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Research paper

# A novel CRISPR-based malaria diagnostic capable of *Plasmodium* detection, species differentiation, and drug-resistance genotyping

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A B S T R A C T

*Background:* CRISPR-based diagnostics are a new class of highly sensitive and specific assays with multiple applications in infectious disease diagnosis. SHERLOCK, or Specific High-Sensitivity Enzymatic Reporter UnLOCKing, is one such CRISPR-based diagnostic that combines recombinase polymerase pre-amplification, CRISPR-RNA base-pairing, and LwCas13a activity for nucleic acid detection.
*Methods:* We developed SHERLOCK assays capable of detecting all *Plasmodium* species known to cause human malaria and species-specific detection of *P. vivax* and *P. falciparum*, the species responsible for the majority of malaria cases worldwide. We further tested these assays using a diverse panel of clinical samples from the Democratic Republic of the Congo, Uganda, and Thailand and pools of *Anopheles* mosquitoes from Thailand. In addition, we developed a prototype SHERLOCK assay capable of detecting the dihydropteroate synthetase (*dhps*) single nucleotide variant A581G associated with *P. falciparum* sulfadoxine resistance.
*Findings:* The suite of *Plasmodium* assays achieved analytical sensitivities ranging from 2•5-18•8 parasites per reaction when tested against laboratory strain genomic DNA. When compared to real-time PCR, the *P. falciparum* assay achieved 94% sensitivity and 94% specificity during testing of 123 clinical samples. Compared to amplicon-based deep sequencing, the *dhps* SHERLOCK assay achieved 73% sensitivity and 100% specificity when applied to a panel of 43 clinical samples, with false-negative calls only at lower parasite densities.
*Interpretation:* These novel SHERLOCK assays demonstrate the versatility of CRISPR-based diagnostics and their potential as a new generation of molecular tools for malaria diagnosis and surveillance.
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## 1. Introduction

Timely and accurate diagnosis is an important component of malaria control and elimination efforts. The current generation of rapid diagnostic tests (RDTs) that detect *Plasmodium falciparum* histidine-rich protein 2 (PfHRP2) are widely deployed, accounting for 74% of all malaria testing in Africa in 2015, but they have shortcomings (https://www.who.int/malaria/publications/world-malaria-report-2016/report/en/). Conventional PfHRP2-based RDTs only detect *P. falciparum* and miss low density infections, under approximately 200 parasites/$\mu$L. Because PfHRP2 antigenemia can persist for weeks, they can produce false-positive results well after resolution of infection [1]. Additionally, increasing reports of false-negative RDT results due to *P. falciparum* parasites that escape detection due to deletion mutations of the *pfhrp2* and *pfhrp3* genes raise concern that PfHRP2-based RDTs may be threatened in select regions of Africa (https://www.who.int/malaria/publications/atoz/information-note-hrp2-based-rdt/en/) [2]. While microscopy is the traditional gold-standard

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**Research in context**

*Evidence before this study*

CRISPR-based diagnostics are a new family of assays that combine the base-pairing recognition of CRISPR RNAs with the varied function of Cas effector proteins to detect nucleic acid targets with high sensitivity and specificity. SHERLOCK, or Specific High-Sensitivity Enzymatic Reporter UnLOCKing, is one of two major classes of CRISPR-based diagnostics. The initial manuscript characterizing SHERLOCK described it as capable of detecting DNA and RNA from pathogens at attomolar concentrations and detecting single nucleotide variants conferring antimicrobial resistance. We identified 20 publications describing the use of SHERLOCK for molecular diagnosis in PubMed as of January 19, 2021 (search terms: "SHERLOCK CRISPR" and "Cas13a"). The majority involved assays for viral targets including SARS-CoV-2, but proof-of-principle assays for human genotyping and species identification of fish were also described. One manuscript published during review applied a CRISPR-based diagnostic approach to *Plasmodium spp.* detection and species differentiation using Cas12a.

The majority of malaria diagnostic testing worldwide relies upon rapid diagnostic tests (RDTs) that detect parasite antigens. Widely deployed malaria RDTs fail to detect low density infections, especially in non-falciparum infections, and *Plasmodium falciparum* parasites with deletions of the *P. falciparum histidine-rich protein 2* and/or 3 genes. Parasites with these deletion mutations have now been reported in at least 24 countries worldwide, leading to renewed calls for new malaria diagnostic approaches.

*Added value of this study*

We developed novel proof-of-concept SHERLOCK assays for malaria that are capable of robust detection, species identification, and drug-resistance genotyping of *Plasmodium spp.* We validated these assays using diverse clinical samples collected in Africa and Asia. This is one of the first manuscripts describing the SHERLOCK method outside of its original group of authors and is its first use for a eukaryotic pathogen in a large cohort of human clinical samples. We demonstrate SHERLOCK's potential as a sensitive and specific diagnostic for malaria and its versatility for a range of applications.

*Implications of all the available evidence*

This manuscript demonstrates that SHERLOCK is a sensitive, specific, and highly adaptable nucleic acid detection platform that can be applied for a variety of uses in infectious disease detection and drug resistance surveillance. While additional streamlining and optimization is needed for field-ready implementation, the novel assays described herein have potential as a new generation of malaria diagnostics.

for malaria diagnosis, it is only sporadically available throughout much of Africa because performance is highly operator dependent, it is labor-intensive, and it requires careful, sustained training of personnel. RDTs that detect alternative antigens such as parasite lactate dehydrogenase (pLDH) are less sensitive and heat-stable.

To address the shortcomings of antigen and microscopy-based diagnostics, molecular methods have been developed to detect parasite nucleic acid such as polymerase chain reaction (PCR), loop-mediated isothermal amplification (LAMP), and recombinase polymerase amplification (RPA) [3–5]. These modalities have proven themselves highly sensitive and specific and can detect different *Plasmodium* species and single nucleotide variants (SNVs), making it possible to monitor the emergence and spread of drug resistance in a timely manner [6–8]. However, most molecular methods have important shortcomings, including relatively expensive instrument requirements and reagent costs. To overcome these challenges, a new generation of field-deployable malaria diagnostics capable of detecting diverse species and drug-resistant variants is needed.

SHERLOCK is a CRISPR-based diagnostic that combines RPA, *in vitro* transcription, and RNA target detection using custom-designed CRISPR RNA (crRNA) oligonucleotides and Cas13a derived from the bacteria *Leptotrichia wadei* (LwCas13a) (Figure 1) [9]. Briefly, the first step of SHERLOCK is an RPA reaction performed with primers tagged with a T7 promoter sequence to generate short, double-stranded DNA (dsDNA) amplicons of a target sequence. *In vitro* transcription of the RPA product by T7 polymerase produces single-stranded (ssRNA) targets, which are recognized by base-pairing interactions with LwCas13a:crRNA complex. CrRNA-ssRNA target base-pairing interactions activate collateral RNAse activity of LwCas13a, which cleaves fluorescent or colorimetric RNA reporter molecules in the reaction to produce a detectable signal. SHERLOCK can also detect SNVs; crRNA-ssRNA target base-pairing interactions are perturbed by mismatches in the target region, leading to differential LwCas13a activation depending on the presence or absence of a variant (Figure 2). Reaction components can be combined into either one or two reactions, with detection on a simple fluorimeter, lateral flow strip, or gold nanoparticle-based colorimetry [10,11]. The range of potential applications for SHERLOCK is broad; reported applications include distinguishing Zika and dengue virus strains, genotyping human cancers and transgenic rice, detecting antimicrobial resistance SNVs, and detecting several agricultural viruses as well as the SARS-CoV-2 coronavirus (https://doi.org/10.1101/2020.05.04.20091231) [9-13].

Here, we describe novel proof-of-concept SHERLOCK assays that enable robust detection of all five *Plasmodium* species known to be pathogenic to humans and species-specific detection of the primary causes of human disease *P. falciparum* and *P. vivax.* We apply these assays to a panel of laboratory parasite strains, diverse clinical isolates from Africa and Asia, and pools of *Anopheles* mosquitoes collected in the field. We then describe an assay for detection of the A581G SNV in the *P. falciparum* dihydropteroate synthase (*dhps*) gene associated with resistance to sulfadoxine, one of the two components of the primary antimalarial medication used in intermittent preventive treatment of malaria in pregnancy (IPTp) throughout Africa [14]. While further optimization and streamlining is necessary before these assays can be used clinically, we demonstrate the potential of SHERLOCK-based diagnostics for the detection of malaria and other human pathogens.

## 2. Materials and methods

### 2.1. RPA primer design

We designed RPA primers capable of genus-specific amplification of the *Plasmodium* 18S ribosomal RNA gene, building upon existing TaqMan real-time PCR assays [3]. PCR primers were extended to 30-35 nt in length and modified to include nucleotide favorability criteria described in the TwistAmp Assay Design Manual (https://www.twistdx.co.uk/docs/default-source/RPA-assay-design/twistamp-assay-design-manual-v2-5.pdf?sfvrsn=29). These RPA primers target regions of the 18S rRNA gene that are conserved across human-infecting *Plasmodium* species, allowing "plug and play" crRNA design for species identification during the crRNA:LwCas13a base-pairing step of SHERLOCK. RPA primers for the *dhps* SNV-detection SHERLOCK assays were designed using NCBI Primer Basic Local Alignment Search Tool (BLAST) (RRID:SCR_004870) with default parameters and the following modifications: amplicon size 100-140 nt, melting temperatures between 54-67°C, and length between 30 and 35

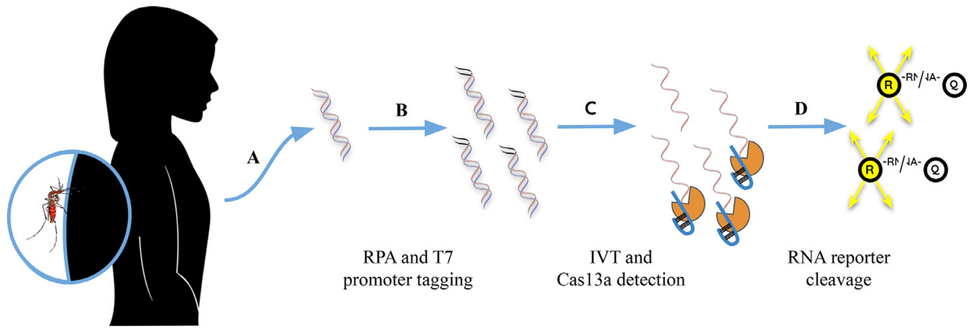


**Fig. 1.** SHERLOCK reaction workflow. (A) DNA extracted from a patient with malaria or infected mosquito is subjected to (B) RPA including T7 promoter-tagged primers for amplification of the *Plasmodium* 18S rRNA or *P. falciparum dhps* genes, followed by (C) IVT and LwCas13a:crRNA complex binding to genus-, species-, or genotype-specific target RNA. This binding triggers collateral (D) activation of LwCas13a RNAse activity and cleavage of reporter RNA, separating the fluorescent reporter from its quencher and producing a signal. Abbreviations: RPA, recombinase polymerase amplification; IVT, in vitro transcription; R, reporter; Q, quencher.

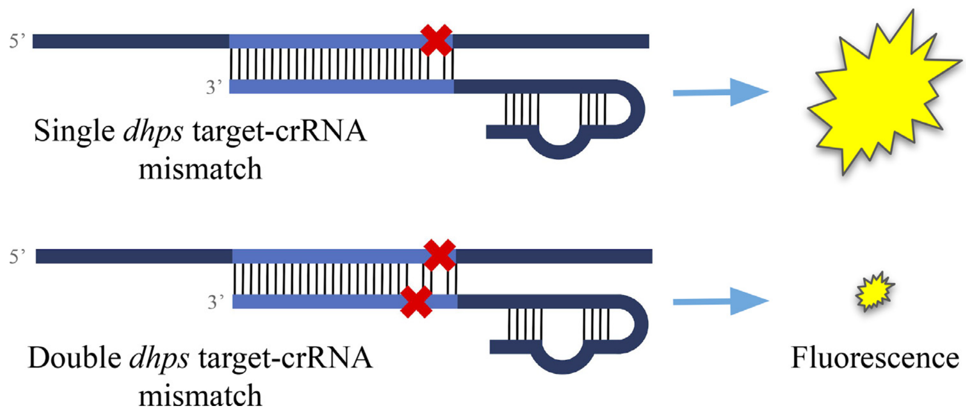


**Fig. 2.** SHERLOCK is capable of distinguishing the *dhps* A581G SNV associated with antimalarial drug resistance. Engineered target-crRNA mismatches produce differential Cas13a activation and fluorescence output.

nucleotides [15]. We then added a T7 promoter sequence (5'-GAAATTAATACGACTCACTATAGGG-3') to the 5' end of one RPA primer in each set to enable *in vitro* transcription by T7 polymerase during the LwCas13a detection step. All primers with off-target complementarity predicted by BLAST were excluded. Salt-free primers were synthesized by Integrated DNA Technologies (Coralville, Indiana). The full list of oligonucleotides designed and used in this manuscript can be found in **Supplementary Table 1.**

### 2.2. crRNA design

Oligonucleotides for crRNA synthesis were designed as two complementary ssDNA oligonucleotides and then *in vitro* transcribed to produce single-stranded 67 nt crRNAs. Each ssDNA oligonucleotide was composed of three parts: a variable spacer region (to facilitate recognition of the RNA target molecule), a constant region (to facilitate crRNA association with LwCas13a), and a T7 promoter sequence (to facilitate crRNA *in vitro* transcription).

Spacers are 28 nt long and recognize the *in vitro* transcribed RNA product of the RPA reaction through complementary base-pairing [9]. For the *P. falciparum* and *P. vivax* species-specific SHERLOCK assays, spacers were designed to bind a species-specific region of the 18S rRNA gene previously targeted for amplification during real-time PCR assays [3]. Spacers were selected to have at least three mismatches between species for the *P. falciparum* and *P. vivax*-specific assays to minimize off-target activity. For the pan-*Plasmodium* SHERLOCK assay, a spacer was selected in a region of the 18S rRNA gene conserved between *Plasmodium* species.

Spacers for *dhps* SHERLOCK assays were designed to maximize the difference in signal between a specific SNV and alternate motifs as previously described [9]. To accomplish this, spacers were designed so the SNV is recognized at position 3 or 6, and a synthetic mismatch between the spacer and target was present at position 5 or 4, respectively (Supplementary Table 2). The resulting spacer has one mismatch with the SNV of interest and two mismatches with the alternate variant, producing a measurable difference in signal between the two variants (Figure 2). Spacers were queried through BLAST to search for any off-target complementarity [15].

The same constant LwCas13a-associating region was used as described in Gootenberg et al. (2017), 5'-GGGGAUUUAGACUACCCCAAAAACGAAGGGGACUAAAAC-3', and the T7 promoter sequence used was 5'-GAAATTAATACGACTCACTATAGGG-3' [9]. Put together, two 89 nt ssDNA oligonucleotides (appended to spacer sequences) for each crRNA were designed in the format 5'-GAAATTAATACGACTCACTATAGGGGATTTAGACTACCCCAAAAACGAAGGGGACTAAAAC-spacer-3' and 5'-spacer reverse complement-GTTTTAGTCCCCTTCGTTTTTGGGGTAGTCTAAATCCCCTATAGTGAGTCGTATTAATTTC-3'. These oligonucleotides were ordered from Integrated DNA Technologies (Coralville, IA). For each SHERLOCK assay, multiple crRNA designs were screened, and crRNAs with the greatest signal:background and/or SNV signal:alternate signal were selected for all future experiments (**Supplemental Figures 1-3**).

### 2.3. crRNA synthesis

Complementary ssDNA oligonucleotides were annealed (final concentration 10 $\mu$M each) in 10 mM Tris–HCl, 50 mM KCl, and 1·5 mM $MgCl_2$ pH 8·3 for 5 minutes at 95°C followed by a 5°C/minute temperature decrease to 25°C. Annealed dsDNA templates were then *in vitro* transcribed using the HiScribe T7 Quick High Yield RNA Synthesis Kit (New England Biolabs, Ipswich, MA) using overnight incubation as described in the protocol for <0·3kb transcripts (https://www.neb.com/products/e2050-hiscribe-t7-quick-high-yield-rna-synthesis-kit#Protocols,%20Manuals%20&%20Usage). CrRNAs were purified using RNAClean XP magnetic bead purification (Beckman Coulter, Indianapolis, IN) and quantified by NanoDrop spectrophotometry (ThermoFisher, Waltham, MA).

### *2.4. LwCas13a synthesis and purification*

LwCas13a protein was synthesized and purified at the University of North Carolina at Chapel Hill (UNC) Protein Expression and Purification Core as previously described with several modifications [9]. Briefly, the pET His6-TwinStrep-SUMO-LwCas13a (NovoPro, Shanghai, China) expression vector was transformed into Rosetta$^{TM}$ 2 (DE3) pLysS Singles Competent Competent Cells (Millipore) in autoinduction media and grown at 37°C until OD=0·6, when the temperature was lowered to 18°C and grown overnight. The pellet was harvested and stored at -80°C until purification. For purification, the pellet was resuspended in lysis buffer (50 mM $NaPO_4$, 500 mM NaCl, 20 mM Imidazole, 1 mM PMSF, 50 $\mu$g/mL lysozyme) and mixed at 4°C for 30 min. The solution was then sonicated on ice and centrifuged for 1 hour at 10,000 x g at 4°C. Clarified supernatant was filtered using a 0·45 micron filter. Filtered supernatant was then loaded over Nickel Sepharose 6 Fast Flow resin (GE). The column was washed with 5 CV Buffer A (50 mM $NaPO_4$, 500 mM NaCl, 20 mM imidazole) and eluted with 10 CV Buffer B (50 mM $NaPO_4$, 500 mM NaCl, 500 mM imidazole). Buffer exchange was performed via dialysis to a final solution of 600 mM NaCl, 50 mM Tris-HCl pH 7·5, 5% glycerol, and 2mM DTT.

### *2.5. Recombinase polymerase amplification*

RPA reactions were conducted as described in TwistAmp Basic Instruction Manual (TwistDx, Cambridge, UK) with several modifications (https://www.twistdx.co.uk/en/products/product/twistamp-basic). Each 50 $\mu$L TwistAmp reaction was subdivided by first creating a 45 $\mu$L mastermix and aliquoting five 5 $\times$ 9 $\mu$L reactions before adding 1 $\mu$L of sample input. Magnesium acetate was added to the mastermix before adding the sample input. To simulate human background in clinical samples, 40 ng human gDNA from buffy coat (Sigma Aldrich, St. Louis, MO) was added in all experiments except those using clinical isolates. RPA reactions were incubated at 37°C for 30 minutes in a thermal cycler with a 40°C heated lid. After the first 4 minutes of incubation, samples were removed, vortexed briefly, and then returned to the thermal cycler for the remainder of the incubation period.

### *2.6. Detection using LwCas13a*

LwCas13a detection reactions were performed as previously described with several modifications. We chose LwCas13a rather than *Leptotrichia shahii* Cas13a because LwCas13a has been demonstrated to have higher RNA-guided RNase activity than LsCas13a and is better characterized in the context of SHERLOCK assays [11]. Each reaction was performed using 25 $\mu$L total volume and contained 45 nM LwCas13, 100-500 ng crRNA, 125 nM fluorescent RNA reporter (RNAse Alert v2, ThermoFisher), 0·625 $\mu$L murine RNase inhibitor (New England Biolabs), 31·25 ng background RNA (isolated from Burkitt's Lymphoma (Raji), ThermoFisher), 1 mM rNTP mix (New England Biolabs), 0·75 $\mu$L T7 polymerase (New England Biolabs), 3mM $MgCl_2$, and 1·25 uL of RPA product in nuclease assay buffer (20 mM HEPES, 60 mM NaCl, 6 mM MgCl2, pH 6·8) [10]. Detection reactions were conducted in 96-well black half-area microplates (PerkinElmer, Waltham, MA) and sealed with MicroAmp optical adhesive film (ThermoFisher). Detection reactions were incubated for 3 hours at 37°C on a VICTOR Nivo fluorescent plate reader (PerkinElmer) with fluorescence measurements taken every 5 minutes. Background subtracted fluorescence for each sample was calculated by subtracting the average fluorescence intensity of the human gDNA controls at 180 minutes of the human gDNA control from each sample.

### *2.7. Analytical sensitivity and specificity estimation*

To determine the analytical sensitivity of the SHERLOCK assays, we performed each assay in triplicate using serially diluted template DNA from *P. falciparum* strain Dd2 (MRA-150G) for *P. falciparum, P. vivax* 18S rRNA plasmid DNA (MRA-178), *P. ovale* 18S rRNA plasmid DNA (MRA-180), *P. malariae* 18S rRNA plasmid DNA (MRA-179), and P. knowlesi genomic DNA (MRA-456G) (Bei Resources, Manassas, VA). To facilitate ease of interpretation, we assumed six copies of 18S rRNA targets per genome when calculating parasite genome-equivalents [16]. Parasite DNA ranging from $10^5$ and $10^{-2}$ parasite genomes/$\mu$L was used to simulate expected parasite densities observed during human infection, including clinical malaria (typically $10^2$ to $10^5$ parasites/$\mu$L) and subclinical infection. Differences in mean background-subtracted fluorescence were assessed using the student's unpaired t-test. We then performed additional biological replicates using 2-fold serial dilutions near the observed limit of detection to improve the precision of our analytical sensitivity estimates. Positive SHERLOCK calls were made if background-subtracted fluorescence was positive. Finally, we assessed each assay's analytical specificity using high-concentration DNA for all human-infecting *Plasmodium* species, including gDNA and 18S rRNA plasmid DNA as described above and containing 100,000 parasite genome-equivalents per microliter.

### *2.8. Clinical sensitivity and specificity estimation*

We assessed the clinical sensitivity and specificity of our assays using a panel of dried blood spot (DBS) samples collected in the Democratic Republic of the Congo (DRC), Uganda, and Thailand. Samples were chosen out of convenience; a summary of the sources of clinical samples is provided in **Supplementary Table 3**. DRC samples were collected from subjects presenting to government health facilities with symptoms of malaria in Kinshasa, South Kivu, and Bas-Uele Provinces in 2017 as part of a separate study of malaria rapid diagnostic test performance [17]. Ugandan samples were collected from febrile children presenting to public health facilities located in the Kasese District of western Uganda over the period November 2017 to June 2018 [18]. Thai samples were collected from patients presenting to public health clinics and found to be smear-positive for *P. vivax* and as part of ongoing entomological surveillance in the Mae Sod district of Tak Province, northwest Thailand in 2010. All enrolled subjects provided informed consent. Ethical approvals were obtained by the Kinshasa School of Public Health, Mbarara University of Science and Technology, Uganda National Council for Science and Technology, Walter Reed Army Institute of Research, Ministry of Public Health in Thailand, and the University of North Carolina at Chapel Hill.

The presence and species of *Plasmodium* in each sample was determined using real-time PCR. Prior to DNA extraction, DBS were stored at -20 or -80°C in sealed plastic bags with desiccant. Briefly, DNA was extracted from three 6 mm DBS punches using Chelex-100 and saponin for DRC samples [19] Chelex-100 and Tween for Uganda samples [20], and the QIAamp DNA mini kit (QIAGEN, Hilden, Germany) for Thailand samples prior to storage at -20°C. DRC samples were first subjected to a pan-*Plasmodium* real-time PCR assay targeting the 18S rRNA gene [21]. Positive samples were then subjected to four species-specific, semi-quantitative 18S rRNA real-time PCR assays for *P. falciparum,*[22] *P. ovale,*[23] *P. malariae,*[22] and *P. vivax,* [24] respectively. Parasite densities for all DRC and Uganda samples were determined using quantitative real-time PCR (qPCR) targeting the single-copy *P. falciparum* lactate dehydrogenase (*pfldh*) gene as previously described [25,26]. *P. vivax* infection was diagnosed and parasite densities determined in Thailand using microscopy at the time of DBS collection and later confirmed using qualitative, species-specific 18S rRNA real-time PCR for *P. vivax* [22,24]. PCR primers, probes, and reaction conditions are described in the **Supplementary File**.

We first assessed the performance of all three assays using a panel of 11 DNA samples from real-time PCR-confirmed *Plasmodium* infections, including the four major human species (no *P. knowlesi* clinical isolates were available) and a range of parasite densities. We further characterized the performance of the *P. falciparum* assay using a panel of 62 PCR-positive and 50 PCR-negative samples collected in the DRC and Uganda. *Plasmodium* SHERLOCK assays were performed in triplicate for the panel of 11 DNA samples and once for the 112 samples collected in the DRC and Uganda. To improve specificity, positive SHERLOCK calls were made if the absolute fluorescence was at least 20% above background fluorescence. Clinical sensitivity, clinical specificity, and Cohen's Kappa coefficient were calculated using real-time PCR as the gold standard.

### *2.9. Parasite DNA detection in mosquito pools using SHERLOCK*

Parasite DNA was extracted from insectary-reared *Anopheles dirus* mosquitoes fed on blood from gametocytemic *P. falciparum*-infected volunteers in Cambodia. Mosquitoes were saved 9 and 16 days post-feeding to capture oocyst-stage and sporozoite-stage infection, respectively. They were pooled in groups of 10 and preserved in 95% ethanol before undergoing DNA extraction via a simplified Chelex protocol as previously described [27]. SHERLOCK assays were performed on DNA from mosquito pools in triplicate. Comparison of mean background-subtracted fluorescence values was performed using the t-test as described above.

### *2.10. Deep sequencing of clinical samples and dhps genotyping*

We selected 463 DNA samples from the DRC with *P. falciparum* mono-infection by real-time PCR for *dhps* genotyping using a multiplex real-time PCR assay that discriminates wild-type from 581G mutants (see Supplementary File for details) [7]. We then selected samples for amplicon-based deep sequencing, including a subset of 92 wild-type and 581G mutant DNA samples identified during *dhps* real-time PCR screening of DRC samples and 92 *P. falciparum* histidine-rich protein 2-based RDT-positive samples from Uganda. Each of these 184 samples was amplified using a barcode-labeled forward and reverse primer (Supplementary Table 4). PCR reactions were carried out in a total volume of 50 $\mu$L and using 5 $\mu$L of input DNA. The reaction mixture contained 1X Accuprime PCR buffer II (Invitrogen, Carlsbad, CA), 1 unit Accuprime HiFi taq, 1 $\mu$L of 0·0025 mg/$\mu$L BSA, and 400 nM of forward and reverse primer. The reaction conditions were 95°C for 2 minutes, followed by 40 cycles of 95°C for 30 seconds, 60°C for 30 seconds and 68°C for 1 minute, with a final extension of 68°C for 10 minutes. PCR products were quantified using picogreen on a microplate reader. Amplicons were pooled, prepared for sequencing using Kappa HyperPrep Kits (Roche, Indianapolis, IN) and sequenced on a single MiSeq (Illumina, San Diego, CA) run using 250 bp paired-end chemistry at the UNC High Throughput Sequencing Facility.

Sequencing data was analyzed using SeekDeep v2·6·5 using default parameters [28]. Briefly, data were demultiplexed using the *dhps* primers and a dual barcoding scheme, and final haplotypes for each sample were determined by removing low per-base quality scores and low frequency mismatches. Final haplotypes were further filtered by removing haplotypes with <2,000 reads within samples. We then selected DNA from samples containing a single variant consistent with a wild-type or A581G mutant parasite for SHERLOCK SNV testing. Mixed infections were excluded. Wild-type parasites were stratified by sampling location and selected for SHERLOCK testing using a random number generator (Excel, Microsoft, Seattle, WA).

### *2.11. Dhps SNV detection by SHERLOCK*

All *dhps* SNV-detection SHERLOCK assays were performed in triplicate using the conditions described above. Positive SHERLOCK calls were made if background-subtracted fluorescence was positive. Clinical sensitivity, clinical specificity, and Cohen's Kappa coefficient were calculated using deep sequencing calls as the gold standard.

### *2.12. Statistical analysis*

Statistical analyses were performed in Prism (RRID:SCR_002798, GraphPad, San Diego, CA) and R (R Core Team, Vienna, Austria). Comparisons were made using the t-test for continuous variables. Probit analysis was used to determine the 95% limit of detection and 95% confidence intervals for each SHERLOCK assay. Samples were not randomized and experimenters were not blinded in conducting these experiments.

## 3. Results

We translated SHERLOCK to malaria by designing and screening candidate RPA primers and crRNAs, optimizing assay conditions, and validating them on clinical and mosquito samples collected in diverse sites (Figure 1). Assay development included the design and testing of two broad categories of RPA primers and crRNAs. The first design targeted conserved and variable regions of the *Plasmodium* 18S rRNA gene and enabled detection and species identification of *Plasmodium* parasites in clinical samples and mosquitoes. The second design included engineered crRNA-target mismatches to enable detection of the *P. falciparum* A581G mutation associated with resistance to sulfadoxine (Figure 2). A range of reaction parameters were tested during pilot testing. While we were unable to replicate the "one-pot" reaction approach (combining RPA, IVT, and Cas13a detection) previously described for viral targets,[10] we achieved best performance using a two-step reaction format (RPA followed by IVT and Cas13a detection) and 10-50-fold higher crRNA concentrations than those originally described by Gootenberg *et al* [9].

### *3.1. Plasmodium SHERLOCK achieves robust analytical sensitivity and specificity*

Our SHERLOCK assays demonstrated attomolar analytical sensitivity for *Plasmodium* DNA, equivalent to 95% lower limits of detection (LODs) of 2·5-18·8 parasite genomes per reaction (4·2-31·3 aM; Figure 3, Table 1) for all three assays. Using only 1 $\mu$L of initial DNA input, this is roughly equivalent to 2·5-18·8 parasites/$\mu$L. These sensitivities approach those of commonly deployed malaria real-time PCR assays,[3] which achieved 0·3-2·5 parasites/$\mu$L analytical sensitivities during head-to-head testing in one controlled setting [29]. The pan-*Plasmodium* and *P. falciparum* SHERLOCK assays achieved superior LODs compared to *P. vivax*, but all three assays achieved LODs well below those detected by commonly used malaria RDTs (Table 1).

To test the specificity of our SHERLOCK assays against different *Plasmodium* species, we conducted assays using high concentration 18S rRNA plasmid DNA or gDNA from all five human-infecting *Plasmodium* species (Figure 4). The pan-*Plasmodium* SHERLOCK assay detected all five *Plasmodium* species, while the *P. falciparum* SHERLOCK assay only displayed activity against *P. falciparum* gDNA. The *P. vivax* SHERLOCK assay detected *P. vivax* 18S rRNA plasmid DNA, but also demonstrated low-level cross-reactivity for *P. knowlesi* gDNA.

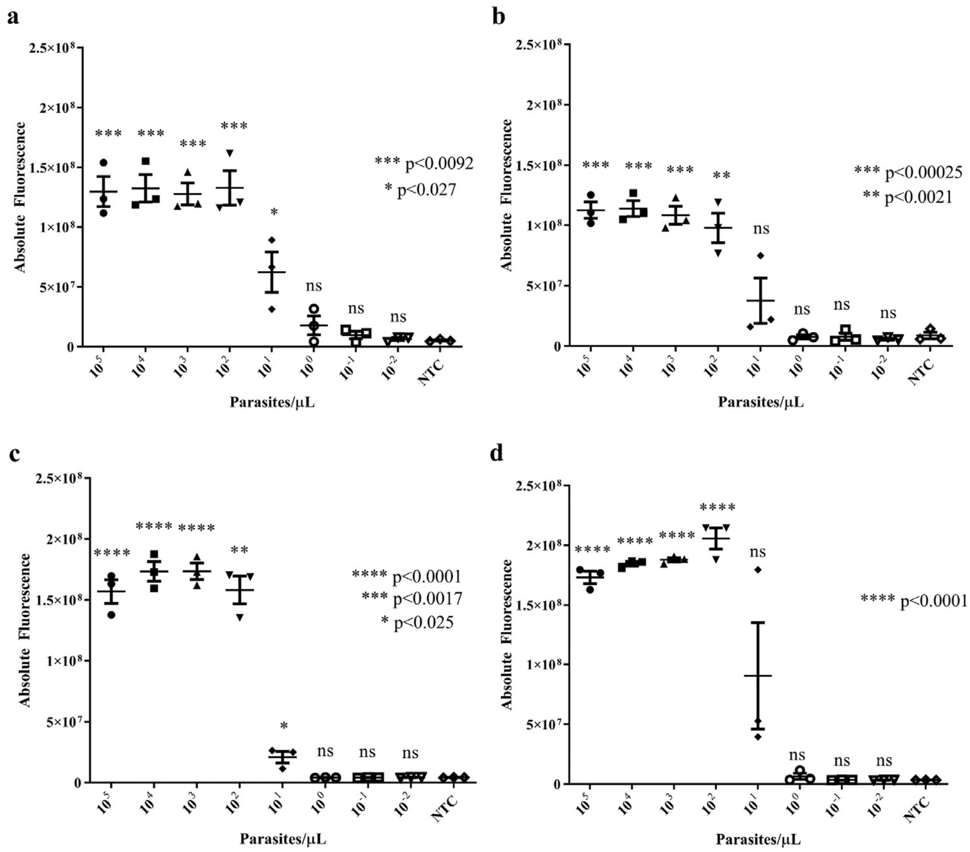


**Fig. 3.** Analytical sensitivity of *Plasmodium spp.* SHERLOCK assays. (a) Pan-*Plasmodium* assay vs. *P. falciparum* strain 3D7 gDNA (b) Pan-*Plasmodium* assay vs. *P. vivax* 18S rRNA plasmid DNA (c) *P. falciparum assay* vs. *P. falciparum* strain 3D7 gDNA (d) *P. vivax* assay vs. *P. vivax* 18S rRNA plasmid DNA. N = 3 technical replicates, n = 1 biological replicate. Unpaired student's T test vs. NTC, ± SEM. Abbreviations: NTC, no template control.

**Table 1**
95% lower limits of detection (LOD) for *Plasmodium spp.* SHERLOCK assays. Input is 1 $\mu$L of DNA template per reaction.

| | | Parasites/$\mu$L (replicates detected / replicates tested) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| SHERLOCK Assay | LOD (95% CI) | $10^5$ | $10^4$ | $10^3$ | $10^2$ | 20 | 15 | 10 | 5 | 2·5 | 1·25 |
| Pan-*Plasmodium* | 2·5 (1·9-21·3) | 3/3 | 3/3 | 3/3 | 3/3 | - | - | 20/20 | 20/20 | 19/20 | 14/20 |
| *P. falciparum* | 6·8 (5·3-11·6) | 3/3 | 3/3 | 3/3 | 3/3 | - | - | 20/20 | 17/20 | 9/20 | 8/20 |
| *P. vivax* | 18·8 (13·3-146·3) | 3/3 | 3/3 | 3/3 | 3/3 | 20/20 | 20/20 | 10/20 | 19/20 | - | - |

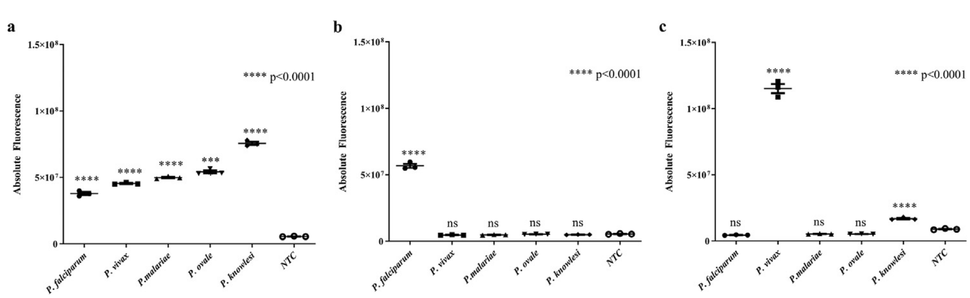


**Fig. 4.** (a) Pan-*Plasmodium* SHERLOCK assay can detect all five *Plasmodium* species in humans, while (b) *P. falciparum*, and (c) *P. vivax* SHERLOCK assays can differentiate between *Plasmodium* species. Input for all species was the equivalent of 100,000 parasites/$\mu$L as either species-specific 18S plasmid or gDNA n (*P. knowlesi* only). N = 3 technical replicates, n = 1 biological replicate. Unpaired student's T test vs. NTC, ± SEM. Abbreviations: NTC, no template control.

### 3.2. SHERLOCK performs well in clinical samples and infected mosquitoes

Our SHERLOCK assays successfully detected and differentiated the four most common *Plasmodium* spp. known to cause human malaria from clinical samples across a range of parasite densities. When applied to a panel of real-time PCR-positive clinical isolates from the DRC and Thailand, the species-specific *P. falciparum* and *P. vivax* assays demonstrated complete clinical specificity (Figure 5). When applied to a larger panel of 62 PCR-positive and 50 PCR-negative samples from the DRC and Uganda, the *P. falciparum* SHERLOCK assay's sensitivity and specificity were 94% and 94%, respectively, compared to real-time PCR (Supplementary Figure 4, Supplementary Table 5). The four PCR-positive/SHERLOCK-negative samples had

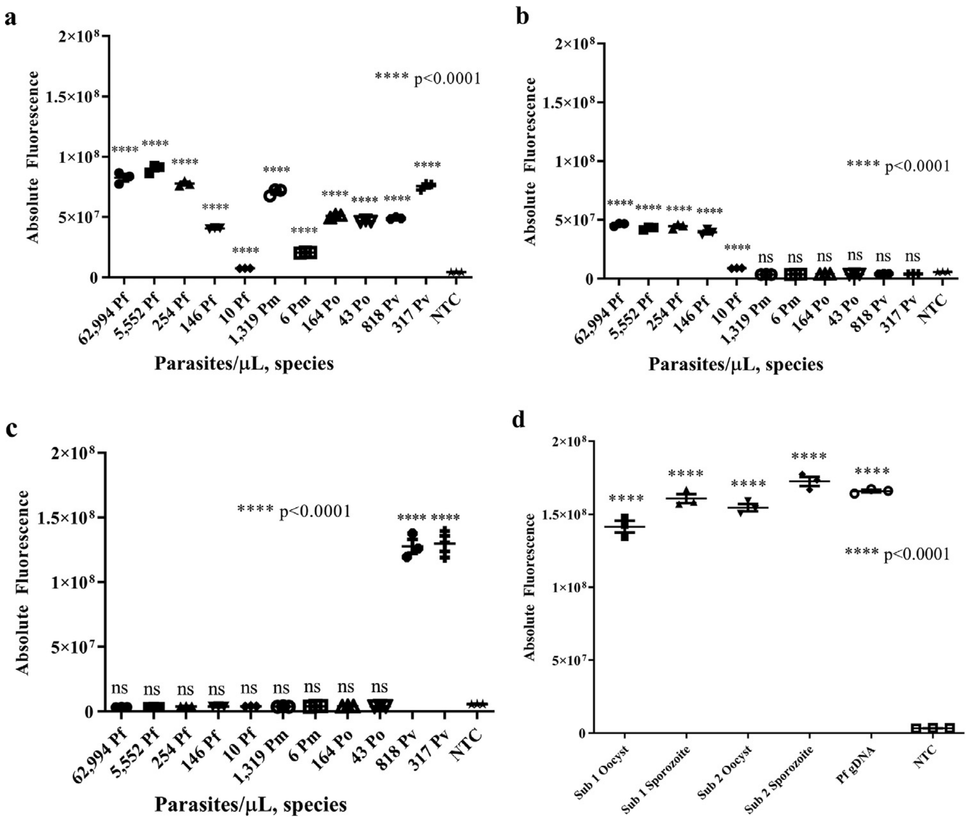


**Fig. 5.** (a) Pan-*Plasmodium*, (b) *P. falciparum*, and (c) *P. vivax* SHERLOCK assays successfully detected and differentiated *Plasmodium* infections in diverse clinical isolates. (d) *P. falciparum* SHERLOCK assay detected both the oocyst and sporozoite stages of DNA extracted from infected mosquitos. Pf gDNA in the mosquito assay was 100,000 parasites/$\mu$L. Parasite densities were determined by qPCR for Pf, Pm, and Po and by microscopy for Pv. N = 3 technical replicates, n = 1 biological replicate. Unpaired student's T test vs. NTC, ± SEM. Abbreviations: Pf, *P. falciparum;* Pv, *P. vivax*; Pm, *P. malariae;* Po, *P. ovale*; NTC, no template control; gDNA, genomic DNA; Sub, subject.

positive SHERLOCK results upon repeat testing, suggesting operator error or stochastic amplification failure during the initial assay. When SHERLOCK was performed in singleton, Cohen's kappa was 0·87, consistent with excellent agreement between methods.

The *P. falciparum* SHERLOCK assay also performed well when applied to DNA extracted from pooled, infected mosquitoes (n=4) (Figure 5). In all cases, the *P. falciparum* SHERLOCK assay was able to detect the presence of parasites, including both the sporozoite and oocyst stages within the sporogonic life cycle.

### 3.3. *SHERLOCK can be used to detect SNVs associated with antimalarial drug resistance*

Amplicon-based deep sequencing was performed to distinguish wild-type and A581G mutant *dhps P. falciparum* infections in 185 samples from the DRC and Uganda collected from patients with symptomatic malaria. The A581G mutation is associated with high-level resistance to sulfadoxine. The median read count per sample was 81,775. After filtering, only two variants were observed across all samples, corresponding to wild-type and A581G mutant *dhps*. Forty-three samples that were confirmed to be mono-infections bearing only wild-type or A581G mutant *dhps* mutations were selected for testing by SHERLOCK.

When applied to 22 wild-type and 21 A581G mutant *dhps* samples from Uganda, the sensitivity and specificity of the *P. falciparum dhps* SHERLOCK assay were 73% and 100%, respectively, using *dhps* deep sequencing calls as the gold standard. Cohen's kappa was 0·72, consistent with good agreement between SHERLOCK and amplicon-based deep sequencing. When restricted to field samples with parasite densities of $\geq$420 parasites/$\mu$L, the *dhps* SHERLOCK assay's clinical sensitivity and specificity were 100% and 100%, respectively. Among these higher parasite density samples, Cohen's kappa was 1.0, consistent with perfect agreement between the *dhps* SHERLOCK assay and amplicon-based deep sequencing in these samples with higher parasite densities.

## 4. Discussion

We describe a suite of novel SHERLOCK malaria assays and highlight the versatility of CRISPR-based diagnostics for a range of infectious disease applications. The new assays achieved robust clinical sensitivity and specificity when applied to well-characterized clinical samples and were easily adapted for parasite detection in mosquitoes and for drug-resistance genotyping. New diagnostics that can be used to detect, identify species, and genotype *P. falciparum* infections rapidly and reliably are especially needed in sub-Saharan Africa, where 94% of malaria deaths occur (https://www.who.int/publications/i/item/9789241565721).

These SHERLOCK malaria assays outperform HRP2-based RDTs, the predominant malaria diagnostic modality deployed throughout Africa, and achieve similar performance to recently developed ultra-sensitive HRP2-based RDTs, which have achieved clinical sensitivities of roughly 3 parasites/$\mu$L [30,31]. Increasing reports of *P. falciparum* with deletion mutations of the histidine rich 2 and/or 3 (*pfhrp2/3*) genes raise concerns about reliance on HRP2 as the primary diagnostic target for RDTs [2]. RDTs that detect alternative antigens such as pLDH are available, but they are less sensitive and less heat-stable than HRP2-based RDTs. Thus, there is a need for novel malaria diagnostics that detect other targets. While both SHERLOCK and other CRISPR-based diagnostic assays currently in development need additional optimization prior to field deployment, we demonstrate that they are easily adapted for specific use cases and represent a promising avenue for malaria diagnostic development.

The analytical and clinical sensitivity of these SHERLOCK malaria assays approached that of commonly used real-time PCR assays, but the ability to conduct SHERLOCK without thermocycling allows for reduced laboratory infrastructure requirements and enables simplified isothermal approaches. More recently developed “ultrasensitive PCR” assays that target RNA and/or multicopy genes have achieved analytical sensitivities as low as 0·02 parasites/$\mu$L, but their use in low-resource laboratory settings is limited by the need to protect against RNA degradation and/or the requirement for large-volume samples [32,33]. LAMP is an alternative nucleic acid detection method that has been used for malaria diagnosis in field settings with similar sensitivity and specificity to SHERLOCK [34]. LAMP shows promise as a field-deployable molecular diagnostic, but its use for bespoke applications has been hampered by the complexity of target selection and primer design. CRISPR-based diagnostic approaches like SHERLOCK are an emerging technology that can be rapidly adapted in response to malaria’s evolving epidemiology [35].

Species discrimination was excellent across *Plasmodium spp.* SHERLOCK assays, with the exception of the *P. vivax* SHERLOCK assay that demonstrated cross-reactivity with *P. knowlesi*. The *P. vivax* spacer and the analogous region on *P. knowlesi* differ by four nucleotides, so this cross-reactivity was unexpected. One explanation is that these nucleotides are towards the 3’ end of the crRNA spacer (positions 20, 26, 27 in the 28), which have been shown to have a smaller influence on crRNA binding efficiency [9]. Another explanation is unappreciated homology between *P. vivax* and *P. knowlesi* spacer binding sites in the setting of incomplete understanding of *P. knowlesi*’s genetic diversity. These findings highlight the importance of using high-quality genomic data when designing target sequences for CRISPR-based diagnostics and consideration of sequence variability within targets. Future SHERLOCK assays will be strengthened by efforts to improve our understanding of parasite genomic diversity [36,37].

Our novel SNV detection SHERLOCK assay for the *dhps* 581G variant in *P. falciparum* represents one of the first uses of SHERLOCK to detect SNVs in clinical isolates outside of its original description [9,10]. We chose to develop a SHERLOCK SNV detection assay for *Plasmodium dhps* variants due to their public health importance in the prevention of malaria during pregnancy. Variants in the *dhps* gene can confer resistance to sulfadoxine, which is used in combination with pyrimethamine (SP) as the primary antimalarial drug for intermittent preventive treatment in pregnancy (IPTp) to prevent malaria in pregnancy throughout much of sub-Saharan Africa [38]. Our *dhps* SHERLOCK assays demonstrated parasite-density dependent sensitivity and perfect specificity when compared to amplicon-based deep sequencing. When applied to a wide range of parasite inputs, the assay only produced false-negative results in samples with low parasite densities (6/7 samples with $\leq$319 parasites/$\mu$L sample input). While the SNV detection assay is concentration dependent, it performed well at parasite densities typically associated with clinically significant malaria [39]. In future, mixed infections involving both wild-type and 581G mutant strains could be identified by pairing the 581G-specific assay described here with a wild-type-specific assay in a multiplexed format. SHERLOCK’s SNV detection capabilities are a promising tool for malaria control programs in low-resource settings where sequencing facilities are not available to support surveillance. For example, high prevalence of resistance-associated *dhps* variants detected by SHERLOCK could trigger deployment of alternative drug regimens for IPTp in affected regions.

Our SHERLOCK assays are now one of two published CRISPR-based diagnostic modalities for *Plasmodium* detection; in a recently published manuscript, Lee *et. al.* describe a suite of highly sensitive and specific assays for *P. falciparum, P. vivax, P. malariae,* and *P. ovale spp.* [40]. These assays employ a detection approach most similar to the DNA endonuclease-targeted CRISPR trans reporter (DETECTR) methodology, which uses the DNA endonuclease Cas12 [41]. While our methods differ in this regard, both approaches highlight the potential uses for SHERLOCK and other CRISPR-based diagnostics for malaria diagnosis, research, and drug resistance surveillance.

The assays described by Lee *et al* have several advantages and disadvantages compared to our assays. First, the analytical sensitivity achieved by Lee et. al. is superior. The 95% lower LODs of their *P. falciparum* and *P. vivax* assays were 0·36 (0·23-1·0) and 1·2 (0·52-6·2) parasites/$\mu$L compared to our 95% lower LODs of 6·8 (5·3-11·6) and 18·8 (13·3-146·3), respectively. One explanation for this improved performance is that Lee *et al.* chose gene targets that are present in higher copy numbers: Pfr364 subtelomeric repeat DNA for *P. falciparum* (41 copies) and mitochondrial DNA for *P. vivax* (up to 20 copies) vs. 18s rRNA in our assays (~6 copies). While the use of 18S rRNA targets reduces assay sensitivity, it provides opportunities for streamlined, multiplexed assays that employ a single, shared RPA primer set targeting regions that are conserved across *Plasmodium* species and bespoke crRNAs for species identification. Second, Lee *et al.* describe streamlined approaches to nucleic acid extraction and reaction set-up that are better suited for field use. These advances make it clear that some of the challenges we encountered with our Cas13a-based SHERLOCK approach can be overcome. Third, the clinical performance of the assays described by Lee *et al.* is not yet defined. They conducted validation experiments using a panel of simulated samples derived from cultured parasites and a small number of serum samples, whereas we validated our assays on dried blood spot samples collected in malaria endemic countries, including a large panel of 112 samples for our *P. falciparum* assay. This type of clinical validation is important because samples collected under field conditions is necessary to define real-world assay performance, both in terms of sample preparation/integrity and parasite genetic diversity. Genetic diversity of sites targeted by these assays can only be partly deduced from publicly available sequencing data, especially in the case of non-falciparum species like *P. ovale* and *P. malariae* that are not well represented in existing databases. Despite these differences, the performance of both sets of CRISPR-based diagnostic assays demonstrates that they can detect parasite concentrations well below what is expected in clinical malaria and supports future studies to adapt and validate these assays for use in research and clinical settings.

We chose LwCas13a-based detection over a Cas12a-based approach to take advantage of Cas13a’s minimal protospacer-adjacent motif (PAM) site nucleotide requirements compared to Cas12a. LwCas13a requires only H (not G) adjacent to the spacer region, which makes guide design and SNV detection for LwCas13a easier than for Cas12a, which has a PAM requirement of “TTTN” [41,42]. Additionally, multiplexing with SHERLOCK has been demonstrated using Cas13a isolated from multiple bacterial species, which have different collateral activities that can be used to activate different RNA reporters, enabling multiplexed assays [10]. These properties of Cas13a provide opportunities for further assay development, including translation of our malaria SHERLOCK assays into a single, multiplexed assay that enables species identification of multiple *Plasmodium* species and drug-resistance SNVs in a single reaction. We experienced success using SHERLOCK to detect *Plasmodium spp.* and drug-resistance variants; however, several limitations must be overcome for successful translation of SHERLOCK from the laboratory to the field. First, we could not achieve the sub-attomolar limits of detection or “one-pot” reaction conditions previously described for viral targets [10]. We also observed that our assays performed best using higher crRNA concentrations than those originally described [9]. Though this may reflect differences in crRNA synthesis or lot-to-lot variation in other reagents, we observed improved reaction performance with higher crRNA concentrations across multiple experiments.

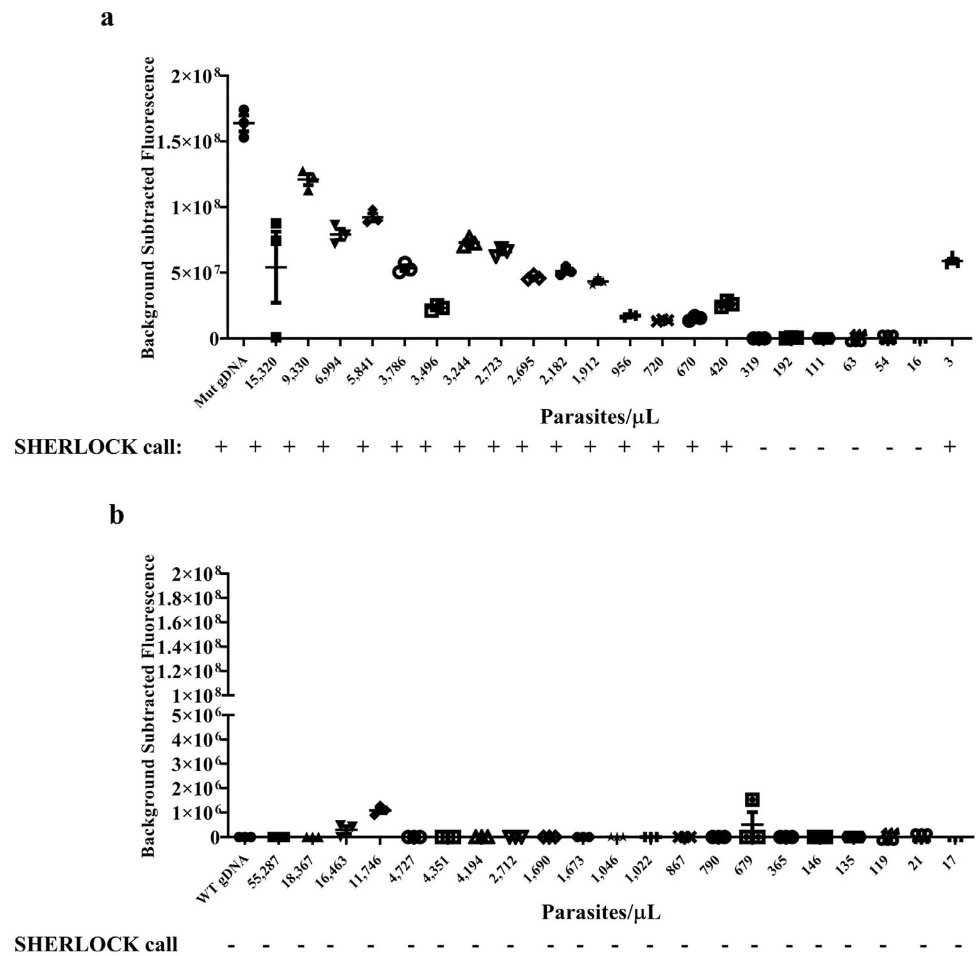


**Fig. 6.** The *dhps* SHERLOCK assay detects *P. falciparum* parasites with (a) mutant (581G) but not (b) wild-type (A581) alleles. Parasite densities were determined by qPCR. N = 3 technical replicates, n = 1 biological replicate,± SEM. Mutant (Mut) and WT gDNA standard inputs were 100,000 parasites/$\mu$L.

Second, the cost of designing and validating a SHERLOCK assay is not trivial. In a reaction volume of 25 $\mu$L used for 96-well plates and using fluorescent output, the cost was roughly equivalent to PCR at over $2·00 of reagent costs per technical replicate, plus upfront costs of crRNA optimization and synthesis and the need for a fluorescence plate-reader. These costs can be reduced for assays brought to scale but are an important consideration during assay design. Finally, SHERLOCK requires multiple reagents, custom crRNAs, and Cas13a. Recent progress has been made to reduce its complexity and make these assays more easily accessible [43]. Indeed, the approach described by Lee et. al includes a streamlined workflow from sample collection to Cas12a-based detection; including an optimized nucleic acid extraction step, one-pot lyophilized reaction, reduced reaction time, and a handheld fluorometer and lateral-flow readout. Other groups have sought to replace RPA preamplification with LAMP, or eliminate it altogether in the case of one Cas13a-based SARS-CoV-2 diagnostic (https://doi.org/10.1101/2020.12.14.20247874)[44]. While these advances confirm rapid improvements in CRISPR-based assay performance and feasibility, implementation of SHERLOCK as a point-of-care molecular diagnostic will require further streamlining including the development of commercially-available mastermixes and the combination of nucleic acid extraction, Cas effector activation, and signal readout into a single device.

While these limitations must be overcome before immediate field applications, SHERLOCK is a promising technology with multiple potential uses in malaria diagnosis, research, and drug resistance surveillance. In particular, our SNV detection SHERLOCK assays demonstrate applications beyond infectious disease diagnosis and include genotyping pathogens at the nucleotide level. With further optimization, our SNV detection assays could facilitate drug resistance surveillance for malaria control programs without the need for PCR or sequencing. Additionally, our pilot application of SHERLOCK to detect *P. falciparum* in infected mosquitoes demonstrates potential beyond clinical diagnostics and other uses for research in low malaria transmission or near elimination settings. Continued development of new Cas effectors, streamlined workflows, and point-of-care read-outs will open new opportunities for CRISPR diagnostics in clinical, surveillance, and research applications. These novel malaria SHERLOCK assays confirm the promise of CRISPR-based diagnostics for diverse applications and in resource-limited settings (Fig. 6).

## Declaration of Competing Interest

JBP reports research support from Gilead Sciences and non-financial support from Abbott Laboratories for providing reagents and laboratory testing in-kind for studies of viral hepatitis and financial support from the World Health Organization, outside the scope of the current work.

## Author Contributions

Clark Cunningham: Conceptualization, Methodology, Validation, Formal Analysis, Investigation, Visualization, Writing - Original Draft. Christopher Hennelly, Kyaw Thwai: Investigation. Jessica Lin, Ratawan Ubalee, Ross Boyce, Edgar Mulogo, Fernandine Phanzu, Albert Kalonji, Kashamuka Mwandagalirwa, Antoinette Tshefu. Nicholas

Hathaway: Software, Formal Analysis. Jonathan Juliano: Conceptualization, Supervision, Funding Acquisition. Jonathan Parr: Conceptualization, Supervision, Project Administration, Funding Acquisition, Visualization, Resources, Writing - Original Draft. All authors: Writing - Review and Editing.


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## Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.ebiom.2021.103415.

## References


[1] Markwalter CF, Gibson LE, Mudenda L, Kimmel DW, Mbambara S, Thuma PE, et al. Characterization of Plasmodium Lactate Dehydrogenase and Histidine-Rich Protein 2 Clearance Patterns via Rapid On-Bead Detection from a Single Dried Blood Spot. Am J Trop Med Hyg 2018;98:1389–96.

[2] Berhane A, Anderson K, Mihreteab S, Gresty K, Rogier E, Mohamed S, et al. Major Threat to Malaria Control Programs by Plasmodium falciparum Lacking Histidine-Rich Protein 2, Eritrea. Emerg Infect Dis 2018;24:462–70.

[3] Rougemont M, Van Saanen M, Sahli R, Hinrikson HP, Bille J, Jaton K. Detection of Four Plasmodium Species in Blood from Humans by 18S rRNA Gene Subunit-Based and Species-Specific Real-Time PCR Assays. J Clin Microbiol 2004;42:5636–43. doi: 10.1128/jcm.42.12.5636-5643.2004.

[4] Lalremruata A, McCall MBB, Mombo-Ngoma G, Agnandji ST, Adegnika AA, Lell B, et al. Recombinase polymerase amplification and lateral flow assay for ultrasensitive detection of low-density Plasmodium falciparum infection from controlled human malaria infection studies and naturally acquired infections. J Clin Microbiol 2020;58.

[5] Hopkins H, González IJ, Polley SD, Angutoko P, Ategeka J, Asiimwe C, et al. Highly sensitive detection of malaria parasitemia in a malaria-endemic setting: Performance of a new loop-mediated isothermal amplification kit in a remote clinic in Uganda. J Infect Dis 2013;208:645–52.

[6] Vachot-Ganée L, Khim N, Iannello A, Legrand E, Kim S, Eam R, et al. A novel field-based molecular assay to detect validated artemisinin-resistant k13 mutants. Malar J 2018;17:1–12.

[7] Kamau E, Alemayehu S, Feghali KC, Tolbert LS, Ogutu B, Ockenhouse CF. Development of a TaqMan Allelic Discrimination assay for detection of single nucleotides polymorphisms associated with anti-malarial drug resistance. Malar J 2012;11:23.

[8] Mohon AN, Menard D, Alam MS, Perera K, Pillai DR. A Novel Single-Nucleotide Polymorphism Loop Mediated Isothermal Amplification Assay for Detection of Artemisinin-Resistant Plasmodium falciparum Malaria. Open Forum Infectious Diseases 2018;5. doi: 10.1093/ofid/ofy011.

[9] Gootenberg JS, Abudayyeh OO, Lee JW, Essletzbichler P, Dy AJ, Joung J, et al. Nucleic acid detection with CRISPR-Cas13a/C2c2. Science 2017;356:438–42.

[10] Gootenberg JS, Abudayyeh OO, Kellner MJ, Joung J, Collins JJ, Zhang F. Multiplexed and portable nucleic acid detection platform with Cas13, Cas12a, and Csm6. Science 2018;360:439–44.

[11] Yuan C-Q, Tian T, Sun J, Hu M-L, Wang X-S, Xiong E-H, et al. Universal and naked-eye gene detection platform based on CRISPR/Cas12a/13a system n.d. https://doi.org/10.1101/615724. 2021

[12] Sullivan TJ, Dhar AK, Cruz-Flores R, Rapid Bodnar AG. CRISPR-Based, Field-Deployable Detection Of White Spot Syndrome Virus In Shrimp. Sci Rep 2019;9:19702.

[13] Chang Y, Deng Y, Li T, Wang J, Wang T, Tan F, et al. Visual detection of porcine reproductive and respiratory syndrome virus using CRISPR-Cas13a. Transbound Emerg Dis 2019. doi: 10.1111/tbed.13368.

[14] van Eijk AM, Larsen DA, Kayentao K, Koshy G, Slaughter DEC, Roper C, et al. Effect of Plasmodium falciparum sulfadoxine-pyrimethamine resistance on the effectiveness of intermittent preventive therapy for malaria in pregnancy in Africa: a systematic review and meta-analysis. Lancet Infect Dis 2019;19:546–56.

[15] Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol 1990;215:403–10.

[16] Mercereau-Puijalon O, Barale J-C, Bischoff E. Three multigene families in Plasmodium parasites: facts and questions. Int J Parasitol 2002;32:1323–44.

[17] Parr JB, Kieto E, Phanzu F, Mansiangi P, Mwandagalirwa K, Mvuama N, et al. Analysis of false-negative rapid diagnostic tests for symptomatic malaria in the Democratic Republic of the Congo. Sci Rep 2021;11:6495.

[18] Boyce RM, Collins M, Muhindo R, Nakakande R, Ciccone EJ, Grounds SC, et al. Dengue in western Uganda: A prospective cohort of children presenting with undifferentiated febrile illness. Infectious Diseases (except HIV/AIDS) 2020. doi:10.1101/2020.08.21.20179002.

[19] Plowe CV, Djimde A, Bouare M, Doumbo O, Wellems TE. Pyrimethamine and proguanil resistance-conferring mutations in Plasmodium falciparum dihydrofolate reductase: polymerase chain reaction methods for surveillance in Africa. Am J Trop Med Hyg 1995;52:565–8.

[20] Teyssier NB, Chen A, Duarte EM, Sit R, Greenhouse B, Tessema SK. Optimization of whole-genome sequencing of Plasmodium falciparum from low-density dried blood spot samples. Malar J 2021;20:116.

[21] Kamau E, Alemayehu S, Feghali KC, Saunders D, Ockenhouse CF. Multiplex qPCR for detection and absolute quantification of malaria. PLoS One 2013;8:e71539.

[22] Veron V, Simon S, Carme B. Multiplex real-time PCR detection of P. falciparum, P. vivax and P. malariae in human blood samples. Exp Parasitol 2009;121:346–51.

[23] Srisutham S, Saralamba N, Malleret B, Rénia L, Dondorp AM, Imwong M. Four human Plasmodium species quantification using droplet digital PCR. PLoS One 2017;12:e0175771.

[24] Perandin F, Manca N, Calderaro A, Piccolo G, Galati L, Ricci L, et al. Development of a real-time PCR assay for detection of Plasmodium falciparum, Plasmodium vivax, and Plasmodium ovale for routine clinical diagnosis. J Clin Microbiol 2004;42:1214–9.

[25] Mwandagalirwa MK, Levitz L, Thwai KL, Parr JB, Goel V, Janko M, et al. Individual and household characteristics of persons with Plasmodium falciparum malaria in sites with varying endemicities in Kinshasa Province, Democratic Republic of the Congo. Malar J 2017;16:456.

[26] Pickard AL, Wongsrichanalai C, Purfield A, Kamwendo D, Emery K, Zalewski C, et al. Resistance to Antimalarials in Southeast Asia and Genetic Polymorphisms in pfmdr1. Antimicrob Agents Chemother 2003;47:2418–23. doi: 10.1128/aac.47.8.2418-2423.2003.

[27] Lin JT, Ubalee R, Lon C, Balasubramanian S, Kuntawunginn W, Rahman R, et al. Microscopic Plasmodium falciparum gametocytemia and infectivity to mosquitoes in Cambodia. J Infect Dis 2015;213:1491–4.

[28] Hathaway NJ, Parobek CM, Juliano JJ, Bailey JA. SeekDeep: single-base resolution de novo clustering for amplicon deep sequencing. Nucleic Acids Res 2018;46:e21.

[29] Alemayehu S, Feghali KC, Cowden J, Komisar J, Ockenhouse CF, Kamau E. Comparative evaluation of published real-time PCR assays for the detection of malaria following MIQE guidelines. Malar J 2013;12:277.

[30] Landier J, Haohankhunnatham W, Das S, Konghahong K, Christensen P, Raksuansak J, et al. Operational Performance of a Plasmodium falciparum Ultrasensitive

Rapid Diagnostic Test for Detection of Asymptomatic Infections in Eastern Myanmar. J Clin Microbiol 2018;56. doi: 10.1128/JCM.00565-18.
[31] Das S, Peck RB, Barney R, Jang IK, Kahn M, Zhu M, et al. Performance of an ultra-sensitive Plasmodium falciparum HRP2-based rapid diagnostic test with recombinant HRP2, culture parasites, and archived whole blood samples. Malar J 2018;17:118.
[32] Hofmann N, Mwingira F, Shekalaghe S, Robinson LJ, Mueller I, Felger I. Ultra-sensitive detection of Plasmodium falciparum by amplification of multi-copy subtelomeric targets. PLoS Med 2015;12:e1001788.
[33] Imwong M, Hanchana S, Malleret B, Rénia L, Day NPJ, Dondorp A, et al. High-throughput ultrasensitive molecular techniques for quantifying low-density malaria parasitemias. J Clin Microbiol 2014;52:3303–9.
[34] Patel JC, Lucchi NW, Srivastava P, Lin JT, Sug-Aram R, Aruncharus S, et al. Field evaluation of a real-time fluorescence loop-mediated isothermal amplification assay, RealAmp, for the diagnosis of malaria in Thailand and India. J Infect Dis 2014;210:1180–7.
[35] Caliendo AM, Hodinka RL. A CRISPR Way to Diagnose Infectious Diseases. N Engl J Med 2017;377:1685–7.
[36] Lapp SA, Geraldo JA, Chien J-T, Ay F, Pakala SB, Batugedara G, et al. PacBio assembly of a Plasmodium knowlesi genome sequence with Hi-C correction and manual annotation of the SICAvar gene family. Parasitology 2018;145:71–84.
[37] MalariaGEN Ahouidi A, Ali M, Almagro-Garcia J, Amambua-Ngwa A, Amaratunga C, et al. An open dataset of Plasmodium falciparum genome variation in 7,000 worldwide samples. Wellcome Open Res 2021;6:42.
[38] Gutman J, Kalilani L, Taylor S, Zhou Z, Wiegand RE, Thwai KL, et al. The A581 G mutation in the gene encoding Plasmodium falciparum dihydropteroate synthetase reduces the effectiveness of sulfadoxine-pyrimethamine preventive therapy in Malawian pregnant women. J Infect Dis 2015;211:1997–2005.
[39] White NJ, Pukrittayakamee S, Hien TT, Abul Faiz M, Mokuolu OA, Dondorp AM. Malaria. Lancet North Am Ed 2014;383:723–35. doi: 10.1016/s0140-6736(13)60024-0.
[40] Lee RA, Puig HD, Nguyen PQ, Angenent-Mari NM, Donghia NM, McGee JP, et al. Ultrasensitive CRISPR-based diagnostic for field-applicable detection of Plasmodium species in symptomatic and asymptomatic malaria. Proc Natl Acad Sci U S A 2020;117:25722–31.
[41] Chen JS, Ma E, Harrington LB, Da Costa M, Tian X, Palefsky JM, et al. CRISPR-Cas12a target binding unleashes indiscriminate single-stranded DNase activity. Science 2018;360:436–9.
[42] Abudayyeh OO, Gootenberg JS, Konermann S, Joung J, Slaymaker IM, Cox DBT, et al. C2c2 is a single-component programmable RNA-guided RNA-targeting CRISPR effector. Science 2016;353:aaf5573.
[43] Kellner MJ, Koob JG, Gootenberg JS, Abudayyeh OO, Zhang F. SHERLOCK: nucleic acid detection with CRISPR nucleases. Nat Protoc 2019;14:2986–3012.
[44] Fozouni P, Son S, Díaz de León Derby M, Knott GJ, Gray CN, D'Ambrosio MV, et al. Amplification-free detection of SARS-CoV-2 with CRISPR-Cas13a and mobile phone microscopy. Cell 2021;184 323-333.e9.