ACS Infectious Diseases

pubs.acs.org/journal/aidcbc

Article

Ex Vivo Plasmodium malariae Culture Method for Antimalarial Drugs Screen in the Field

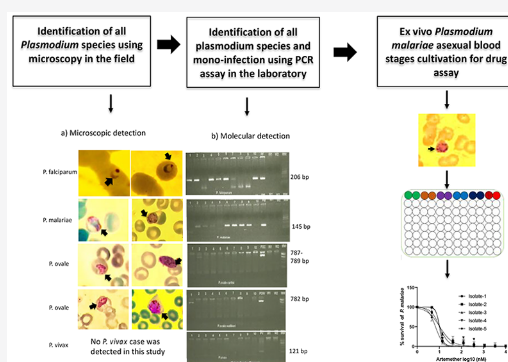
Laurent Dembele,* Nouhoum Diallo, Fanta Sogore, Bintou Diarra, Fatoumata I. Ballo, Amadou Daou, Ousmaila Diakite, Yacouba Bare, Cheick Papa Oumar Sangare, Aboubecrin Sedhigh Haidara, Seidina A. S. Diakite, Amadou Niangaly, Mahamadou Diakite, Brice Campo, Gordon A. Awandare, Yaw Aniweh, and Abdoulaye A. Djimde*

Cite This: *ACS Infect. Dis.* 2021, 7, 3025−3033

Read Online

ACCESS | Metrics & More | Article Recommendations

ABSTRACT: *In vitro* and *ex vivo* cultivation of *Plasmodium (P) falciparum* has facilitated active research into the malaria parasite toward the quest for basic knowledge and the discovery of effective drug treatments. Such a drug discovery program is currently difficult for *P. malariae* simply because of the absence of *in vitro* and *ex vivo* cultivation system for its asexual blood stages supporting antimalarial evaluation. Despite availability of artemisinin combination therapies effective on *P. falciparum*, *P. malariae* is being increasingly detected in malaria endemic countries. *P. malariae* is responsible for chronic infections and is associated with a high burden of anemia and morbidity. Here, we optimized and adapted *ex vivo* conditions under which *P. malariae* can be cultured and used for screening antimalarial drugs. Subsequently, this enabled us to test compounds such as artemether, chloroquine, lumefantrine, and quinine for *ex vivo* antimalarial activity against *P. malariae*.



KEYWORDS: *malaria, nonfalciparum, Plasmodium malariae, ex vivo culture, drug discovery*

Routine *in vitro* cultivation system of *P. falciparum* developed in 1976[1] has led to a greater understanding of the parasite biology and subsequently to the development of effective interventions such as drug treatment against the parasite that is responsible for the highest malaria-specific mortality rates globally. Six species of malaria parasites, *P. falciparum, P. knowlesi, P. vivax, P. ovale curtisi, P. ovale wallikeri,* and *P. malariae*, have been shown to infect humans.[2] Recently, the simian *P. cynomolgi*[3,4] was detected in humans. However, *P. falciparum* is the only species for which most life cycle stages have been successfully cultured[5] to enable research into the different aspects of malaria elimination such as drug screening. Efforts at cultivating *Plasmodium* parasites *in vitro* are complicated as each *Plasmodium* species has its own life cycle and set of physiological, metabolic, and nutritional requirements.[6,7]

As an alternative to the *in vitro* continuous culture, *ex vivo* culture techniques have been established to support major research activities, such as drug sensitivity testing. *Ex vivo* cultures are usually developed using fresh or cryopreserved field isolates of *Plasmodium* species. During culture adaptation the parasite undergo changes that may impair growth of the *ex vivo* cultured parasites.[6] Tireless attempts have however enabled development of an improved *ex vivo* cultivation methods for the second most prevalent and the most widespread species *P. vivax*.[8] Such *ex vivo* culture is currently providing useful data on *P. vivax*, such as sensitivity to antimalarial drugs,[8−10] parasite biology, and invasion mechanisms.[11]

While artemisinin-based combination therapy has helped to significantly decrease *P. falciparum* transmission, morbidity, and mortality, persistent transmission of *P. malariae* is widely reported.[12−14] *P. malariae* is highly prevalent in malaria endemic areas such as the Amazon[13] area and sub-Saharan Africa.[12,14,15] *P. malariae* is the second dominant species in sub-Saharan African and has been shown to be able to cause life threatening chronic infections and severe anemia, similar to the deadliest *P. falciparum*.[15,16] These have all been shown to have negative socio-economic impact in the affected countries.[15,16] However, no cultivation method has been

Received: May 13, 2021
Published: October 28, 2021

ACS Publications

© 2021 American Chemical Society

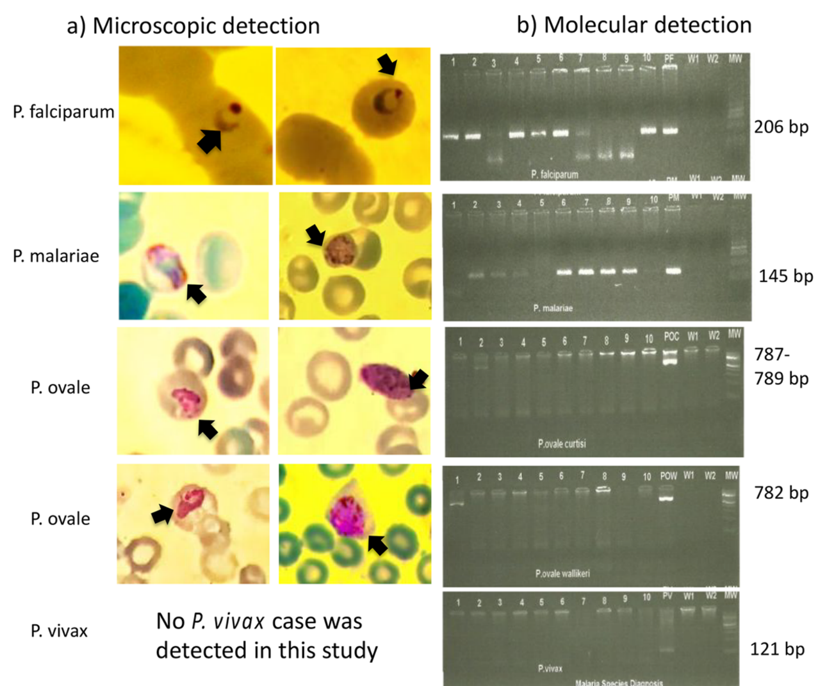


Figure 1. Microscopy and molecular detection of Human malaria species. (A) Giemsa-stained asexual blood stages of Human malaria parasites and (B) molecular detection of human malaria parasites species in test samples 1−10 along with control for *Pf* = *Plasmodium falciparum*, *Pm* = *Plasmodium malariae*, *Poc* = *Plasmodium ovale curtisi*, *Pow* = *Plasmodium ovale wallikeri*, *Pv* = *Plasmodium vivax*. W1 = Control 1, W2 = Control 2, bp = Base pairs, and MW = Molecular weight.

Table 1. Summary of the List of Samples and the Parasitic Species Present in Each Sample

samples/ species	1	2	3	4	5	6	7	8	9	10
P. falciparum (*Pf*)	+	+	−	+	+	+	+	−	−	+
P. malariae (*Pm*)	−	+	+	+	−	+	+	+	+	−
P. ovale curtisi (*Poc*)	−	+	−	−	−	−	−	−	−	−
P. ovale wallikeri (*Pow*)	+	−	−	−	−	−	−	−	−	−
P. vivax (*Pv*)	−	−	−	−	−	−	−	−	−	−
species per samples	*Pf* + *Pow*	*Pf* + *Pm* + *Poc*	*Pm*	*Pf* + *Pm*	*Pf*	*Pf* + *Pm*	*Pf* + *Pm*	*Pm*	*Pm*	*Pf*

developed to contribute to the development of new antimalarial drugs against *P. malariae* or providing insight into the parasites' biology, such as the mechanism of invasion and mechanism of drug resistance. In our current study, we defined conditions in which *P. malariae* has grown and displayed increased parasitemia at 72 h time point. Using this as a tool, we have screened known antimalarial compounds such as chloroquine, lumefantrine, artemether, and quinine for their activity against *P. malariae* fresh field clinical isolates. This, we believe, will facilitate the testing of new lead compounds as well as facilitate further development in the protocol to allow for the study of the parasite's biology.

■ RESULTS

Morphological and Molecular Detection of Plasmodium Malariae. The samples obtained from the patients were Giemsa stained for the identification of the different parasite species using light microscopy. The microscopic screen identified *P. falciparum*, *P. malariae*, and *P. ovale* (Figure 1a). Morphologically, only ring and early trophozoite stages of *P. falciparum* were observed in the thin smears (Figure 1a, top panel), while other late blood stages were detected for the other nonfalciparum species (Figure 1a). Morphologically, trophozoites of *P. malariae* and *P. ovale* were slightly bigger than those of *P. falciparum* (Figure 1a top and second panel). Multiple infected erythrocytes were absent for nonfalciparum species. Erythrocytes infected with *P. ovale* trophozoite or schizont stages were enlarged and exhibited Schüffner's dots (Figure 1a, third and fourth panel). The trophozoites of *P. malariae* were more compact than those of *P. ovale*. The host erythrocytes infected with *P. malariae* trophozoite or schizont were not enlarged (Figure 1a, second panel). An equatorial band characteristic of *P. malariae* trophozoite dividing the red blood cell was visible (Figure 1a, second panel). The samples were also screened for any possible *P. vivax* infection, but none were seen as expected based on its geographical distribution (Figure 1a). *P. knowlesi* was not monitored in the current study as the geographical distribution of this parasite makes unlikely its presence in Mali.

To further confirm the species observed in the samples, DNA was extracted from the samples. Species specific primers were used to amplify the different samples and genotype all different species (Figure 1b and Table 1). The primers for *P. falciparum* yielded a 206bp product as observed on the top panel relative to the positive control *Pf*. The additional bands observed in *P. falciparum* gel are result of primer dimerization appearing under 100bp of our reference molecular weight (MW) that first band appear at 100bp. *P. malariae* yielded a

https://doi.org/10.1021/acsinfecdis.1c00262
ACS Infect. Dis. 2021, 7, 3025−3033

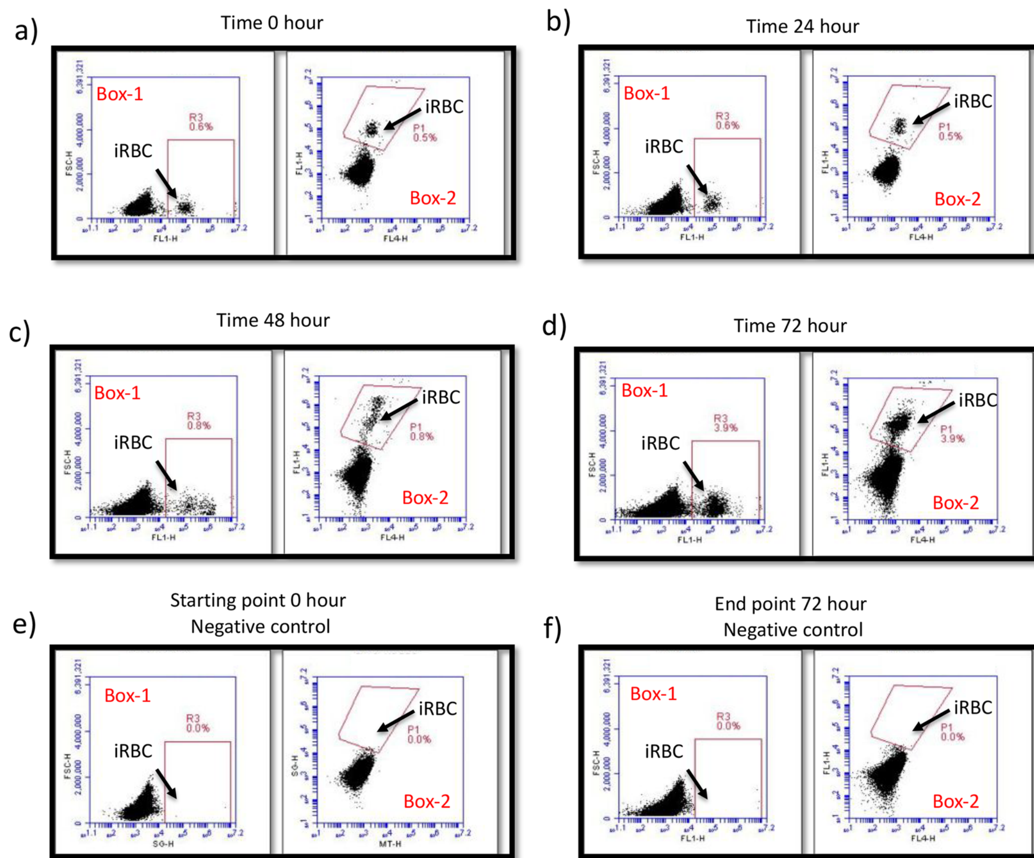


Figure 2. *P. malariae* maturation and growth monitoring in *ex vivo* culture using flow cytometry. (a) Time 0 h freshly collected *P. malariae* asexual stages, (b) time 24 h cultured *P. malariae* asexual blood stages, (c) time 48 h cultured *P. malariae* asexual blood stages, and (d) time 72 h cultured *P. malariae* asexual blood stages, (e) culture starting 0 h time point negative control of parasite free red blood cells, (f) culture time 72 h end point negative control of parasite free red blood cells. iRBC = infected red blood cells.

145 band (Figure 1b, second panel) compared to the positive control band *Pm*, *P. ovale curtisi* yielded 787−789 bp relative to *Poc*, while *P. ovale wallikeri* gave a product of size 782 bp relative to *Pow*. The genotyping for *P. vivax* yielded no product relative to the positive control at 121 bp. The PCR assay revealed that samples 5 and 10 were monoinfections of *P. falciparum* while samples 3, 8, and 9 were *P. malariae* monoinfections (Figure 1b and Table 1). Samples 1 and 2 were respectively mixed infections of *P. ovale wallikeri* with *P. falciparum* and *Plasmodium ovale curtisi* together with both *P. malariae* and *P. falciparum* (Figure 1b). *P. malariae* mixed infection with *P. falciparum* was detected in samples 4, 6, and 7 (Figure 1b and Table 1).

***P. malariae* Has Successfully Matured and Grown in an *Ex Vivo* Culture System.** We have used freshly collected PCR confirmed clean *P. malariae* field isolates cultured *in P. falciparum's* routine media lacking extra glucose for 72 h. When culture media were supplemented with 10% glucose, *P. malariae* parasites died in the culture within 24−48 h. With the growth media lacking the extra 10% glucose supplement, *P. malariae* viability, maturation, and growth was monitored daily using flow cytometry for 3 days as shown in Figure 2 and Figure 3c. Time zero hour (0 h) corresponds to the freshly collected field isolates displaying small nucleic acid content in FL1, box 1 and less mitochondrial activity signal in FL4, box 2 (Figure 2a) resulting from the very low parasitemia of this parasite. The clustering was typical of early asexual blood stage that usually circulate in the bloodstream (box 1 and box 2 Figure 2a). The DNA staining showed 0.6% positivity of the acquired cells (Box 1 Figure 2a) while the mitochondria staining (Box 2 Figure 2a) indicated that 0.5% of the 0.6% parasites were viable. Thus, only 0.1% of the parasites were not viable at the time 0 h of the culture. After 24 hours (24 h) in culture, no parasite maturation has been observed and viability remained the same as observed in time 0 h (Figure 2a,b). Interestingly following 48 and 72 h cultures, a clear maturation of the parasites was observed (Figure 2c,d boxes 1 and 2). Thus, *P. malariae* has successfully matured in *ex vivo* culture. A slight increase of parasitemia was also observed during the 48 h culture period (Figure 2c) but was higher (7.8-fold) in the 72 h (Figure 2d) when compared to the time 0 h parasitemia 0.5% (Figure 2a) and the parasite free controls (Figure 2e,f). In addition to flow cytometry approach, we assessed parasites maturation and growth using standard microscopy (Figure 3a). Ring stage was detected for the different parasite isolates at initiation of the assay at time 0 h, while *P. malariae* typical trophozoite stages were detected at the 24 h time point culturing. At the 48 h time point, late trophozoites and early schizonts were detected (Figure 3a) indicating maturation of *P. malariae ex vivo*. By the 72 h time point of maintaining the culture, new ring stages (>90%) and rare bursting schizonts were observed (Figure 3a). Microscopic assessment of *P. malariae* cultures showed no increase of the parasitemia from 0 to 48 h while high parasitemia was observed in 72 h cultures for five different isolates (Figure 3b). To finally confirm that *P. malariae* was successfully grown in *ex vivo* culture, 19 field

https://doi.org/10.1021/acsinfecdis.1c00262
ACS Infect. Dis. 2021, 7, 3025−3033

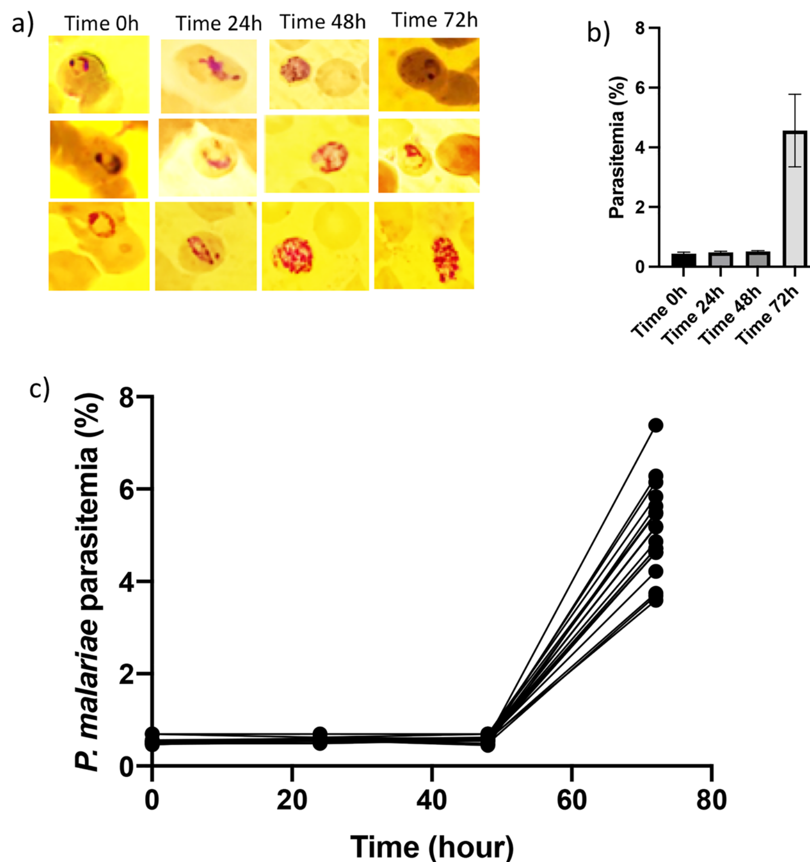


Figure 3. *Plasmodium malariae* maturation and growth. (a) Giemsa-stained *P. malariae* parasites at defined time points of the culture. (b) Five *P. malariae* isolates parasitemia at defined time points of the culture measured using light microscopy. (c) Nineteen field isolates of *P. malariae* comparative growth measured using flow cytometry. At time 0 h, 0.5% parasitemia of *P. malariae* asexual blood stages are cultured for 24, 48, and 72 h, and times respective parasitemia of viable parasites were evaluated using flow cytometry. Parasites were detected with Sybr Green while viability was measured using deep red mito-tracker.

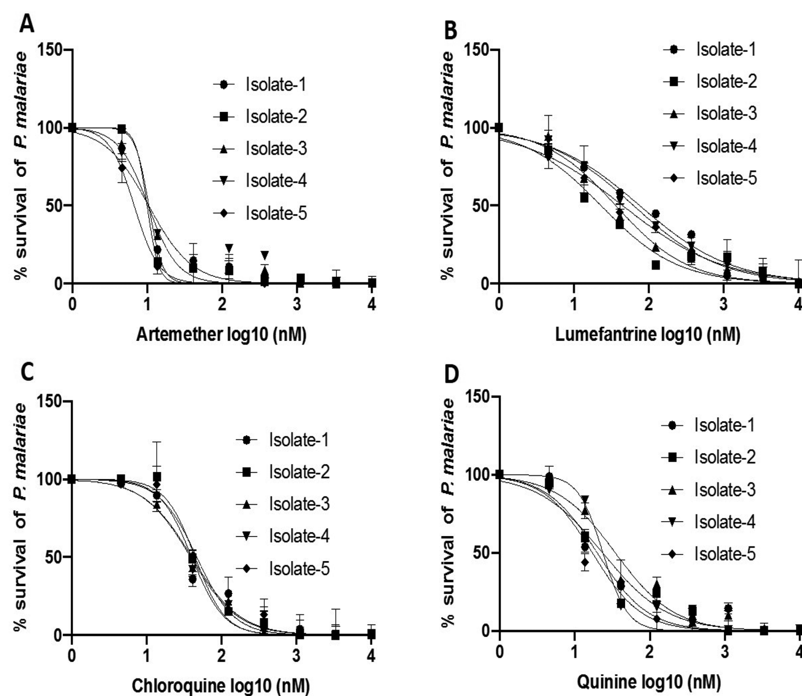


Figure 4. *P. malariae* sensitivity to references antimalarial drugs. (A) Dose response curves of artemether against *P. malariae* asexual blood stages. (B) Dose response curves of lumefantrine against *P. malariae* asexual blood stages. (C) Dose response curves of chloroquine against *P. malariae* asexual blood stages. (D) Dose response curves of quinine against *P. malariae* asexual blood stages.

https://doi.org/10.1021/acsinfecdis.1c00262
ACS Infect. Dis. 2021, 7, 3025−3033

Table 2. IC_{50} Values of Selected Antimalarial Drug Compounds against *P. malariae*[a]

	IC_{50} (nM) against *P. malariae*					
compounds	isolate 1	isolate 2	isolate 3	isolate 4	isolate 5	std deviation
artemether	3.51	9.971	5.31	10.46	6.71	2.98
lumefantrine	75.7	23.03	36.32	62.84	44.89	20.94
chloroquine	38.81	44.48	40.19	40.1	46.84	3.41
quinine	23.6	19.95	34.83	23.78	17.24	6.69

[a]These data are from five independent experiments done with five different field isolates of *P. malariae*. std deviation = standard deviation.

isolates were cultivated and parasitemia of viable parasites were monitored for the period using FACS for 3 days (Figure 3c). The starting parasitemia were estimated at 0.5% at time 0 h. The percentage parasitemia at 24 and 48 h following culture were similar to that of time 0 h, 0.5% (Figure 3b). Similarly, in Figure 2d and Figure 3b the 72 h cultures showed a significant increase of parasitemia for all 19 field isolates (Figure 3c). Thus, *P. malariae* has fully matured and grown in *ex vivo* culture.

***P. malariae Ex Vivo* Culture System Supported Screening of Antimalarial Drug.** When establishing the *ex vivo* culture conditions of *P. malariae* field isolates, we set to evaluate the potential for antimalarial screen. *P. malariae* fresh field isolates were cultured and incubated with serially diluted concentrations of Artemether, lumefantrine, chloroquine, and quinine for 72 h (Figure 4). Dose response curves of artemether, lumefantrine, chloroquine and quinine are displayed in Figure 4a−d, respectively. It was observed that the different isolates obtained from different patients showed differential susceptibility to artemether, lumefantrine, chloroquine, and quinine (Table 2). All antimalarials potently inhibited *P. malariae* within 72 h drug exposure Figure 4 and Table 2.

■ DISCUSSION

In this current study, we established suitable conditions for the *ex vivo* culture of *P. malariae* that have successfully supported screening of antimalarial drugs against this parasite (Figures 2, 3, and 4). We first combined previously reported PCR conditions[17] and light microscopy to accurately detect all plasmodium species. Morphologically, *Plasmodium* ring stages are similar and difficult to distinguish for all species while trophozoites and mature forms can be used to discriminate Plasmodium species. Trophozoites of *P. falciparum* are slightly smaller than those of other species. The presence of a few number of mature stages together with more trophozoite forms, as well as the absence of multiple infected erythrocytes, are highly suggestive of nonfalciparum species.[18]

After accurate detection of all *Plasmodium* species from field isolated samples, we set to identify *ex vivo* culture conditions for *P. malariae*. We used several modified *P. falciparum*'s culture conditions and only when standard culture conditions of *P. falciparum* lacking extra glucose supplement were used *P. malariae* was able to grow successfully. Interestingly, previous attempts to cultivate *P. malariae in vitro* that managed to observe reinvasion all have used culture media lacking extra glucose supplements.[19,20] *P. malariae* maturation and growth *ex vivo* were monitored using flow cytometry combining DNA and mitochondrial[21,22] staining to determine viable parasites from all parasites (dead + live), respectively, with deep red mito-tracker and Sybr Green. Giemsa-stained thin blood smears slides examination using microscope remains the gold standard approach used for the detection, quantification, speciation, and staging of *Plasmodium* blood stages.[23] However, this technique relies on the microscopists skills that should be well-trained to correctly assess parasitemia and also accurately identify the different parasite species and stages.[24] This is also a time-consuming and very tedious approach often marred with human errors. Several reports indicated high concordance between flow cytometry and microscopy in determining parasitemia and *Plasmodium* maturation.[25−30] Flow cytometry has the ability to resolve microscopic bias and challenges in assessing malaria parasites maturation and growth accurately and therefore make this technique the preferred approach.[28,30] Within 24 h cultivation of *P. malariae*, no evidence of maturation was observed (Figure 2a,b). After 48 h in culture, a clear maturation evidenced by the flow cytometry data was observed (Figure 2c) with a slight increase of parasitemia from 0.5% 24 h to 0.8% 48 h (Figure 2b,c). When blood is collected from *P. malariae* infected patients, some rare schizont stage can still be observed and this slight increase in parasitemia might be due to those rare schizont bursts. In agreement with the 72 h asexual life cycle of *P. malariae*,[18] substantial increase of parasitemia was observed after time 72 h in culture (Figure 2d and Figure 3b,c). Reports have indicated that *P. malariae* may produce from 6 to 14 merozoites per schizont with an average number of 8[18] *ex vivo*. Interestingly, 7.8-fold increase in *P. malariae* parasitemia in our study indicated that the merozoites average was 8 per schizont (Figure 2d and Figure 3b,c) *ex vivo*, which indicates that our observed growth rate was within the expected range. Siddiqui et al. observed that only at the end of 72 h the majority of *P. malariae* parasites (62 to 64%) matured to segments and a new generation of rings (8−10%) was formed.[20] Like in our current study, *P. malariae* entire asexual cycle was shown to be 72 h.[19,20] Siddiqui et al. cultured *P. malariae* in *Aotus trivirgatu* blood while *Lingnau et al.* cultured *P. malariae* in human blood group B Rh-positive. Both authors have used RPMI media containing low glucose and assessed parasite using microscopy only. None of these methods have been tested to support antimalarial evaluation and discovery. The current article is developed as a tool for drug discovery using human blood samples assessing parasite culture by combining microscopy as in a previous report[31] with SYBR GREEN and Mitotracker readouts; both were not used in the previous reports. Thus, viable parasites can be assessed accurately in our current article using the unbiased flow cytometry approach and the mitotracker unlike the previous report. The current article has shown that drug IC_{50} can be predicted using the method presented herein.

New antimalarial drugs are mainly identified and developed after extensive testing against *P. falciparum,* which is the most important species that can be continuously grown *in vitro*. Recent studies provided some drug susceptibility profile for *P. vivax*[8,32] and *P. knowlesi*.[33] The parasite drug susceptibility

studies are very important to inform lead optimization toward discovery of drug that can inhibit all *Plasmodium* species. Drug sensitivity profiles against *P. malariae* have been unavailable as this species had not been adapted to *ex vivo* and/or *in vitro* culture supporting drug screening. Having established *ex vivo* culture conditions of *P. malariae*, we set to evaluate the culture method potential for antimalarial screening against *P. malariae*. We show that our method allowed blood stage drug screens of the currently used antimalarials artemether, lumefantrine, chloroquine and quinine (Figure 4). The drug IC_{50} values measured for tested antimalarial against *P. malariae* were comparable[34] to that reported earlier against *P. falciparum*.[35,36] However, there are reports of *P. malariae* treatment failures using current *P. falciparum* treatments.[12,37−40] In our recent work,[31] many *P. malariae* field isolates also displayed a decreased susceptibility to currently used antimalarial artemether and lumefantrine as well as to chloroquine that is no longer used in Mali because of *P. falciparum* resistance. These differences could be due to factors such as host related pharmacokinetic and pharmacodynamic effects which are absent *ex vivo* or the extended 72 h intraerythrocytic stage life cycle of *P. malariae* that may lead to a treatment designed to treat *P. falciparum* being nonoptimal for *P. malariae*.[31] A further rational that could explain *P. malariae* decreased susceptibility to currently used antimalarial would be true drug resistance occurring in this parasite in which molecular markers remain to be well determined and characterized. Thus, this report would further enable one to conduct comparative drug susceptibility between *P. malariae* and *P. falciparum* in other malaria endemic countries as recently reported in Mali.[31] Large data from such comparative drug susceptibility study across malaria species could help various malaria control programs to target eliminate any Plasmodium specie.

CONCLUSION

In this study, *P. malariae* was successfully grown in *P. falciparum*'s culture conditions lacking extra 10% glucose supplement. This culture method allowed screening of antimalarial drugs against *P. malariae* clinical field isolates. This *ex vivo* culturing method of *P. malariae* offers a wealth of opportunities to study the parasite biology as well as screening for active novel antimalarial against *P. malariae*. Finally, flow cytometry may be a better approach than light microscopy in assessing *P. malariae* maturation.

METHODS

Ethical Considerations. The current study protocol was reviewed and approved by the ethical committee of the Faculties of Medicine-Odonto-Stomatology and Pharmacy, University of Science, Techniques and Technologies of Bamako, Mali with the reference N°2O17/141/CE/FMPOS and N°2O19/168/CE/FMPOS/FAPH. Only participants or their parent/guardian who provided written informed consent, plus children able to understand the study and who gave assent were enrolled in this study. All patients with malaria that consented to participate in the study were enrolled and treated using recommended AL therapy or quinine in case of AL failure to clear the parasite.

Study Design, Site, and Population Screen. We conducted a cross-sectional screening and detection of all *Plasmodium* malaria cases during a longitudinal prospective study aimed at assessing *ex vivo* efficacy of panel of antimalarials against *P. malariae*. Thus, only freshly collected field isolates of *P. malariae* monoinfection parasites were used in the current study drug assay while PCR assay was used to detect *P. malariae* monoinfection samples from other *Plasmodium* species infection samples.

For PCR assay, DNA was extracted from dried blood spots using the QIAamp DNA Mini Kit (QIAgen, Valencia, CA, U.S.A.) following the manufacturer's instruction. Using the small subunit rRNA 18s gene[41] and the most advanced and reliable PCR techniques for the diagnosis of human malaria,[17] we performed two nested PCR assays. The first for the diagnosis of the genus *Plasmodium* using the following genus-specific set of primers.[17] Set 1 was rPLU1 (TCAAAGATTAAGCCATGCAAGTGA) and rPLU5 (CCTGTTGTTGCCTTAAACTTC). Set 2 was rPLU3 (TTTTTATAAGGATAACTAC GGAAAAGCTGT) and rPLU4 (TACCCGTCATAGCCATGTTAGGCCAATACC). The second nested PCR was to determine specific *Plasmodium species* using the following set of primers: Set 1 as above and Set 3 was rFAL1 (TTAAACTG GTTTGGGAAAACCAAATATATT) and rFAL2 (ACACAATGAACTCAATCATGAC TACCCGTC) for *P. falciparum* (206 bp); rVIV1 (CGCTTCTAGCTTAATCCACATA ACTGATAC) and rVIV2 (ACTTCCAAGCCGAAGCAAAGAAAGTCCTTA) for *P. vivax* (121 bp); rMAL1 (ATAACATAGTTGTACGTTAAGAATAACCGC) and rMAL2 (AAAATTCCCATGCATAAAAAATTATACAAA) for *P. malariae* (145 bp); rOVA1 (ATCTCTTTTGCTATTTTTTAGTATTGGAGA) and rOVA2 (ATCTAAGAATTTCACC TCTGACATCTG) for *P. ovale curtisi* (787−789bp); and rOVA1v (ATCTCCTTTACTTTTTGTACTGGAGA) and rOVA2v (GGAAAAGGACACTATAATGTATCCTAATA) for *P. ovale wallikeri* (782 bp).

Compounds tested included chloroquine, lumefantrine, artemether, and quinine all from Sigma (Sigma-Aldrich, France). This study was conducted from September 2018 to October 2019 in Faladje, a rural village located in Koulikoro region at 80 km from Bamako. Malaria is hyperendemic and highly seasonal in Faladje. Children older than 1 year and nonpregnant adults with uncomplicated or asymptomatic malaria were enrolled if written informed consent was obtained from patients, parents, or guardians of children. Thick and thin smears were Giemsa-stained and immediately read for initial parasitemia screening, quantification, and for parasite speciation to identify *P. malariae* carriage. Patients with severe malaria, chronic HIV, or tuberculosis infection or who had taken antimalarial drugs during the past 14 days were excluded.

Samples Blood Collection and Processes. For *ex vivo* drug assay and parasite culture, we were using 3−6 h freshly collected *P. malariae* samples in this study. Three to 10 mL venous blood were collected into acid-citrate-dextrose vacutainers (Becton-Dickinson, Franklin Lakes, NJ, U.S.A.) from *P. malariae* carriers before any antimalarial medication. Blood samples were transported on ice packs between 2 and 4 °C from the field site to the laboratory. Thick and thin smears were prepared and stained with 10% Giemsa for double parasite quantification and speciation. Dried blood spots were made for plasmodial DNA extraction and amplification using polymerase chain reaction (PCR) assay to confirm monoinfection of *P. malariae* as previously described[17] while blood samples were centrifuged to save plasma for further assessment. Red blood cell pellets were washed three times with incomplete RPMI-1640 (10.43 g of RPMI-1640, 5.96 g of

HEPES, 2.5 g of $NaHCO_3$, 2.5 mL of gentamicin 50 mg/mL for 1 L in H_2O). Erythrocyte pellets were split into two by using one half for cryopreservation in glycerolyte and immediately stored at −80 °C. The other half was transferred into complete RPMI medium for *ex vivo* culture and drug assay. Complete RPMI medium contained 10.43 g of RPMI-1640, 5.96 g of HEPES, 2.5 g of $NaHCO_3$, 1 mL of hypoxanthine, 5 g of Albumax, 2.5 mL of gentamicin 50 mg/mL in 1L of H_2O lacking extra glucose supplement. The hematocrit of the red blood cell pellets in complete RPMI-1640 was 2% and the parasitemia 0.5%. Monitoring of the *ex vivo* cultures of *P. malariae* parasites was done timely as shown in Figure 1 from time 0 h to time 72 h. The *ex vivo* sensitivity of *P. malariae* parasites to chloroquine, lumefantrine, artemether, and quinine was assessed using 72 h combined Sybr Green and Mitotracker assay as previously reported[21,22,31] in plates of 96 wells. Nineteen *P. malariae* isolates with ring stage >80% were used for the *ex vivo* cultivation, while 5 isolates with ring stage >90% were used for the *ex vivo* drug assay. Only PCR confirmed monoinfection cases of *P. malariae* were used to validate isolates used in this study. All compounds were prepared in 100% DMSO and diluted in complete RPMI-1640 with drug concentration ranging from 10 to 0.004 μM (8 points of 3-fold serial dilutions). Drug assay plates of freshly collected *P. malariae* contained compounds tested; 0.02% dimethyl sulfoxide (0.02% DMSO) and uninfected erythrocytes were incubated in a final volume of 120 μL and cultured at 37 °C in a gas atmosphere of 5% CO2 for 72 h. After 72 h, drug assay plates were washed (HBSS 1X + FBS 2%) and double stained with Sybr Green and deep red mitotracker. Plates stained were read on Accuri flow cytometer with 200 000 events. Sybr Green and deep red mitotracker were respectively used to measure acid nuclei content in FL1, boxes 1−2, and mitochondrial activity in FL4 box 2 (Figure 1 and 2). Parasite maturation and growth are both determined by the acid nuclei content while viability is measured though mitochondrial activity.

Dose−response curves and inhibitory concentrations 50% (IC50) were calculated by nonlinear regression analysis using GraphPad Prism software version 8 with the data previously normalized to the untreated controls. Statistical test was done using GraphPad Prism software version 8 and *t* test. A *p* value <0.05 was considered as significant.

AUTHOR INFORMATION

Corresponding Authors

Laurent Dembele − *Faculté de Pharmacie, Malaria Research and Training Center (MRTC), Université des Sciences des Techniques et des Technologies de Bamako, 1805 Bamako, Mali*; orcid.org/0000-0001-9087-8439; Email: laurent@icermali.org

Abdoulaye A. Djimde − *Faculté de Pharmacie, Malaria Research and Training Center (MRTC), Université des Sciences des Techniques et des Technologies de Bamako, 1805 Bamako, Mali*; Email: adjimde@icermali.org

Authors

Nouhoum Diallo − *Faculté de Pharmacie, Malaria Research and Training Center (MRTC), Université des Sciences des Techniques et des Technologies de Bamako, 1805 Bamako, Mali*

Fanta Sogore − *Faculté de Pharmacie, Malaria Research and Training Center (MRTC), Université des Sciences des Techniques et des Technologies de Bamako, 1805 Bamako, Mali*

Bintou Diarra − *Faculté de Pharmacie, Malaria Research and Training Center (MRTC), Université des Sciences des Techniques et des Technologies de Bamako, 1805 Bamako, Mali*

Fatoumata I. Ballo − *Faculté de Pharmacie, Malaria Research and Training Center (MRTC), Université des Sciences des Techniques et des Technologies de Bamako, 1805 Bamako, Mali*

Amadou Daou − *Faculté de Pharmacie, Malaria Research and Training Center (MRTC), Université des Sciences des Techniques et des Technologies de Bamako, 1805 Bamako, Mali*

Ousmaila Diakite − *Faculté de Pharmacie, Malaria Research and Training Center (MRTC), Université des Sciences des Techniques et des Technologies de Bamako, 1805 Bamako, Mali*

Yacouba Bare − *Faculté de Pharmacie, Malaria Research and Training Center (MRTC), Université des Sciences des Techniques et des Technologies de Bamako, 1805 Bamako, Mali*

Cheick Papa Oumar Sangare − *Faculté de Pharmacie, Malaria Research and Training Center (MRTC), Université des Sciences des Techniques et des Technologies de Bamako, 1805 Bamako, Mali*

Aboubecrin Sedhigh Haidara − *Faculté de Pharmacie, Malaria Research and Training Center (MRTC), Université des Sciences des Techniques et des Technologies de Bamako, 1805 Bamako, Mali*

Seidina A. S. Diakite − *Faculté de Pharmacie, Malaria Research and Training Center (MRTC), Université des Sciences des Techniques et des Technologies de Bamako, 1805 Bamako, Mali*

Amadou Niangaly − *Faculté de Pharmacie, Malaria Research and Training Center (MRTC), Université des Sciences des Techniques et des Technologies de Bamako, 1805 Bamako, Mali*

Mahamadou Diakite − *Faculté de Pharmacie, Malaria Research and Training Center (MRTC), Université des Sciences des Techniques et des Technologies de Bamako, 1805 Bamako, Mali*

Brice Campo − *Medicines for Malaria Venture (MMV), CH-1215 Geneva 15, Switzerland*

Gordon A. Awandare − *West African Centre for Cell Biology of Infectious Pathogens (WACCBIP), Department of Biochemistry, Cell and Molecular Biology, College of Basic and Applied Sciences, University of Ghana, LG 54 Accra, Ghana*

Yaw Aniweh − *West African Centre for Cell Biology of Infectious Pathogens (WACCBIP), Department of Biochemistry, Cell and Molecular Biology, College of Basic and Applied Sciences, University of Ghana, LG 54 Accra, Ghana*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsinfecdis.1c00262

Funding

This project is part of the European and Developing Countries Clinical Trials Partnership (EDCTP2) program supported by the European Union (Grant TMA2017CDF-1892-HypnoBio) and the Medicines for Malaria Venture (RD-18−0067).

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by a clinical research grant from European and Developing Countries Clinical Trials Partnership (EDCTP) (TMA2017CDF-1892-HypnoBio) and Medicines for Malaria Venture (RD-18-0067). L.D., Y.A., N.D., F.S., B.D., F.I.B., A.D., O.D., Y.B., C.P.O.S., A.S.H., A.N., and S.D. codesigned and performed the experiments. B.C. critically supported the assay conduction with important input in data analysis and manuscript preparation. L.D. and Y.A. analyzed the data. L.D., A.D., Y.A., and G.A.A., codesigned the project and wrote the manuscript. M.D. provided access to some important lab facilities. All authors critically revised the manuscript.

■ REFERENCES

(1) Trager, W.; Jensen, J. B. Human Malaria Parasites in Continuous Culture. *Science (Washington, DC, U. S.)* **1976**, *193* (4254), 673−675.
(2) Calderaro, A.; Piccolo, G.; Gorrini, C.; Rossi, S.; Montecchini, S.; Dell'Anna, M. L.; De Conto, F.; Medici, M. C.; Chezzi, C.; Arcangeletti, M. C. Accurate Identification of the Six Human Plasmodium Spp. Causing Imported Malaria, Including Plasmodium Ovale Wallikeri and Plasmodium Knowlesi. *Malar. J.* **2013**, *12*, 321.
(3) Grignard, L.; Shah, S.; Chua, T. H.; William, T.; Drakeley, C. J.; Fornace, K. M. Natural Human Infections With Plasmodium Cynomolgi and Other Malaria Species in an Elimination Setting in Sabah, Malaysia. *J. Infect. Dis.* **2019**, *220* (12), 1946−1949.
(4) Imwong, M.; Madmanee, W.; Suwannasin, K.; Kunasol, C.; Peto, T. J.; Tripura, R.; Von Seidlein, L.; Nguon, C.; Davoeung, C.; Day, N. P. J.; Dondorp, A. M.; White, N. J. Asymptomatic Natural Human Infections with the Simian Malaria Parasites Plasmodium Cynomolgi and Plasmodium Knowlesi. *J. Infect. Dis.* **2019**, *219* (5), 695−702.
(5) Hollingdale, M. R. Is Culture of the Entire Plasmodium Cycle, in Vitro, Now a Reality? *Parasitol. Today* **1992**, *8*, 223.
(6) Schuster, F. L. Cultivation of Plasmodium Spp. *Clin. Microbiol. Rev.* **2002**, *15* (3), 355−364.
(7) Grande, R.; Antinori, S.; Meroni, L.; Menegon, M.; Severini, C. A Case of Plasmodium Malariae Recurrence: Recrudescence or Reinfection? *Malar. J.* **2019**, *18*, 169.
(8) Russell, B.; Suwanarusk, R.; Malleret, B.; Costa, F. T. M.; Snounou, G.; Kevin Baird, J.; Nosten, F.; Rénia, L. Human Ex Vivo Studies on Asexual Plasmodium Vivax: The Best Way Forward. *Int. J. Parasitol.* **2012**, *42*, 1063−1070.
(9) Aguiar, A. C. C.; Pereira, D. B.; Amaral, N. S.; De Marco, L.; Krettli, A. U. Plasmodium Vivax and Plasmodium Falciparum Ex Vivo Susceptibility to Anti-Malarials and Gene Characterization in Rondônia, West Amazon, Brazil. *Malar. J.* **2014**, *13*, 73.
(10) Chaorattanakawee, S.; Lon, C.; Chann, S.; Thay, K. H.; Kong, N.; You, Y.; Sundrakes, S.; Thamnurak, C.; Chattrakarn, S.; Praditpol, C.; Yingyuen, K.; Wojnarski, M.; Huy, R.; Spring, M. D.; Walsh, D. S.; Patel, J. C.; Lin, J.; Juliano, J. J.; Lanteri, C. A.; Saunders, D. L. Measuring Ex Vivo Drug Susceptibility in Plasmodium Vivax Isolates from Cambodia. *Malar. J.* **2017**, *16* (1), 392.
(11) Rangel, G. W.; Clark, M. A.; Kanjee, U.; Lim, C.; Shaw-Saliba, K.; Menezes, M. J.; Mascarenhas, A.; Chery, L.; Gomes, E.; Rathod, P. K.; Ferreira, M. U.; Duraisingh, M. T. Enhanced Ex Vivo Plasmodium Vivax Intraerythrocytic Enrichment and Maturation for Rapid and Sensitive Parasite Growth Assays. *Antimicrob. Agents Chemother.* **2018**, *62* (4), e02519−17.
(12) Betson, M.; Clifford, S.; Stanton, M.; Kabatereine, N. B.; Stothard, J. R. Emergence of Nonfalciparum Plasmodium Infection Despite Regular Artemisinin Combination Therapy in an 18-Month Longitudinal Study of Ugandan Children and Their Mothers. *J. Infect. Dis.* **2018**, *217* (7), 1099−1109.
(13) Camargo-Ayala, P. A.; Cubides, J. R.; Niño, C. H.; Camargo, M.; Rodríguez-Celis, C. A.; Quiñones, T.; Sánchez-Suárez, L.; Patarroyo, M. E.; Patarroyo, M. A. High Plasmodium Malariae Prevalence in an Endemic Area of the Colombian Amazon Region. *PLoS One* **2016**, *11* (7), e0159968.
(14) Yman, V.; Wandell, G.; Mutemi, D. D.; Miglar, A.; Asghar, M.; Hammar, U.; Karlsson, M.; Lind, I.; Nordfjell, C.; Rooth, I.; Ngasala, B.; Homann, M. V.; Färnert, A. Persistent Transmission of Plasmodium Malariae and Plasmodium Ovale Species in an Area of Declining Plasmodium Falciparum Transmission in Eastern Tanzania. *PLoS Neglected Trop. Dis.* **2019**, *13* (5), e0007414.
(15) Roucher, C.; Rogier, C.; Sokhna, C.; Tall, A.; Trape, J. F. A 20-Year Longitudinal Study of Plasmodium Ovale and Plasmodium Malariae Prevalence and Morbidity in a West African Population. *PLoS One* **2014**, *9* (2), e87169.
(16) Langford, S.; Douglas, N. M.; Lampah, D. A.; Simpson, J. A.; Kenangalem, E.; Sugiarto, P.; Anstey, N. M.; Poespoprodjo, J. R.; Price, R. N. Plasmodium Malariae Infection Associated with a High Burden of Anemia: A Hospital-Based Surveillance Study. *PLoS Neglected Trop. Dis.* **2015**, *9* (12), e0004195.
(17) Fuehrer, H.-P.; Noedl, H.; Doern, G. V. Recent Advances in Detection of Plasmodium Ovale : Implications of Separation into the Two Species Plasmodium Ovale Wallikeri and Plasmodium Ovale Curtisi. *J. Clin. Microbiol.* **2014**, *52* (2), 387−391.
(18) Collins, W. E.; Jeffery, G. M. Plasmodium Malariae: Parasite and Disease. *Clin. Microbiol. Rev.* **2007**, *20* (4), 579−92.
(19) Lingnau, A.; Doehring-Schwerdtfeger, E.; Maier, W. A. Evidence for 6-Day Cultivation of Human Plasmodium Malariae. *Z. Parasitenkd.* **1994**, *80* (3), 265−266.
(20) Siddiqui, W. A.; Schnell, J. V.; Geiman, Q. M. In Vitro Cultivation of Plasmodium Malariae. *J. Parasitol.* **1972**, *58* (4), 804.
(21) Dembele, L.; Ang, X.; Chavchich, M.; Bonamy, G. M. C.; Selva, J. J.; Lim, M. Y.-X.; Bodenreider, C.; Yeung, B. K. S.; Nosten, F.; Russell, B. M.; Edstein, M. D.; Straimer, J.; Fidock, D. A.; Diagana, T. T.; Bifani, P. The Plasmodium PI(4)K Inhibitor KDU691 Selectively Inhibits Dihydroartemisinin-Pretreated Plasmodium Falciparum Ring-Stage Parasites. *Sci. Rep.* **2017**, *7*, 2325.
(22) Dembele, L.; Gupta, D. K.; Lim, M. Y.-X.; Ang, X.; Selva, J. J.; Chotivanich, K.; Nguon, C.; Dondorp, A. M.; Bonamy, G. M. C.; Diagana, T. T.; Bifani, P. IImidazolopiperazines Kill Both Rings and Dormant Rings in Wild-Type and K13 Artemisinin-Resistant Plasmodium Falciparum In Vitro. *Antimicrob. Agents Chemother.* **2018**, *62* (5), e02235−17.
(23) Giemsa, G. Eine Vereinfachung Und Vervollkommung Meiner Methylenazur-Methylenblau-Eosin-Fa¨rbemethode Zur Erzielung Der Romanowsky-Nacht'schen Chromatin-Fa¨ Rbung. *Cent. fur Bakteriol. I Abteilung* **1904**, *37*, 307−313.
(24) Maguire, J. D; Lederman, E. R; Barcus, M. J; O'Meara, W. A P.; Jordon, R. G; Duong, S.; Muth, S.; Sismadi, P.; Bangs, M. J; Prescott, W R.; Baird, J K.; Wongsrichanalai, C. Production and Validation of Durable, High Quality Standardized Malaria Microscopy Slides for Teaching, Testing and Quality Assurance during an Era of Declining Diagnostic Proficiency. *Malar. J.* **2006**, *5*, 92.
(25) Li, Q.; Gerena, L.; Xie, L.; Zhang, J.; Kyle, D.; Milhous, W. Development and Validation of Flow Cytometric Measurement for Parasitemia in Cultures of P. Falciparum Vitally Stained with YOYO-1. *Cytometry, Part A* **2007**, *71A* (5), 297−307.
(26) Grimberg, B. T.; Erickson, J. J.; Sramkoski, R. M.; Jacobberger, J. W.; Zimmerman, P. A. Monitoring Plasmodium Falciparum Growth and Development by UV Flow Cytometry Using an Optimized Hoechst-Thiazole Orange Staining Strategy. *Cytometry, Part A* **2008**, *73A* (6), 546−554.
(27) Bei, A. K.; DeSimone, T. M.; Badiane, A. S.; Ahouidi, A. D.; Dieye, T.; Ndiaye, D.; Sarr, O.; Ndir, O.; Mboup, S.; Duraisingh, M. T. A Flow Cytometry-Based Assay for Measuring Invasion of Red Blood Cells by Plasmodium Falciparum. *Am. J. Hematol.* **2010**, *85* (4), 234−7.
(28) Malleret, B.; Claser, C.; Ong, A. S. M.; Suwanarusk, R.; Sriprawat, K.; Howland, S. W.; Russell, B.; Nosten, F.; Rénia, L. A

Rapid and Robust Tri-Color Flow Cytometry Assay for Monitoring Malaria Parasite Development. *Sci. Rep.* **2011**, *1*, 118.

(29) Lelliott, P. M.; Lampkin, S.; McMorran, B. J.; Foote, S. J.; Burgio, G. A Flow Cytometric Assay to Quantify Invasion of Red Blood Cells by Rodent Plasmodium Parasites in Vivo. *Malar. J.* **2014**, *13*, 100.

(30) Tiendrebeogo, R. W; Adu, B.; Singh, S. K; Dodoo, D.; Dziegiel, M. H; Mordmuller, B.; Nebie, I.; Sirima, S. B; Christiansen, M.; Theisen, M. High-Throughput Tri-Colour Flow Cytometry Technique to Assess Plasmodium Falciparum Parasitaemia in Bioassays. *Malar. J.* **2014**, *13*, 412.

(31) Dembele, L.; Aniweh, Y.; Diallo, N.; Sogore, F.; Sangare, C. P. O.; Haidara, A. S.; Traore, A.; Diakité, S. A. S.; Diakite, M.; Campo, B.; Awandare, G. A.; Djimde, A. A. Plasmodium Malariae and Plasmodium Falciparum Comparative Susceptibility to Antimalarial Drugs in Mali. *J. Antimicrob. Chemother.* **2021**, *76* (8), 2079−2087.

(32) Lu, F.; Gao, Q.; Chotivanich, K.; Xia, H.; Cao, J.; Udomsangpetch, R.; Cui, L.; Sattabongkot, J. In Vitro Anti-Malarial Drug Susceptibility of Temperate Plasmodium Vivax from Central China. *Am. J. Trop. Med. Hyg.* **2011**, *85* (2), 197−201.

(33) van Schalkwyk, D. A.; Blasco, B.; Davina Nuñez, R.; Liew, J. W. K.; Amir, A.; Lau, Y. L.; Leroy, D.; Moon, R. W.; Sutherland, C. J. Plasmodium Knowlesi Exhibits Distinct in Vitro Drug Susceptibility Profiles from Those of Plasmodium Falciparum. *Int. J. Parasitol.: Drugs Drug Resist.* **2019**, *9*, 93−99.

(34) Cheruiyot, A. C.; Auschwitz, J. M.; Lee, P. J.; Yeda, R. A.; Okello, C. O.; Leed, S. E.; Talwar, M.; Murthy, T.; Gaona, H. W.; Hickman, M. R.; Akala, H. M.; Kamau, E.; Johnson, J. D. Assessment of the Worldwide Antimalarial Resistance Network Standardized Procedure for in Vitro Malaria Drug Sensitivity Testing Using SYBR Green Assay for Field Samples with Various Initial Parasitemia Levels. *Antimicrob. Agents Chemother.* **2016**, *60* (4), 2417−2424.

(35) Wong, R. P. M.; Lautu, D.; Tavul, L.; Hackett, S. L.; Siba, P.; Karunajeewa, H. A.; Ilett, K. F.; Mueller, I.; Davis, T. M. E. In Vitro Sensitivity of Plasmodium Falciparum to Conventional and Novel Antimalarial Drugs in Papua New Guinea. *Trop. Med. Int. Health* **2010**, *15* (3), 342−349.

(36) Tinto, H.; Bonkian, L. N.; Nana, L. A.; Yerbanga, I.; Lingani, M.; Kazienga, A.; Valéa, I.; Sorgho, H.; Kpoda, H.; Guiguemdé, T. R.; Ouédraogo, J. B.; Mens, P. F.; Schallig, H.; D'Alessandro, U. Ex Vivo Anti-Malarial Drugs Sensitivity Profile of Plasmodium Falciparum Field Isolates from Burkina Faso Five Years after the National Policy Change. *Malar. J.* **2014**, *13*, 1−7.

(37) Kugasia, I. R.; Polara, F. K.; Assallum, H. Recrudescence of Plasmodium Malariae after Quinine. *Case Rep. Med.* **2014**, 1.

(38) Visser, B. J.; Wieten, R. W.; Kroon, D.; Nagel, I. M.; Bélard, S.; Van Vugt, M.; Grobusch, M. P. Efficacy and Safety of Artemisinin Combination Therapy (ACT) for Non-Falciparum Malaria: A Systematic Review. *Malar. J.* **2014**, *13*, 463.

(39) Rutledge, G. G.; Marr, I.; Huang, G. K. L.; Auburn, S.; Marfurt, J.; Sanders, M.; White, N. J.; Berriman, M.; Newbold, C. I.; Anstey, N. M.; Otto, T. D.; Price, R. N. Genomic Characterization of Recrudescent Plasmodium Malariae after Treatment with Artemether/Lumefantrine. *Emerging Infect. Dis.* **2017**, *23* (8), 1300−1307.

(40) Sagara, I.; Beavogui, A. H.; Zongo, I.; Soulama, I.; Borghini-Fuhrer, I.; Fofana, B.; Traore, A.; Diallo, N.; Diakite, H.; Togo, A. H.; Koumare, S.; Keita, M.; Camara, D.; Somé, A. F.; Coulibaly, A. S.; Traore, O. B.; Dama, S.; Goita, S.; Djimde, M.; Bamadio, A.; Dara, N.; Maiga, H.; Sidibe, B.; Dao, F.; Coulibaly, M.; Alhousseini, M. L.; Niangaly, H.; Sangare, B.; Diarra, M.; Coumare, S.; Kabore, M. J. T.; Ouattara, S. M.; Barry, A.; Kargougou, D.; Diarra, A.; Henry, N.; Soré, H.; Bougouma, E. C.; Thera, I.; Compaore, Y. D.; Sutherland, C. J.; Sylla, M. M.; Nikiema, F.; Diallo, M. S.; Dicko, A.; Picot, S.; Borrmann, S.; Duparc, S.; Miller, R. M.; Doumbo, O. K.; Shin, J.; Gil, J. P.; Björkman, A.; Ouedraogo, J. B.; Sirima, S. B.; Djimde, A. A. Pyronaridine−Artesunate or Dihydroartemisinin−Piperaquine versus Current First-Line Therapies for Repeated Treatment of Uncomplicated Malaria: A Randomised, Multicentre, Open-Label, Longitudinal, Controlled, Phase 3b/4 Trial. *Lancet* **2018**, *391* (10128), 1378−1390.

(41) Snounou, G.; Viriyakosol, S.; Jarra, W.; Thaithong, S.; Brown, K. N. Identification of the Four Human Malaria Parasite Species in Field Samples by the Polymerase Chain Reaction and Detection of a High Prevalence of Mixed Infections. *Mol. Biochem. Parasitol.* **1993**, *58* (2), 283−292.

https://doi.org/10.1021/acsinfecdis.1c00262
ACS Infect. Dis. 2021, 7, 3025−3033