD), with loop mediated isothermal amplification (LAMP) on a smartphone-enabled device to detect low levels of parasitemia often associated with asymptomatic malaria. After amplification, PD quantifies the Brownian motion of fluorescent nanoparticles in the solution during a 30 second video taken on the phone. The resulting diffusion coefficient is used to detect the presence of *Plasmodium* DNA amplicons by comparing to coefficients of negative samples using a one-way ANOVA test.

Results: As few as 1 parasite/μL of blood was detectable in 45 minutes without DNA extraction. We detected *Plasmodium falciparum* (*P. falciparum*) parasites from asymptomatic individuals' whole blood samples with 89% sensitivity and 100% specificity when compared to quantitative polymerase chain reaction (qPCR).

Conclusions: PD-LAMP is of value for the detection of low density parasitemia especially in areas where trained personnel may be scarce. The demonstration of this smartphone biosensor paired with the sensitivity of LAMP provides a proof of concept to achieve widespread asymptomatic malaria testing at the point of care.

1. Background

Malaria is a crucial public health concern in resource-constrained countries. In 2018, there were 228 million cases of malaria and 405,000 malaria related deaths worldwide.(1) Despite the World Health Organization's (WHO) strategic goal to eradicate malaria in 10 countries and reduce global incidence by 40% by 2020, malaria cases have increased in the past several years.(2,3) Countries throughout sub-Saharan Africa carry the greatest percentage of malaria cases (92%), followed by countries in Southeast Asia (5%).(2) One contributing factor to the disproportionate number of malaria cases in sub-Saharan Africa is delayed or inaccurate results along with a lack of access to malarial diagnostic tools that are practical for field use.(4) There is a need for portable, prompt, and easy-to-use diagnostic tools to decrease mortality from such a curable and preventable disease.(5)

Malaria is caused by the protozoan parasite *Plasmodium* with *Plasmodium falciparum* (*P. falciparum)* being the deadliest in Africa.(2,4) When *P. falciparum* malaria is left untreated it can become fatal and accounts for 99% of malaria deaths.(2) Malaria symptoms are nonspecific and often mimic symptoms of common viral and bacterial illnesses.(6) However, a large

proportion of malaria cases are asymptomatic in endemic countries.(7–9) Asymptomatic carriers of *P. falciparum* malaria are largely responsible for persistent transmission by maintaining the parasite life cycle.(8,10,11) Malaria management strategies are needed to control and monitor infections in asymptomatic carriers and are a key step towards elimination.(10)

Rapid and sensitive asymptomatic malaria detection would provide real-time disease surveillance for disease outbreak identification and prevent further transmission. However, current on-site malaria detection approaches often rely on diagnostics that are not sensitive enough for asymptomatic malaria cases.(12,13) The standard method for diagnosing malaria is through microscopic examination of blood smears. Microscopy achieves a sensitivity of 50-100 parasites/μL.(12,14–17) Major drawbacks of microscopy include the need for extensive technical training for skilled personnel and the lack of quality control that is introduced in the diagnostic interpretation.(12) An alternative approach, polymerase chain reaction (PCR), is by far the most sensitive malaria diagnostic on the market with a limit of detection (LOD) of 5 parasites/μL, but requires expensive equipment and reagents not found in local clinical facilities.(18,19) Additionally, rapid diagnostic tests (RDTs) have been developed as a simple point-of-care alternative for malaria diagnosis, which require little technical training and no laboratory infrastructure.(20) However, common RDTs are currently not sensitive enough to accurately detect below 100 parasites/μL of blood, a concentration too high to identify malaria in some asymptomatic individuals.(21–24)

Isothermal amplification methods eliminate the thermal cycling that is needed for highly accurate methods such as PCR and have the ability to robustly amplify nucleic acids in complex matrices while maintaining sensitivity and specificity. (25,26) This simplifies the process and decreases the time from sample to answer. (25,26) The use of one such isothermal technique,

78 loop mediated isothermal amplification (LAMP) is an attractive nucleic acid amplification
79 technique for field use due to its simplicity and robustness in complex matrices in comparison to
80 other isothermal methods.(27) LAMP-based assays have also been deemed appropriate for
81 detection of low-level parasitemia with the commercialized Loopamp^{TM} malaria Pan/Pf kit by
82 having an excellent limit of detection of 2 parasites/μL. (27,28) However, DNA still needs to be
83 extracted from the organism to use the Eiken kit. LAMP detection from blood without sample
84 purification has been done previously with 1000 fold dilution of the blood samples and blood
85 spots using chemical lysis through commercialized Loompamp kits (29,30). However these
86 methods are commonly analyzed based on fluorescence detection adding to testing complexity
87 but can be expounded upon to increase its compatibility for field use.
88 LAMP is often monitored by turbidity, fluorescence, and electrochemical methods
89 (14,31,32). To accurately measure these signals, many research groups have begun to rely on the
90 use of smartphones as sensing instruments. Smartphones are the next leading technology in the
91 medical field as they are an attractive alternative to expensive medical equipment, oftentimes
92 contain a camera, GPS capabilities, and vibrational sensors that can be manipulated for
93 implementation of advanced diagnostics.(33) Smartphone-enabled LAMP-based diagnostics for
94 detection of *Plasmodium* has been performed previously and shows promise for assisted
95 microfluidic lab-on-a-chip devices.(20,34) With developments on previous works, a feasible
96 point-of-care diagnostic can be developed to combat the challenges present in parasite detection
97 including laborious DNA extraction, purification and low-level malaria parasite detection in one
98 device.
99 An alternative highly sensitive detection method applying the optical sensing technique
100 is particle diffusometry paired with LAMP (PD-LAMP). PD-LAMP has previously been

demonstrated by Clayton and Moehling *et al*., for the environmental detection of *Vibrio cholerae*.(35) The presence of a pathogen is detected by measuring the Brownian motion of particles in solution after LAMP. The LAMP assay, with biotinylated primers incorporated, produces approximately 10^9 copies of target DNA amplicons. These amplicons consist of stem-loops with varying lengths; increasing the viscosity of the sample drastically (**Figure 1**A).(16,36) These LAMP amplicons are then combined with 400 nm streptavidin-coated fluorescent particles and placed into a microfluidic chip (**Figure 1**A). The fluorescent particles bind the biotinylated DNA primer that is hybridized into the DNA amplicons resulting in an increase in the hydrodynamic radius of the particles, further slowing their Brownian motion. Movement of the fluorescent particles is captured in a series of images via the smart-phone camera. Correlation-based algorithms of the images are used to calculate the diffusion coefficient of the particles (**Figure 1**B). Combined, the particle size change and increased fluid viscosity yields a significant difference between the diffusion coefficients of particles in positive versus negative samples.(35) Diffusion coefficients are low in the presence of the targeted pathogen and in the absence of pathogen nanoparticles will exhibit higher diffusivity. Clayton and Moehling *et al*. used PD-LAMP to sensitively and specifically detect 10 *V. cholerae* cells in a 25 µL reaction in pond water within 35 minutes.(35) However, this technique was performed using a laboratory epifluorescence microscope, a method that is not easily accessible at the point of care. More recently, Moehling and Lee *et al.* expanded upon the PD-LAMP method, achieving the same limit of detection of *V. cholerae* cells in pond water using a newly developed smartphone-enabled detection platform.(37) Their newly developed portable device miniaturizes a fluorescent microscope and takes advantage of the smartphone camera and computational power needed to perform PD-LAMP.(37)

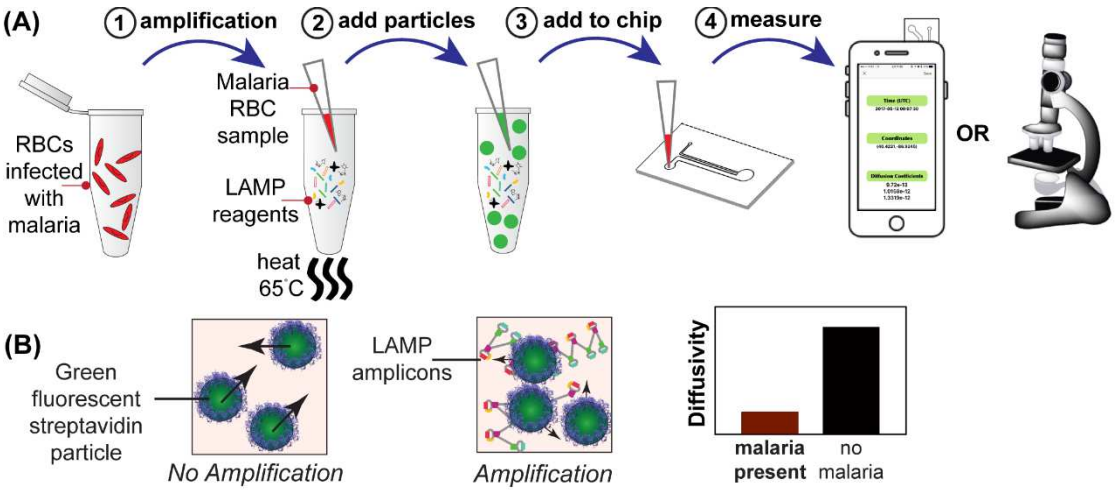

Figure 1. Illustration of PD-LAMP set-up. (A) LAMP was performed with whole blood samples added directly to the assay reagents. (A1) The red blood cells lysed upon heating, releasing the malaria DNA and initiating the LAMP reaction. (A2) After LAMP is completed, the amplicons are combined with fluorescent particles and (A3) the mixture was then added to a microfluidic chip. (A4) Imaging of the fluorescent beads took place using an epifluorescent microscope or the smartphone device (developed by Moehling and Lee *et al.*)(37) (B) The smartphone images the fluorescent particles undergoing Brownian motion for 30 seconds. The particles will exhibit faster Brownian motion in the absence of DNA amplicons. In the presence of malaria DNA, the particle motion will be hindered. The diffusion coefficient value, a numerical measure of Brownian motion, is lower when malaria DNA is amplified than if no malaria DNA is present. Here, we show the detection of low concentrations of *Plasmodium* parasites from unprocessed blood using PD-LAMP. We used a previously published primer set targeting the 18s rRNA gene (38) that is specific to *P. falciparum* and *P. vivax* and further designed a novel 6-primer LAMP assay to amplify pan-*Plasmodium* by targetting the 28s rRNA gene which is conserved across all malaria species. We employ rapid, specific, and robust smartphone-enabled **5. Methods**

Loop Mediated Isothermal Amplification (LAMP)

Purified genomic DNA from *P. falciparum* 3D7 (UK repository) was stored at 66 ng/µL ($3x10^6$ copies/µL) at -20˚C. The stock was diluted 10-fold ($3x10^0$-10^4 copies/reaction) in molecular biology water (Invitrogen, Carlsbad, CA) for experiments. The 28s rRNA LAMP primers were designed using primer explorer to target the *Plasmodium* 28s rRNA, a gene conserved across all *Plasmodium* species a gene conserved across *P. vivax* and *P. falciparum*.

18s rRNA, another conserved gene, primers used in this study were adopted from Lau *et al*.(38) Primer sequences can be found in **Table S1 and S2**.

For all amplification experiments a master mix containing 22.5 µL of master mix (master mix recipe found in **Table S3**) and 2.5 µL of template or NTC were added just prior to heating. The templates were genomic DNA or infected blood; negative template controls (NTCs) were of molecular biology water or uninfected blood, respectively. The reactions were heated at 65°C for 45 minutes for all reactions without blood and all clinical samples with the 18s primer set, 60 minutes for specificity reactions with blood, or 75 minutes for sensitivity reactions with blood using an Applied Biosystems 7500 Real time PCR System (Foster City, CA). Samples were stored at 4°C before PD analysis.

LAMP products were visualized using an ethidium bromide stained 2% agarose gel at 100V for 50 minutes. The gel was imaged using an ultraviolet light gel system (c400, Azure Biosystems, Dublin, CA). Gel images were collected with an exposure time of 15 seconds using the Azure cSeries software at settings of UV302. Gel images were transferred from the Azure as .tiff files and have not been cropped or edited in this manuscript.

PD-LAMP Particle Preparation and Imaging

For PD measurements, 400 nm streptavidin coated Dragon Green polystyrene beads (Bangs Laboratories, Fishers, IN) were added to the LAMP samples at a final concentration of $6x10^9$ particles/mL. After 10 seconds of microcentrifugation 3 µL of the LAMP and bead sample was placed into the microfluidic chip imaging chamber. Each sample was imaged for 30 seconds in the smartphone-enabled platform twice and analyzed using an in-house algorithm.(37) The diffusion coefficients were exported for statistical analysis.

For the microscope images, the samples (N=3) were imaged for 1000 frames using an inverted fluorescence microscope (Carl Zeiss Microscopy, Thornwood, NY).(35) A 40x magnification objective lens was used with the ZEN software and Zeiss camera at 13.5 frames per second. Each sample video from the microscope was analyzed using an in-house MATLAB code to obtain diffusion coefficients.

Chip Preparation

The microfluidic chip for phone measurements was manufactured by pressure and heat with a heat press (Carver Inc. Wabash, IN). The chip consisted of two 188 µm thick cyclic olefin polymer (COP) sheets (Zeon, Tokyo, Japan) that were adhered together at 1.2 metric tons at 120°C for 2 minutes and then rotating the COP sheets 180° and pressing for another 1 minute. Double-sided sticky tape (120 µm thickness iCraft Super Tape) had a 1/8" (3mm) through-hole fabricated with a hole punch. The tape was placed on one side of the pressed 188 µm sheets. The tape acted as the fluid sample well. A 60 µm COP sheet was placed on top of the double-sided sticky tape after adding the sample to prevent evaporation.

For the samples analyzed via microscope, 6 mm punches were used on double-sided sticky tape to act as a sample well. The tape was placed onto a cover glass slide (Thickness No. 1, Thermo Scientific, Erie, NY, USA) 3 µL sample was placed into the wells and sealed with a second cover glass slide to limit evaporation.(35)

Theory of Particle Diffusometry

PD-LAMP is a correlation-based fluid visualization technique that utilizes imaging of Brownian motion of particles post-DNA amplification.(51–53) Diffusion coefficients are calculated by correlating sequential particle images and using autocorrelation and cross-correlation of these images to measure particle pixel displacement. The greater particle displacement between images

creates a broader cross-correlation peak width, s_c. The autocorrelation coefficient s_a is determined by correlating an image captured at time t with itself. Through the use of these correlation coefficients the diffusion coefficient can be calculated by an equation derived by Olsen and Adrian:(54)

$$D = \frac{s_c^2 - s_a^2}{16\, M^2 \Delta t} \quad (1)$$

where M is the magnification of the microscope objective. D is the diffusion coefficient where its theoretical value is calculated using the Stokes-Einstein equation (55,56) .

$$D = \frac{kT}{6\pi\eta a} \quad (2)$$

Here k is the Boltzmann constant, T is the absolute temperature, η is the viscosity and a is the hydrodynamic radius of the imaged fluorescent particles. The **equations 1** and **equation 2** and its use in PD-LAMP have been previously described (35,37,52).

Patient/Study Participant Samples and Malarial DNA

Genomic *P. falciparum* 3D7A DNA was obtained from the European Malaria Reagent Repository. NIAID, NIH: *Plasmodium falciparum*, Strain 3D7, MRA-102, contributed by Daniel J. Carucci, and NIAID, NIH: *Plasmodium vivax*, Strain Chesson, MRA-383, contributed by W. E. Collins, were obtained through BEI Resources. Human blood (Innovative Research Novi, MI) was used to dilute infected blood samples.

Cerebral malaria (CM), severe malarial anemia (SMA), and community control (CC) blood samples were collected as part of a study conducted at Mulago National Referral Hospital in Kampala, Uganda from 2008-2013 as previously described.(39) Children 18 months to 12

years of age were enrolled into the CM group if they had coma (Blantyre Coma Score ≤ 2), *P. falciparum* on blood smear, and no other known cause of coma (e.g. meningitis, a prolonged postictal state, or hypoglycemia-associated coma reversed by a glucose infusion) or into the SMA group if they had *P. falciparum* on blood smear and serum Hgb ≤ 5 mg/dL. CC were healthy children in the same age group and from the same neighborhood, extended household, or nearby neighborhood as a child with CM. Whole blood was also obtained from a Kenyan individual with uncomplicated malaria (UM) and from North American individuals without malaria (IRB protocol 1601403732). DNA was extracted from whole blood samples using the QIAamp DNA Blood Mini Kits (Qiagen, Hilden, Germany) for nPCR and qPCR testing.

Blinded Study

P. falciparum positive or negative patient samples (2.5 µL) were placed into a PCR strip tube labeled 1-7. The master mix was prepared, and the samples added. Samples were heated to 65^0C for 45 minutes for the 18s primer set and 90 minutes for the 28s primer set. The researchers performing amplification and PD experiments were blinded to the parasitemia sample concentration. After PD-LAMP was performed, the diffusion coefficients were then matched with the initial concentrations to obtain unbiased measurements.

Nested Polymerase Chain Reaction

nPCR was used to first amplify the genus specific 18s ribosomal RNA common to all *Plasmodium* species using the rPLU1 and rPLU5 primers as described in the Snounou protocol(57) and the cycling method as described in the Bharti protocol.(58) Then, the product of this first reaction was used as the DNA template for the second, *P. falciparum* species-specific amplification using the rFAL1 and rFAL2 primers as described in the Snounou protocol with the same cycling conditions as the first amplification.

Quantitative Polymerase Chain Reaction

qPCR targeting the multi-copy nuclear *var*ATS gene was performed on the study participant samples that also underwent PD-LAMP. 20 µL reactions were run on the Applied Biosystems™ QuantStudio™ 6 Flex Real-Time PCR System (Foster, CA) using primers and protocol previously described(59) but modified for PowerUp™ SYBR™ Green Master Mix (Applied Biosystems, Foster, CA) (**Table S4**). Parasite density was quantified by comparison to a standard curve of 3D7 parasite cultures. After 2 rounds of synchronization (5% sorbitol), parasite concentration was determined using the parasitemia calculation described below and the RBC concentration via hemocytometer. A 10-fold serial dilution of parasite culture in RPMI diluted in malaria-negative O+ blood was produced and the DNA isolated using the QIAamp DNA Blood Mini Kits (Qiagen, Hilden, Germany) to create the standards (1×10^{5}-1×10^{-1} parasites/µL). All standards, controls, and samples were quantified in duplicate and averaged.

Parasitemia and Parasite Density Calculations

Parasitemia of the NIAID, NIH: *P. falciparum*, Strain 3D7, MRA-102 and NIH: *Plasmodium vivax*, Strain Chesson, MRA-383 containing live parasites, were verified by microscopy. Microscopy was performed on a sample of 1.5 µL, of the *P.f.* or *P.v.* sample, using a thin blood smear to determine % parasitemia and then converted to parasite concentration. The smear was fixed onto a glass slide using methanol and stained with Wright-Giemsa stain for 15 minutes. Parasites were visualized and counted using a 100x oil immersion objective under white light. At least 500 RBC's were counted to determine % parasitemia. The **equation 3** shows the calculation for estimating parasite concentration from the blood smear using an estimated average red cell count of 5,000,000 RBC's per μL.

$$parasites/\mu L = \frac{\#\ of\ parasites}{\#\ of\ RBC's} \times 5{,}000{,}000 \quad (3)$$

Parasite densities for clinical samples were calculated based on the number of asexual parasites per µL of blood on a thick smear stained with 10% Giemsa. Parasites were counted until the field containing the 200th white blood cell (WBC) was reached. Then, density was calculated based on the study participant's WBC count as described by **equation 4** below.

$$\frac{\#\ of\ parasites\ \times\ WBC\ count}{\#\ of\ WBCs\ counted} = \#\ of\ parasites\ per\ \mu l\ of\ blood \quad (4)$$

Statistical Analysis

Statistical tests were used for data analysis of all specificity and sensitivity measurements. The LOD was determined from PD data by using a one-way ANOVA post-hoc Dunnett's compared to the negative controls (NTC) with a 95% confidence interval. Box-and-whisker plots were made for PD measurements at the 10-fold dilutions where the minimum and maximum values were represented by the upper and lower whiskers. Quartiles 25% and 75% were represented by the upper and lower bounds respectively.

PD-LAMP for the detection of asymptomatic malaria in a portable detection platform.

Results

PD-LAMP Comparison in Phone and Microscope

LAMP reactions targeting the 28s rRNA gene were performed across 10-fold serial dilutions from 3x10^4 to 3x10^0 DNA copies/µL of *P. falciparum* DNA. All dilutions amplified in less than 45 minutes as visualized by the sigmoidal increase in real-time fluorescence measurements (**Figure 2A**). Real-time fluorescence visualization shows that the highest initial concentrations of 28s RNA amplified more rapidly. Negative template control (NTC) samples remained at baseline throughout the 45-minute amplification for all instances in the qPCR graphs

(N=4) (Figure S1). Amplification was confirmed with a 2% agarose gel showing banding only in positive samples (**Figure 2B**).

We used the 28s rRNA LAMP products to validate the PD measurements made on an inverted epifluorescent microscope against PD measurements on the smartphone device with an in-house MATLAB code. After performing a one-way ANOVA with Dunnett's post-hoc against the NTC, we found that there were statistically significant differences between sample dilutions $3x10^4$ -$3x10^1$ (****$p<0.0001$) and $3x10^0$ (*$p<0.05$) DNA copies/µL relative to the NTC for both PD measurements on the microscope (**Figure 2C**) and the smartphone device (**Figure 2D**). PD yielded lower diffusion coefficients in positive samples as expected due to the inhibition of particle Brownian motion in the presence of malaria DNA amplicons. To ensure reproducibility each sample was measured in duplicate on each platform after 4 different amplification experiments (N=4) . We determined that there were no significant differences in the measurement efficacy between the microscope and smartphone platform.

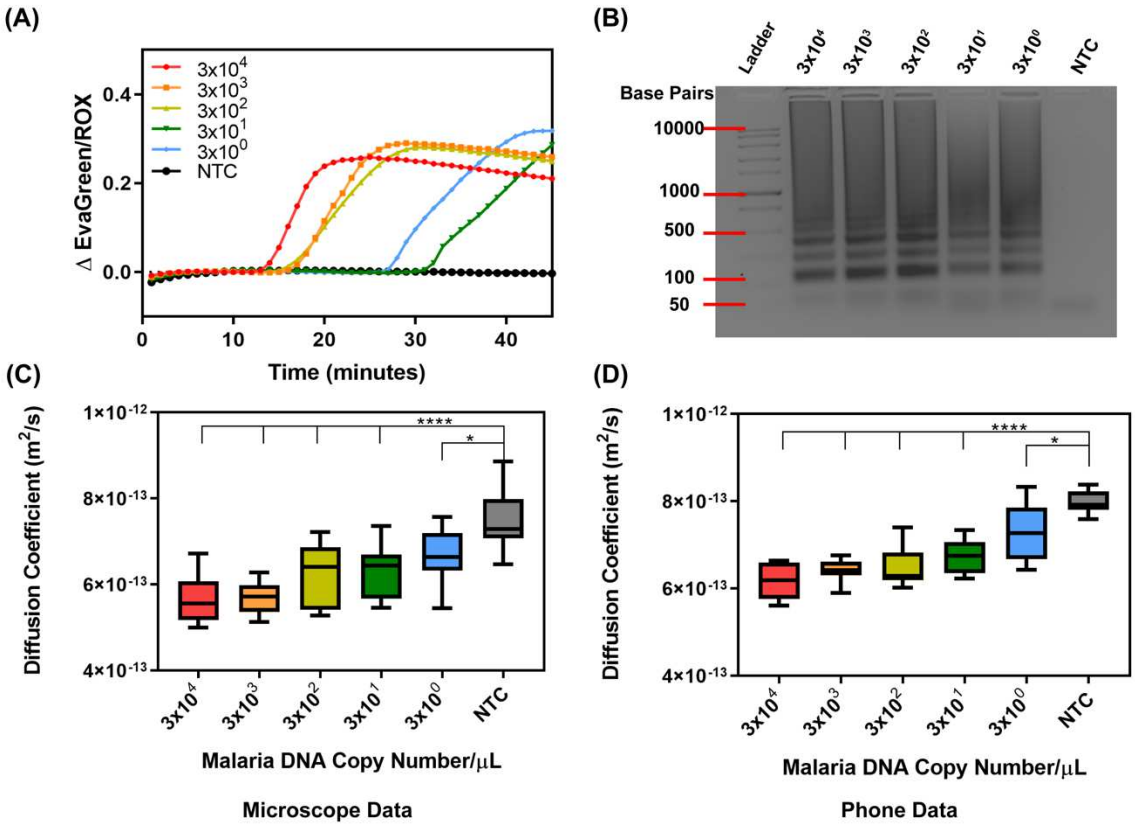

Figure 2. *P. falciparum* genomic DNA detection targeting 28s rRNA. (A) Real-time fluorescence data during a 45-minute LAMP reaction with concentrations ranging from $3x10^4$ to $3x10^0$ DNA copies/µL. (B) The representative LAMP DNA banding pattern for all positive samples are confirmed in 2% agarose gel electrophoresis (note the NTC shows no banding pattern). (C) PD analysis from the microscope images of the samples indicates the change in diffusion coefficient for LAMP samples with a statistically significant difference from the negative template control (NTC) for samples with $3x10^4$ -$3x10^1$ (****$p<0.0001$) and $3x10^0$ (*$p<0.05$) DNA copies/µL. (D) PD analysis on smartphone device indicates statistically significant differences between $3x10^4$ - $3x10^1$ (****$p<0.0001$) and $3x10^0$ (*$p<0.05$) DNA copies/µL and the NTC. NTC here represents water added in place of genomic DNA. (N=4)

28s rRNA PD-LAMP Specificity in Blood

As *Plasmodium* parasites reside in red blood cells, we needed to assess the feasibility of PD-LAMP in whole blood samples. We performed LAMP with *P. falciparum* genomic DNA at a concentration of 10^4 copies/µL in reactions containing several whole blood concentrations (v/v). The amplification time was extended from 45 to 60 minutes due to the inhibition caused by the addition of blood. Samples containing *P. falciparum* genomic DNA amplified when up to 10%

of the reaction volume consisted of blood (**Figure S2**). No amplification occurred in 15% blood or greater. Further, no non-specific amplification occurred with the NTC in the reactions consisting of 10% blood (**Figure S3**). Therefore, 10% blood was the greatest concentration that could be used without inhibiting LAMP or causing non-specific amplification of control samples.

To ensure assay selectivity for malaria, LAMP targeting 28s rRNA was performed with with *P. falciparum* and *P. vivax* blood samples (BEI), alongside *dengue virus* (III) and *chikungunya virus* RNA, which are also mosquito-borne pathogens, but do not contain the 28s rRNA gene. We performed a 60-minute LAMP assay for each sample in a reaction containing 10% blood. Specific amplification occured for the *P. falciparum* and *P. vivax* DNA samples while *dengue virus* (III) and *chikungunya virus* did not amplify, as indicated on an agarose gel (**Figure 3A**). Following amplification, we performed PD on the LAMP samples. Dunnett's post-hoc test was used against each individual sample. We saw no significant change in the PD signal against NTC ($p > 0.5$) in the presence of *dengue virus* (III) or *chikungunya virus* RNA (N=4) (**Figure 3B**). Alternatively, both malaria positive samples (*P. falciparum* and *P. vivax*) were found to be significantly different from dengue and chikungunya virus samples and the NTC (unifected blood) (****$p<0.0001$), but not from each other.

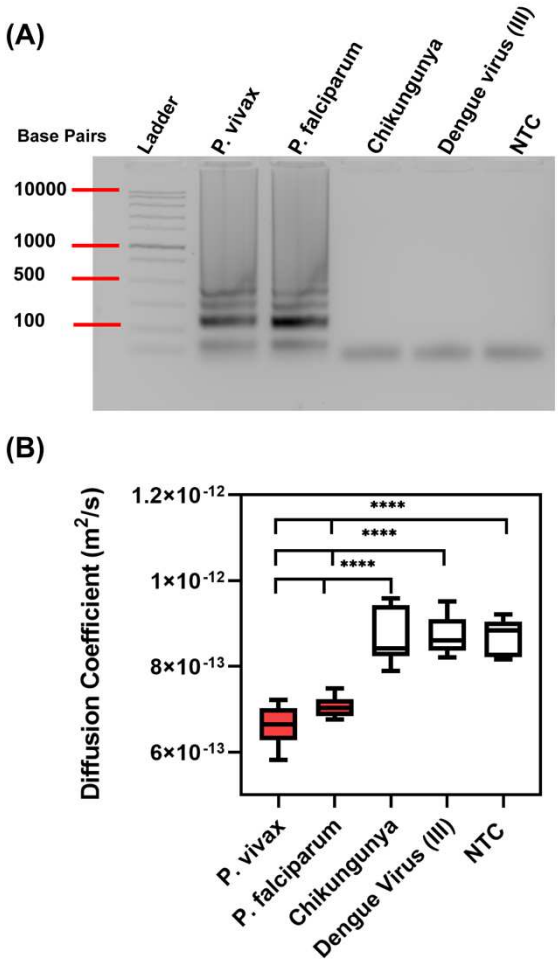

Figure 3. PD-LAMP specificity in 10% blood. (A) A 2% agarose gel from LAMP reactions in blood containing malarial strains *P. falciparum* and *P. vivax* alongside controls *chikungunya and dengue virus* (III) at $6x10^4$ parasites/µL blood after a 60-minute LAMP reaction. Only malaria samples amplified, demonstrated by the DNA banding pattern in the gel. (B) Diffusion coefficients from smartphone PD analysis, where malaria samples showed a significant difference from *chikungunya virus*, *dengue virus* (III) and NTC (One-way ANOVA Dunnett's post-hoc test). NTC represents blood without spiked pathogens. (N=3)

PD-LAMP Sensitivity in 10% Blood (v/v) with Infected RBCs

We determined the LOD of PD-LAMP in 10% blood using commercially available malaria infected blood samples. The stock infected blood was diluted with uninfected blood to obtain 10-fold dilutions between $3x10^5$ to $3x10^0$ parasites/µL of blood for a 75-minute LAMP reaction using 28s rRNA primers. From the LAMP assay, we determined that the LOD was 3

parasites/µL. Amplification was confirmed with a 2% agarose gel (**Figure 4A**). Further, when measured by PD-LAMP, amplification from as few as 3 parasites/µL blood resulted in a significantly reduced diffusion coefficient compared to NTC in blood (N=4) (**Figure 4B**). Dunnett's post-hoc test for all samples confirmed significance from NTC with ****p<0.0001 for $3x10^5$ to $3x10^2$ and **p<0.001 for $3x10^1$ to $3x10^0$.

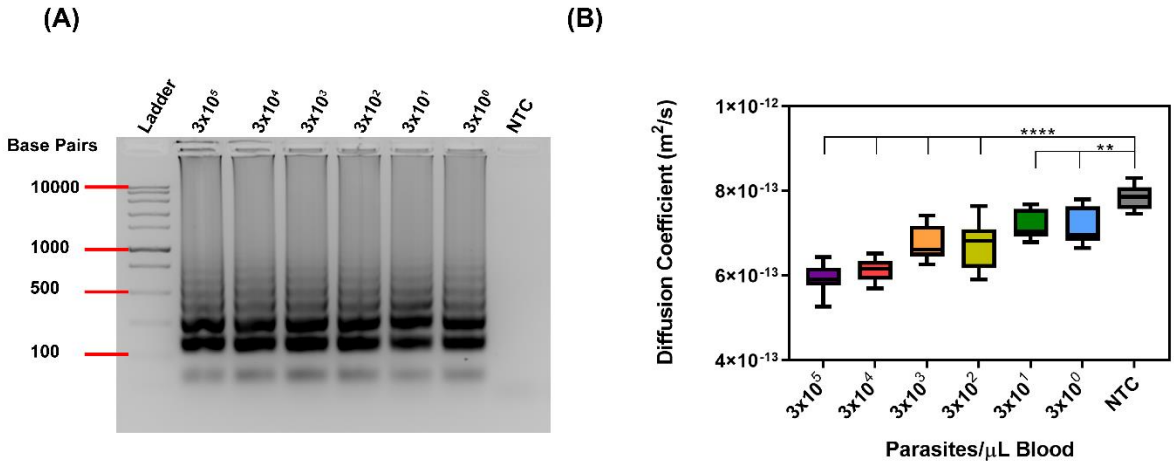

Figure 4. Sensitivity of malaria PD-LAMP with 28s primers using 10% blood. (A) 2% agarose gel confirming amplification in positive samples. (B) Diffusion coefficients measured using smartphone device for dilutions of $3x10^5$-$3x10^0$ of *P. falciparum* infected blood after a 75-minute reaction. PD analysis shows statistical difference from controls for $3x10^5$-$3x10^2$ parasites/µL blood (****p<0.0001) and $3x10^1$-$3x10^0$ parasites/µL blood (**p<0.001) .(N=4).

2.4 Blinded Study of PD-LAMP in Infected Blood Samples with PCR Confirmation

We performed a blinded study with patient blood samples to validate the robustness of the smartphone device. Six previously collected and deidentified malaria samples (labeled patient ID 1-6) with parasite densities ranging from 4 parasites/µL to 265,782 parasites/µL as quantified by qPCR were used for this study.(39) Each sample was analyzed on the smartphone via PD-LAMP in a blinded study where the user of the device did not know the nature of the samples being analyzed. The samples were amplified using two different primer sets, 28s rRNA and 18s rRNA. The 18s rRNA primer set was introduced to this work to reduce the amplification

363 time of the patient samples because there are more copies of the 18s rRNA gene than 28s rRNA
364 in the *P. falciparum* genome.(40) **Table 1** shows quantitative PCR (qPCR) values, microscopy,
365 and diffusion coefficients (PD-LAMP) for each sample using the 18s and 28s rRNA primers.
366 The 18s rRNA PD-LAMP resulted in diffusion coefficients of less than $7.2e^{-13}$ m^2/s for all
367 patients with parasitemia while the NTC resulted in a diffusion coefficient of $9.0e^{-13}$ m^2/s, higher
368 than the positive samples.
369 From the LAMP assay, all 6 patient samples that incorporated primers targeting the 18s
370 rRNA gene amplified within 45 minutes. Samples with the 28s rRNA primer set underwent a 90-
371 minute reaction and two of the patient samples did not amplify. Agarose gel electrophoresis
372 confirmed LAMP amplification of all positive samples at the end of the blinded study using the
373 18s primer set (N=3) (**Figure S5A**). Measuring the LAMP products with PD, we found all of the
374 diffusion coefficient values for patient samples to be significantly different from NTC for 18s
375 rRNA gene target (****$p<0.0001$) (**Figure S5B**). For patient samples targeting the 28s rRNA
376 gene, we observed that Patient ID 3 and 4 both showed inconsistent amplification in the agarose
377 gels while all other patient samples indicate consistent amplification between repeats (N=3)
378 (**Figure S5C**). Targeting the 28s rRNA gene, patient ID 3 and 4 were not found to be
379 significantly different from NTC after a 90-minute amplification. All other patient samples with
380 LAMP targeting the 28s rRNA gene were found to be significant from PD measurements
381 (****$p<0.0001$ for 1 and 5, ***$p<0.001$ for 2, **$p<0.01$for 6) (**Figure S5D**). The nature of this
382 blinded study showed reproducibility in using PD for the detection of LAMP amplicons in the
383 smartphone device as well as the importance of choosing a robust LAMP assay target.

Table 1. Parasite densities and average diffusion coefficients from 18s and 28s rRNA primers of patient samples. Quantitative PCR and PD-LAMP was performed on 6 infected blood samples and a negative control (uninfected blood). A significant difference ($p<0.05$) was shown in the diffusion coefficients of all positive samples compared to the control for 18s rRNA primers. A significant difference ($p<0.05$) was shown in the diffusion coefficients from controls for all samples except IDs 3 and 4 for 28s rRNA primers.

Patient ID	Group	Parasite Density by Microscopy (parasites/μL)	Parasite Density by qPCR (parasites/μL)	28s Diffusion Coeff. ($\pm standard\ dev.$) (m^2/s)	18s Diffusion Coeff. ($\pm standard\ dev.$) (m^2/s)
1	UM[a]	N.D.[e]	265,782	$6.4(\pm0.51)e^{-13}$	$6.1(\pm0.61)e^{-13}$
2	CM[b]	64	126	$6.7(\pm1.31)e^{-13}$	$7.0(\pm0.58)e^{-13}$
3	SMA[c]	524[d]	4	$7.9(\pm0.91)e^{-13}$	$6.9(\pm0.78)e^{-13}$
4	CM	545	2,552	$7.7(\pm0.67)e^{-13}$	$6.9(\pm0.28)e^{-13}$
5	CM	26,643	1,466	$6.5(\pm0.51)e^{-13}$	$7.1(\pm0.45)e^{-13}$
6	CM	511,972	N.D.	$7.05(\pm0.25)e^{-13}$	$6.9(\pm0.35)e^{-13}$
NTC	--	0	–	$8.8(\pm0.73)e^{-13}$	$9.0(\pm1.01)e^{-13}$

[a]Uncomplicated Malaria; [b] Cerebral Malaria; [c]Severe Malaria Anemia;
[d]parasite density not corrected for white blood cell count
[e]not determined

LOD from Diluted Patient Samples

We determined the LOD of PD-LAMP on the smartphone device using patient samples. We performed 10-fold serial dilutions of patient sample ID 2, the 2nd lowest concentration by qPCR, in blood starting with a dilution from stock of 12.6 parasites/μL to 0.0126 parasites/μL and performed PD after a 45-minute LAMP reaction using the 18s rRNA primer set. As shown in **Figure S5**, the 18s rRNA primer set had greater reproducibility and amplified in less time than the 28s rRNA primer set. Therefore, 18s rRNA primer sets were chosen for this sensitivity study. The 2% agarose gel indicated inconsistent amplification between repeats below 12.6 parasites/μL (**Figure 5A**). However, the LOD of PD-LAMP on the smartphone device was found to be 6 parasites/μL (**Figure 5B**). The diffusion coefficient values were significantly different from NTC for 12.6 (****$p<0.0001$), 1.26 and 0.126 (*$p<0.05$) parasites/μL blood using one-way ANOVA

post-hoc Dunnett's test. The lowest concentration, 0.0126 parasites/µL blood, was not significantly different from NTC (uninfected blood) but followed the same trend of having lower diffusivity than the NTC. Thus, PD-LAMP is sensitive enough to detect amplicons even when they cannot be ascertained via agarose gel electrophoresis and a LOD of 0.126 parasites/µL was obtained with the smartphone device from patient samples.

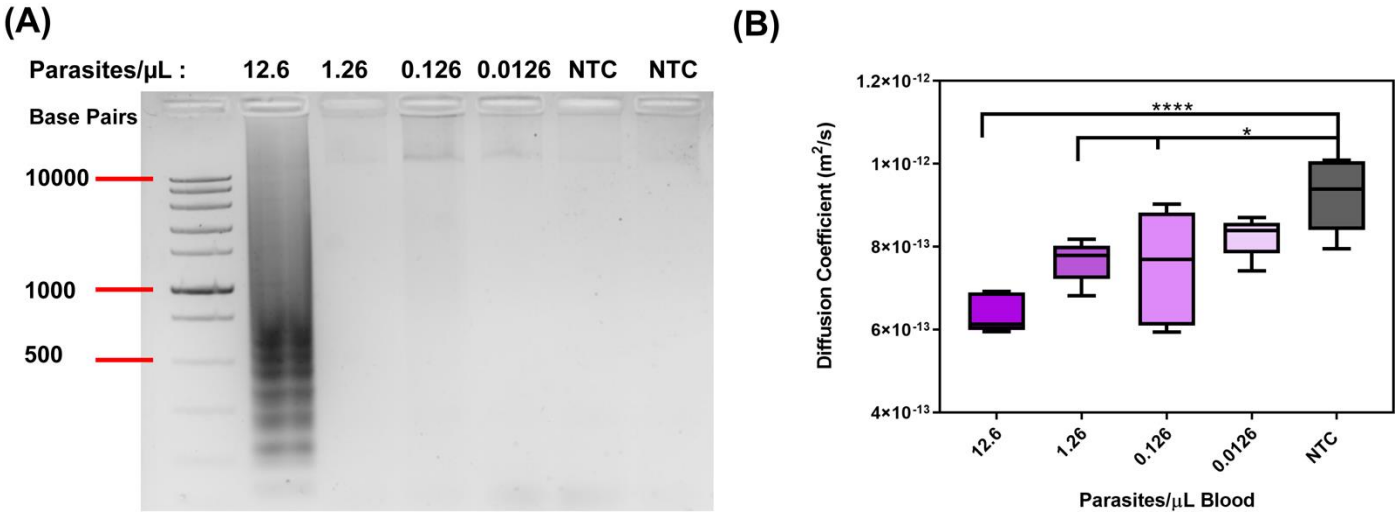

Figure 5. Serial dilutions of patient ID 2 for the determination of the LOD in patient samples using 18s primers. (A) A representative gel from dilutions of ID 2 with concentrations ranging from 12.6 to 0.0126 parasites/µL. Only the 12.6 parasites/µL sample showed consistent amplification in gel represented by the dark smeared banding. (B) Diffusion coefficients from smartphone PD analysis of diluted samples yielded a LOD of 0.126 parasites/µL blood. All dilutions, 12.6 parasites/µL blood (****$p<0.0001$), 1.26 and 0.126 parasites/µL blood (*$p<0.05$), except 0.0126 parasites/µL blood were found to be statistically different from NTC. (N=3)

PD-LAMP from Asymptomatic Samples

We then determined if PD-LAMP could accurately detect *P. falciparum* in asymptomatic study participants. Whole blood samples were collected from 11 healthy asymptomatic individuals (community controls, CC) and 2 symptomatic individuals (Cerebral Malaria, CM and Severe Malaria Anemia, SMA) from Uganda and analyzed via microscopy on site and were stored for nested PCR (nPCR), qPCR, and PD-LAMP testing. Samples were tested in duplicate for qPCR assays targeting *var*ATS gene, and for PD-LAMP assays using the 18s rRNA gene

(N=4) (**Table 2**). For qPCR, the sample was deemed negative if all replicates were negative. Each sample was also run on an agarose gel after a 45-minute LAMP reaction. We found 6 of the 11 asymptomatic samples to be positive for *P. falciparum* via PD-LAMP using Dunnett's multiple comparison test to healthy parasite-free controls, the same was true for the 2 out of 2 symptomatic individuals. PD-LAMP positive results correlated with 8/9 of the positive results by qPCR. Therefore, PD-LAMP was 89% sensitive and 100% specific when compared to qPCR (**Table 2**). The nPCR, targeting 18s rRNA gene, detected 2 more positive cases than PD-LAMP or qPCR (**Table 2**). This discordinance among molecular methods has been documented in samples with very low levels of parasitemia.(41–43)

Table 2. Presence of *P. falciparum* parasitemia as asssessed by nested PCR (nPCR), quantitative PCR (qPCR) and particle diffusion-LAMP (PD-LAMP) in community children with asymptomatic parasitemia (CC), and positive control children with cerebral malaria (CM) or severe malarial anemia (SMA)

Study ID	Group	Parasite Presence (nPCR)	Parasite Density by qPCR (parasites/µL)	PD-LAMP
7	CC[a]	–	0	–
8	CC	–	1	+
9	CC	–	0	–
10	CC	+	0	–
11	CC	+	0	–
12	CC	+	1	–
13	CC	+	56	+
14	CC	+	35	+
15	CC	+	12,749	+
16	CC	+	8.741	+
17	CC	+	100,669	+
18	CM[b]	N.D.[e]	377,406	+
19	SMA[c]	N.D.	136,926	+

[a]Community Controls; [b]Cerebral Malaria; [c]Severe Malaria Anemia; [e]not determined
− = negative for *P. falciparum* DNA, + = positive for *P. falciparum* DNA

Discussion

Many low and middle income countries (LMICs) struggle to maintain laboratory-intensive testing programs that are necessary for accurate malaria diagnoses.(4) However, widescale testing for asymptomatic malaria infections will be a necessary component of WHO malaria reduction and elimination efforts. (44) In this work, we demonstrated the use of PD-LAMP on a portable smartphone-enabled platform for the sensitive, rapid, and robust detection of malaria parasites from unfiltered blood. Through the use of genomic *P. falciparum* DNA, we were able to validate malaria detection on the smartphone first by comparing its results to PD measurements from a fluorescent microscope. Following this, we determined that the PD-LAMP smartphone platform has a LOD of 3 parasites/µL (**Figure 2**) which is comparable to qPCR and Loopamp™ detection limits.[34] Detection at this low concentration is promising for identifying asymptomatic cases that cannot be identified via microscopy. We have demonstrated that PD-LAMP detects as few as 3 parasites/µL in 10% whole blood and is 66-fold more sensitive than currently used RDTs and comparable to the LAMP kit without sample purification.(23)

Further, the PD-LAMP smartphone detection technique is 6-fold more sensitive than even emerging ultrasensitive RDTs, which detect down to 20 parasites/µL.(21,46) By directly adding blood to the LAMP reaction, we have eliminated the need for DNA extraction and pre-processing steps (**Figure 4**). In the future, we foresee that the PD-LAMP smartphone device could be used for point-of-care malaria testing.

We confirmed the selectivity of the pan-*Plasmodium* 28s rRNA LAMP reaction to malaria by testing against *chikungunya virus* and *dengue virus* (III) RNA, spiked into blood.

These viruses are also mosquito-borne and may have similar symptoms as malaria.(47) There are also some regions where there are coinfections of dengue or chikungunya with malaria.(47) Specifically identifying the *Plasmodium* parasite can aid in proper treatment amidst confounding symptoms. In this work, we determined that amplification and a resulting change in diffusivity only occurred in the positive malaria samples (**Figure 3**). Additionally, we confirmed the specificity of the LAMP assay at various concentrations of blood, discovering that none of the negative controls amplified (**Figure S3**). This proves that this malaria LAMP assay is specific to the *Plasmodium* genus.

PD-LAMP can also detect malaria at low concentrations from patient samples in 45 minutes (**Table 1** and **Table 2**). However, from the first 6 patient samples tested, the 28s rRNA primer set failed to amplify patient ID 3 and ID 4 consistently. The amplification inconsistencies with ID 3 and ID 4 may be due to the low copy number or low copies of the 28s target gene. Conversely, the primer set targeting 18s rRNA successfully amplified all 6 of the same patient samples within 45 minutes. Therefore, we identified that the samples were likely not degraded over time, but rather the difference in gene target copies had a greater effect in ID 3 and ID 4. The use of multiple primer sets, such as 28s and 18s rRNA targeted primers, could prove useful for multiplexing and targeting multiple regions to combat emerging mutations in malaria DNA.(48) Although the sensitivity of the PD-LAMP device is not superior to the commercial Eiken kit, our method does not require pre-processing steps of the blood sample and works in complex sample matrices.

We compared the PD-LAMP method to a variety of well-established malaria detection methods. For example, we demonstrated the ability to detect *P. falciparum* parasites from asymptomatic participants with 89% sensitivity and 100% specificity when compared to qPCR

performed on DNA from the same whole blood samples (**Table 2**). qPCR using DNA extracted from whole blood is comparable to the PD-LAMP method. However, nPCR detected 2 more positive cases than PD-LAMP or qPCR, which could also be a result of PCR irreproducibility at ultra-low parasite densities. qPCR sensitivity is positively correlated with template copy number.(43) Low template numbers are subject to the "Monte Carlo" effect, where the success of a primer annealing and replicating during PCR is random.(49) In samples with high parasite densities, this effect is minimal since the template number is high, so the probability of primer binding, and ultimately replicating, is very high. But, in samples with low parasite densities, template copy number is lower and the probability of binding and replication is much lower, resulting in reduced PCR yield and irreproducible results. The differences seen between nPCR , PD-LAMP and qPCR can be attributed to the difference in the targeted regions for amplification. There were variances in repeats for qPCR and PD-LAMP due to the low concentration of DNA, yet we still detected a sample at a concentration of 1 parasite/µL that was undetectable by microscopy.

The use of PD-LAMP on a smartphone is a promising technique for rapid detection of malaria at the point-of-care, because PD-LAMP eliminates the need for DNA extraction steps or the need to rely on antibody-antigen measurements. Ultimately, a smartphone-enabled hardware device could integrate a portable heating element for a standalone, sample-to-answer, portable diagnostic.(26) Total reaction volumes can also be increased to allow for higher input of blood sample while keeping the overall concentration of blood at 10%. Additionlly, malaria PD-LAMP applications could also be extended for use with alternative sample matrices, such as dried blood spots and urine, which would provide users to have alternative sample storage and/or perform

non-invasive screening. Future development of malaria PD-LAMP will involve field-testing in low-resource areas and multiplexing for detection of coinfections.

Conclusion

In this work, we demonstrate that we can detect low concentrations of malaria DNA from unprocessed blood samples through the use of a smartphone-enabled device that is robust, portable, and has potential to be used in low-resource settings. We are able to detect down to 3 copies/µL from *P. falciparum* DNA. We demonstrate that *Plasmodium* parasites can be detected from whole blood specifically and robustly with PD-LAMP at concentrations down to 1 parasite/µL with no need for DNA extraction or pre-processing. Further, we detected *P. falciparum* parasites from asymptomatic participants with 89% sensitivity and 100% specificity when compared to qPCR measurements from the same samples.

Current diagnostics are unable to rapidly and accurately detect parasatimia below 100 parasites/µL, which is one reason why there has been poor progress toward the reduction of malaria transmission.(50) The sensitivity of the PD-LAMP device is competitive against field-based testing techniques such as RDTs, Loopamp^{TM} malaria kit, and white light microscopy. Future work includes incorporating dried reagents on-chip for long term storage and integrating heating into the device to perform the assays all on one handheld platform. Ultimately, a fully integrated PD-LAMP smartphone device could improve public health in malaria endemic areas through rapid low parasitemia detection and aid in process towards eradication of the infectious disease.

Declarations

Competing interests

Steven T. Wereley, Tamara L. Kinzer-Ursem, Katherine N. Clayton, and Jacqueline C. Linnes are co-founders of OmniVis Inc., a spinout company of Purdue University to translate the smartphone PD-LAMP technology. Dr. Clayton is presently the CEO of OmniVis Inc. All others have declared that they have no competing interests.

Ethics Approval and Consent to Participate

Written informed consent was obtained from parents or guardians of study participants. Ethical approval was granted by the institutional review boards for human studies at the Makerere University School of Medicine, the Uganda National Council for Science and Technology, and the University of Minnesota Medical School.

Consent for publication

Not Applicable

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

AC and KC developed the study. AC performed all LAMP studies. KC selected samples for the study. GC and KC carried out all PCR work. AC and DHL performed PD on samples and analyzed the results. AC, KC and KNC drafted the manuscript. JC,SW,CJ and TKU critically revised the manuscript. All authors read and approved the final manuscript.

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Figures

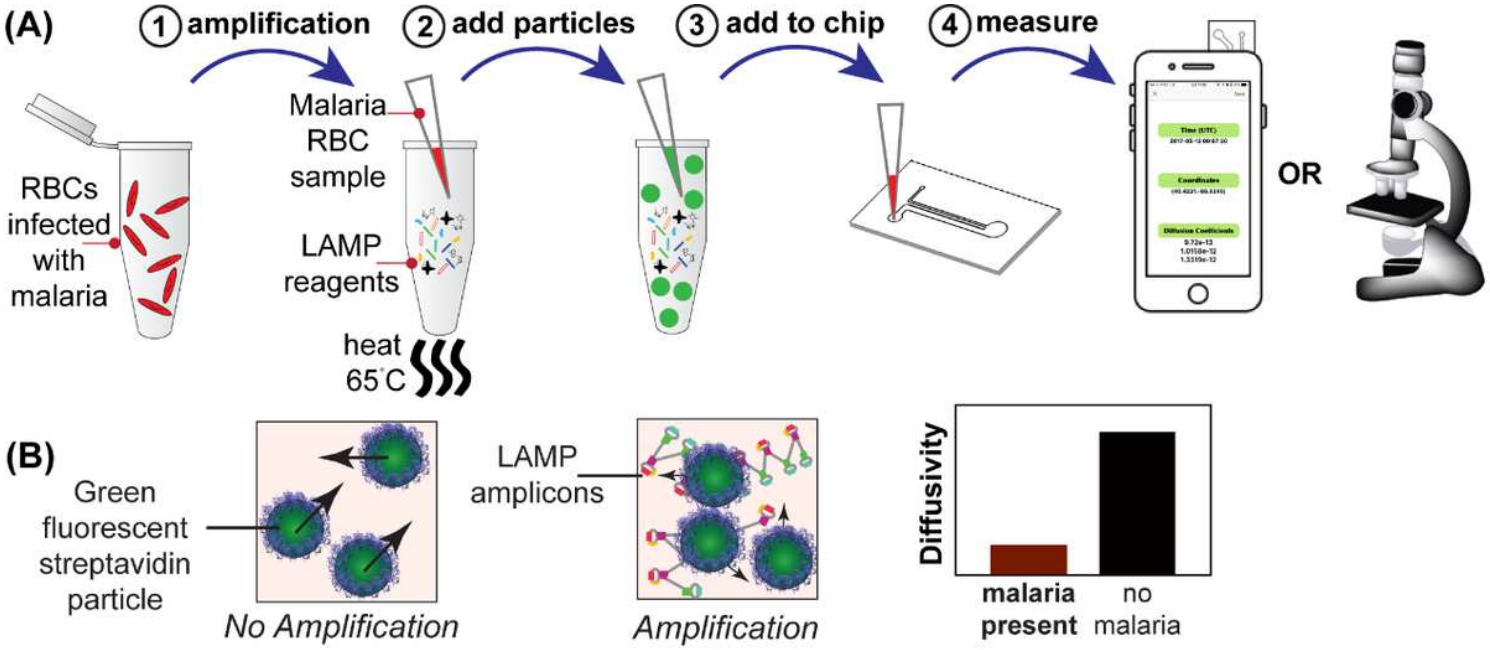

Figure 1

Illustration of PD-LAMP set-up. (A) LAMP was performed with whole blood samples added directly to the assay reagents. (A1) The red blood cells lysed upon heating, releasing the malaria DNA and initiating the LAMP reaction. (A2) After LAMP is completed, the amplicons are combined with fluorescent particles and (A3) the mixture was then added to a microfluidic chip. (A4) Imaging of the fluorescent beads took place using an epifluorescent microscope or the smartphone device (developed by Moehling and Lee et al.)(37) (B) The smartphone images the fluorescent particles undergoing Brownian motion for 30 seconds. The particles will exhibit faster Brownian motion in the absence of DNA amplicons. In the presence of malaria DNA, the particle motion will be hindered. The diffusion coefficient value, a numerical measure of Brownian motion, is lower when malaria DNA is amplified than if no malaria DNA is present.

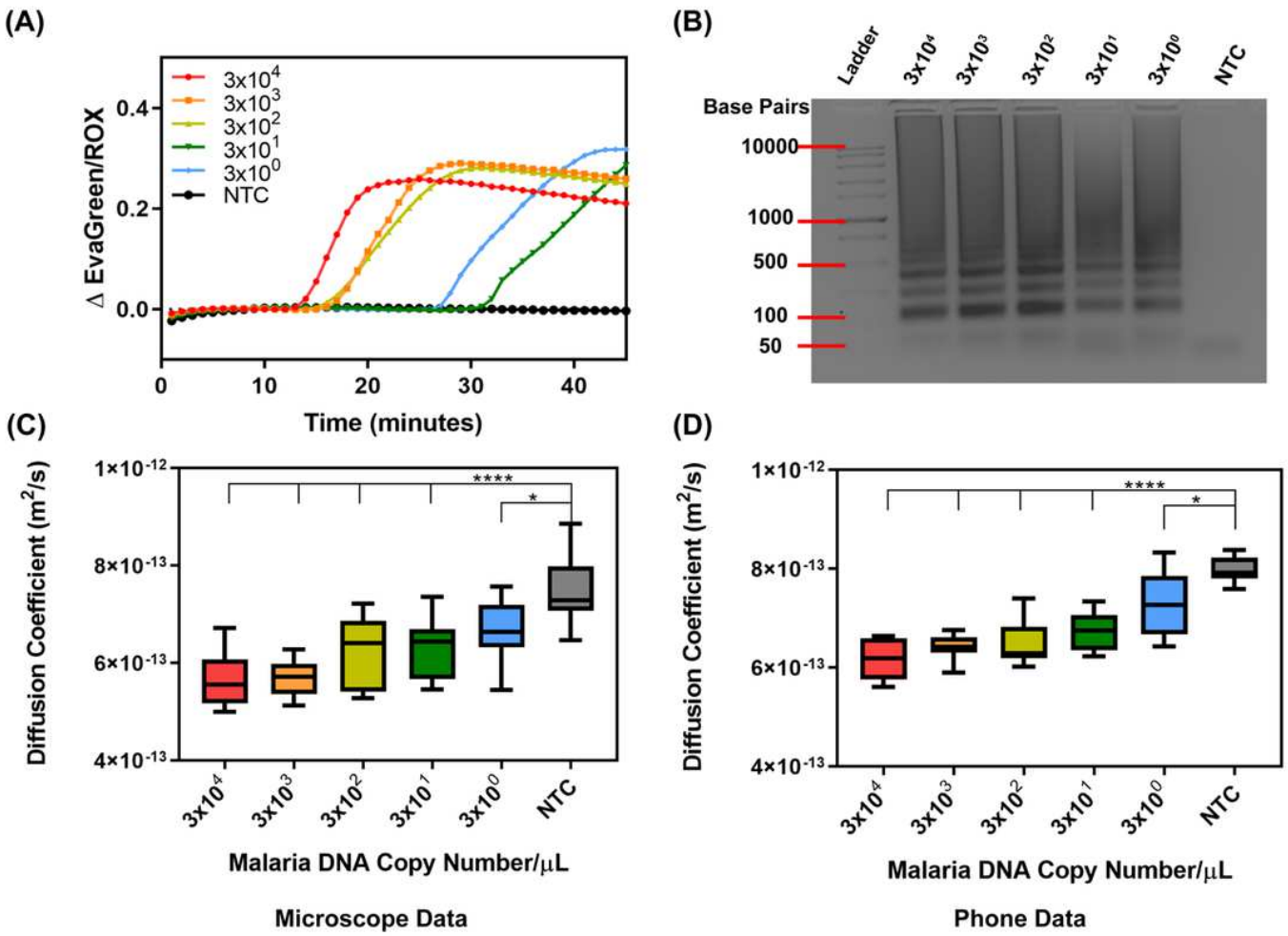

Figure 2

P. falciparum genomic DNA detection targeting 28s rRNA. (A) Real-time fluorescence data during a 45-minute LAMP reaction with concentrations ranging from 3x104 to 3x100 DNA copies/µL. (B) The representative LAMP DNA banding pattern for all positive samples are confirmed in 2% agarose gel electrophoresis (note the NTC shows no banding pattern). (C) PD analysis from the microscope images of the samples indicates the change in diffusion coefficient for LAMP samples with a statistically significant difference from the negative template control (NTC) for samples with 3x104 -3x101 (****p<0.0001) and 3x100 (*p<0.05) DNA copies/µL. (D) PD analysis on smartphone device indicates statistically significant differences between 3x104 -3x101 (****p<0.0001) and 3x100 (*p<0.05) DNA copies/µL and the NTC. NTC here represents water added in place of genomic DNA. (N=4)

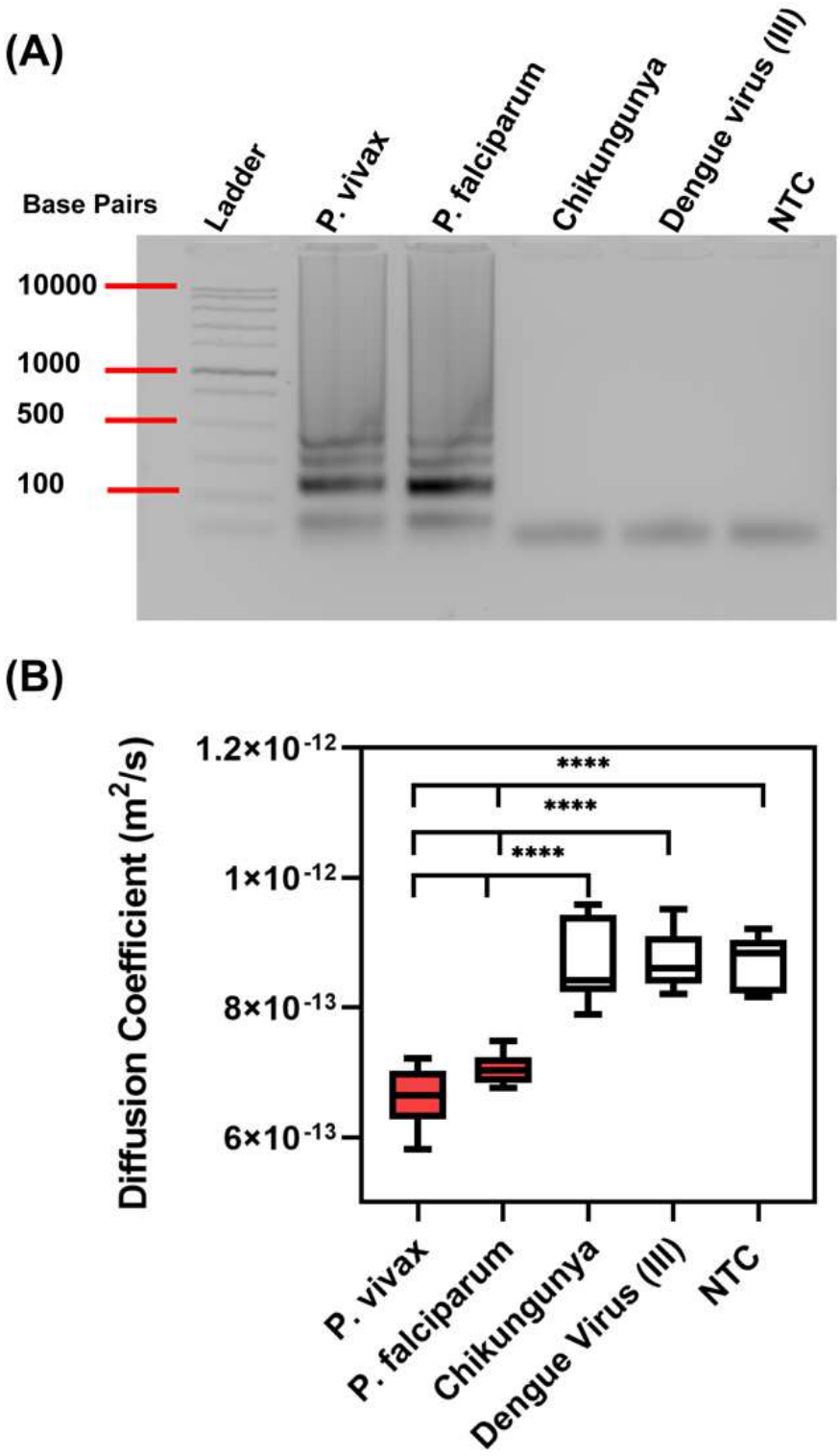

Figure 3

PD-LAMP specificity in 10% blood. (A) A 2% agarose gel from LAMP reactions in blood containing malarial strains P. falciparum and P. vivax alongside controls chikungunya and dengue virus (III) at 6x104 parasites/µL blood after a 60-minute LAMP reaction. Only malaria samples amplified, demonstrated by the DNA banding pattern in the gel. (B) Diffusion coefficients from smartphone PD analysis, where

malaria samples showed a significant difference from chikungunya virus, dengue virus (III) and NTC (One-way ANOVA Dunnett's post-hoc test). NTC represents blood without spiked pathogens. (N=3)

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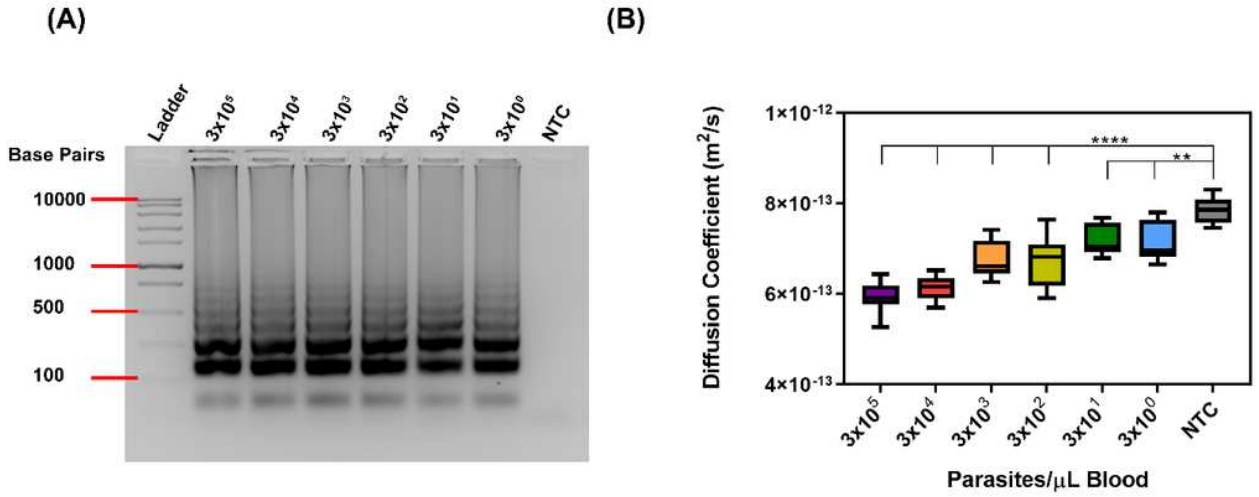

Figure 4

Sensitivity of malaria PD-LAMP with 28s primers using 10% blood. (A) 2% agarose gel confirming amplification in positive samples. (B) Diffusion coefficients measured using smartphone device for dilutions of 3x105-3x100 of P. falciparum infected blood after a 75-minute reaction. PD analysis shows statistical difference from controls for 3x105-3x102 parasites/µL blood (****p<0.0001) and 3x101-3x100 parasites/µL blood (**p<0.001) .(N=4).

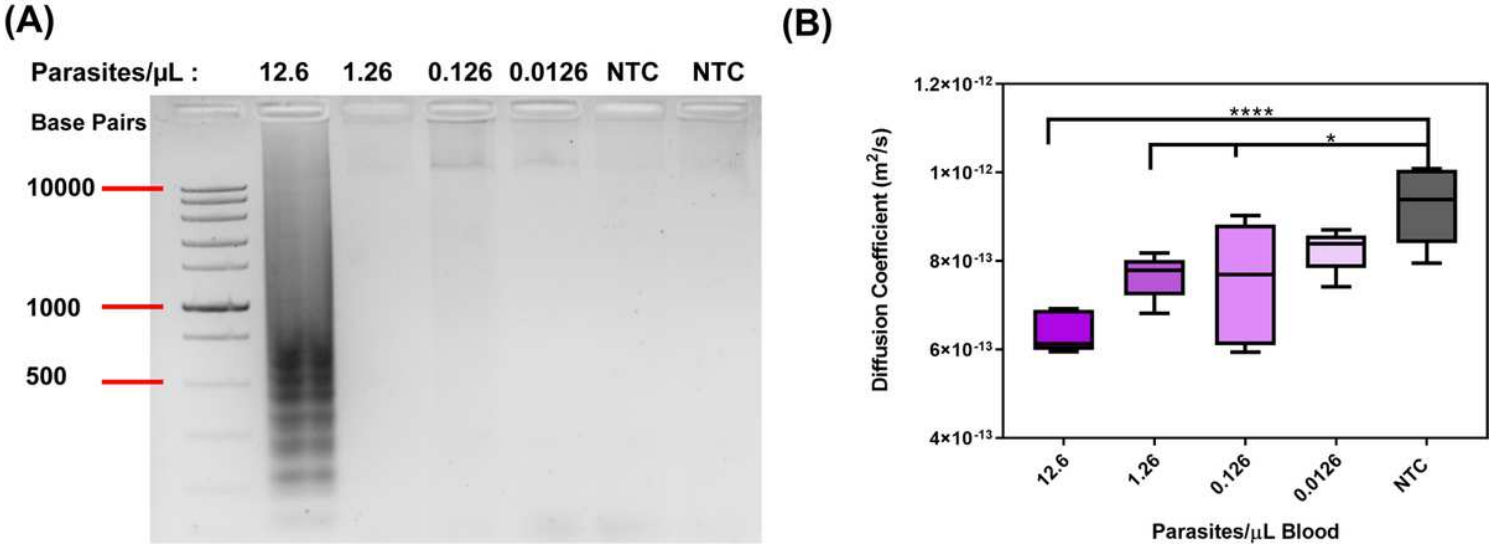

Figure 5

Serial dilutions of patient ID 2 for the determination of the LOD in patient samples using 18s primers. (A) A representative gel from dilutions of ID 2 with concentrations ranging from 12.6 to 0.0126 parasites/µL. Only the 12.6 parasites/µL sample showed consistent amplification in gel represented by the dark smeared banding. (B) Diffusion coefficients from smartphone PD analysis of diluted samples yielded a

LOD of 0.126 parasites/µL blood. All dilutions, 12.6 parasites/µL blood (****p<0.0001), 1.26 and 0.126 parasites/µL blood (*p<0.05), except 0.0126 parasites/µL blood were found to be statistically different from NTC. (N=3)

Supplementary Files

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