Multiple Phenotypic and Genotypic Artemisinin Sensitivity Evaluation of Malian *Plasmodium falciparum* Isolates

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Abstract. We assessed the ex vivo/in vitro sensitivity of 54 Malian *Plasmodium falciparum* isolates to artemisinin for the monitoring of drug resistance in this area. The artemisinin sensitivity of parasites was evaluated using 1) the ex vivo and in vitro parasite recrudescence detection after treatment of the ring stage with 1–200 nM artemisinin for 48 hours and 2) the in vitro parasite recrudescence kinetics assay over 7 days after 6-hour treatment of the ring stage with 700 nM dihydroartemisinin (DHA). In addition, as recommended by the World Health Organization for artemisinin resistance characterization, the ring-stage survival assay (RSA^{0–3h}) was performed and the parasite isolates were sequenced at the kelch 13 propeller locus. No clinical and molecular evidence of artemisinin resistance was observed. However, these isolates present different phenotypic profiles in response to artemisinin treatments. Despite all RSA^{0–3h} values less than 1.5%, six out of 46 (13.0%) isolates tested ex vivo and four out of six (66.7%) isolates tested in vitro were able to multiply after 48-hour treatments with 100 nM artemisinin. Moreover, five out of eight isolates tested showed faster parasite recovery after DHA treatment in kinetic assays. The presence of such phenotypes needs to be taken into account in the assessment of the efficacy of artemisinins in Mali. The assays presented here appear as valuable tools for the monitoring of artemisinin sensitivity in the field and thus could help to evaluate the risk of emergence of artemisinin resistance in Africa.

INTRODUCTION

The current strategies of malaria control have showed a significant decrease in malaria burden worldwide.¹ Despite this major progress, the estimations of malaria unacceptably remain high since the World Health Organization (WHO) reported 212 million cases of malaria with 429,000 deaths in 2015, of which 92% occurred in sub-Saharan Africa alone.² In addition, the spread of artemisinin resistance^{3–9} and its independent emergence in several areas demonstrated in Southeast Asia^{10–12} may compromise the achievement of malaria eradication agenda. Given that artemisinins constitute the core components of the first-line treatments of malaria in endemic countries, emergence of artemisinin resistance in sub-Saharan Africa could more jeopardize thousands of lives in that area and also could lead to increased medical and economic costs estimated to be several hundred million U.S. dollars per year.^{13,14}

Artemisinin resistance critically occurs when parasites are at the ring stage.^{3,15–19} The selection of an in vitro highly artemisinin-resistant strain enabled the characterization of the quiescence-based artemisinin resistance mechanism and identification of *Plasmodium falciparum* kelch 13 propeller domain (*PfK13*) as a molecular marker of artemisinin resistance.^{16,20,21} Several single-nucleotide polymorphisms (SNPs) of the *pfk13* gene were clearly correlated with the prolongation of parasite clearance time after treatment with artemisinins in Southeast Asia.^{9,20,22,23} The recent investigations of artemisinin resistance in sub-Saharan Africa have

been dominated by the use of the *pfk13* genotyping, parasite clearance time, and irrelevant 50% inhibitory concentration (IC₅₀) values obtained by ex vivo and in vitro assays based on the inhibition of parasite proliferation. Today, several SNPs of *pfk13* have been described in sub-Saharan Africa, but none of them was associated with artemisinin resistance in this region.^{10,22,24–26} Parasite clearance time measurement is a useful monitoring tool of artemisinin resistance,^{27,28} but several confounding factors such as host immunity, drug pharmacokinetics, or spleen clearance of infected red blood cells (RBCs) influence its outcomes. In vitro and ex vivo assays are not concerned by these factors as they measure the parasite intrinsic sensitivity by direct contact between the parasites and the drugs.²⁹ These tools are thus essential for malaria drug resistance studies using laboratory strains and field isolates.

However, the standard methods based on the measurement of parasite growth inhibition induced by the drug appear irrelevant for the assessment of artemisinin resistance, which is quiescence based and ring stage specific.^{3,15–19} A few number of novel assays specifically targeting the ring stage and taking into account the quiescence mechanism have been developed, including the ring-stage survival assay (RSA^{0–3h}).^{17,30,31} The increase of parasite survival rates measured in the RSA^{0–3h} was associated with *pfk13*-resistant polymorphisms and delayed parasite clearance in Southeast Asia.^{20,32} Although any *pfk13* polymorphisms were not yet associated with delayed parasite clearance in sub-Saharan Africa,^{10,33} the current sensitivity status of African parasites to artemisinins needs to be urgently clarified.

To contribute to fill this gap, the present study comprising ex vivo and in vitro assays was conducted to precisely determine artemisinin sensitivity phenotypes of Malian isolates collected 5 years after the implementation of artemisinin-based combination therapies (ACTs). These isolates were also sequenced to investigate *pfk13* polymorphisms. All the assays were designed to take into account both the quiescence mechanism and the *P. falciparum* ring-stage

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specificity of artemisinin resistance. The findings presented here with Malian isolates could contribute to guide future monitoring of artemisinin resistance emergence in Africa.

MATERIALS AND METHODS

Sample collection and study sites. From October 2010 to January 2011, *P. falciparum* isolates were collected from patients participating in a 28-day clinical trial of two ACTs, namely, artesunate plus sulfadoxine/pyrimethamine (ARTECOSPE[®], Guilin Pharmaceutical, Shanghai, China) and artemether-lumefantrine (COARTEM[®], Novartis Pharmaceuticals Corporation, East Hanover, NJ), randomly distributed.³³ This study got ethically cleared from the WHO and the Ethics Committee at the University of Sciences, Techniques and Technologies of Bamako, Mali, and was registered under the number 2010/63/FMPOS. Isolate samples were collected either when the parasitemia was comprised between 0.5% and 2% at day 0, corresponding to the inclusion day to the clinical study, or when the patients presented treatment failures to any of both tested drugs until day 28. Five milliliters of blood was collected in heparin tubes from patients after signing the informed consent forms. At the same time, dried blood spots were also prepared on 3-mm filter papers (Whatman[®], Maidstone, United Kingdom) for *pfk13* sequencing. Once collected, the blood samples were transported within 1–5 hours from the study sites to the laboratory located at the Malaria Research and Training Center (MRTC) in Bamako (the capital city of Mali). There were two study sites: the villages of Kolle, located 57 km southwest of Bamako, and Sotuba, a periurban area of Bamako (the isolates were named Kolle-MRTC-Toulouse [KMT] and Sotuba-MRTC-Toulouse [SMT]). These sites have been previously described.³⁴ Briefly, the entomological inoculation rates were 137–167 infective bites/person/year in Kolle and 1–12 infective bites/person/year in Sotuba. The transmission of malaria is seasonal in both sites because of *Anopheles gambiae* and *Anopheles funestus* species complexes.

Parasite sample conservation. In the laboratory, the blood samples were centrifuged to remove the plasma and the RBC pellets were washed twice with Roswell Park Memorial Institute (RPMI) RPMI 1640 medium (GIBCO-Invitrogen[®], Carlsbad, CA) to deplete the leukocytes. Parasitemia, estimated by the ratio of infected RBCs counted in a total of 10,000 RBCs (composed of infected and noninfected RBCs), was verified after blood wash using Giemsa-stained thin smear. For each sample, 2-mL aliquot of washed blood pellet was added to an equal volume of freezing medium (3% sorbitol, 0.65% NaCl, and 28% glycerol in distilled water) and cryopreserved in vapor-phase liquid nitrogen for 4 years before used for later in vitro studies and *pfk13* sequencing. The remaining amount of the fresh isolate sample (approximately 0.5 mL) was used to perform only the ex vivo ring-stage recrudescence assay (RSRA).

Test molecules. Ex vivo and in vitro assays were carried out either with artemisinin (the leading endoperoxide-based compound), which was routinely used in 2010–2011 when the ex vivo experiments were performed to discriminate artemisinin-resistant and artemisinin-sensitive phenotypes,¹⁶ or with dihydroartemisinin (DHA), the active metabolite of artemisinin derivatives (but not of artemisinin itself) presently used in ACTs in the field.^{35,36} Artemisinin was purchased from SIGMA-ALDRICH[®] (St. Louis, MO) and DHA was provided by the WorldWide Antimalarial Resistance Network.

Ex vivo RSRA. Fresh isolate samples at the ring stage were added to a freshly prepared culture medium (RPMI 1640; 4% hematocrit, 5% AB human serum, 0.004% hypoxanthine, and 1% Albumax II) and distributed into 24-well culture plates. Powdered artemisinin was dissolved in dimethyl sulfoxide (DMSO) and diluted in RPMI to obtain 1, 10, 50, and 100 nM of final artemisinin concentrations with a maximum of 0.025% DMSO. Each sample was treated in eight separate wells, including three wells for positive control, that is, parasite culture without any drug, one well for each of the four different doses of artemisinin, and one well for artemisinin-free control but with 0.025% DMSO. Forty-eight hours later, the parasites were washed twice with RPMI to remove drug and were resuspended in a new freshly prepared culture medium for 36-hour incubation. Giemsa-stained thin smears were then performed at hour 84 to check parasite recrudescence and evaluate parasitemia. If no viable parasites were detected, a second Giemsa-stained thin smear was performed 48 hours later (hour 132), as illustrated in Figure 1A.

Isolate adaptation to in vitro culture. After 4-year storage in vapor-phase liquid nitrogen, cryopreserved parasites were thawed and adapted to the continuous in vitro culture as previously described.³⁷ For each isolate, the cryo vial was thawed in 37°C water bath; the content was mixed up with hypertonic saline (3.5% NaCl) for 2 minutes before washing the parasites with RPMI 1640. The pellet was placed in RPMI 1640 culture medium at 2% hematocrit, 20% AB human serum, and 37°C in trigas (5% O₂, 5% CO₂, and 90% N₂). The parasite cultures were patiently and courageously managed for long time by regularly changing the culture medium and adding fresh O-group RBCs until parasite growth became detectable with Giemsa-stained thin smears.

In vitro RSRA. In vitro RSRA was performed in the same conditions as those of ex vivo RSRA using artemisinin as well. However, results obtained from the ex vivo assay allowed minor modifications of this in vitro test (See Figure 1B). Adapted parasite cultures were treated with 5% D-sorbitol to specifically obtain parasites at the ring stage and diluted in RBCs to get 1% parasitemia. Regarding results obtained ex vivo, artemisinin concentrations tested in vitro were elevated to 50 nM, 100 nM, and 200 nM for 48-hour exposure at 37°C in trigas (5% O₂, 5% CO₂, and 90% N₂). Parasite recrudescence was verified at hour 72 and potentially at hour 120.

In vitro RSA^{0–3 h}. In vitro RSA^{0–3 h} was performed following the previously described protocol³² and summarized in Figure 1C. Briefly, in 48-well culture plates, the adapted isolates were exposed to 700 nM DHA at the stage of 0–3-hour-old ring (obtained after Percoll-sorbitol treatments) for 6 hours at 37°C in trigas (5% O₂, 5% CO₂, and 90% N₂). In parallel, control wells (exposed to 0.1% DMSO) were also prepared and processed identically as DHA-exposed wells. After drug exposure, the parasites were washed with RPMI and placed in a new culture medium for 66-hour incubation. Therefore, at hour 72, the Giemsa-stained smears were performed in DHA-exposed and DMSO-exposed wells to evaluate parasite survival rates. The parasite survival rates to drug exposure were estimated as follows:

$$\frac{\text{Parasitemia of DHA exposed wells}}{\text{Parasitemia of DMSO exposed wells}} \times 100$$

Parasitemias were calculated from a total of at least 40,000 RBCs per assay. Slides were read from the two duplicate wells

FIGURE 1. Diagram of ex vivo and in vitro phenotyping assays. (A) Ex vivo ring-stage recrudescence assays (RSRA). (B) In vitro RSRA. (C) In vitro ring-stage survival assay (RSA^{0-3h}). (D) In vitro ring-stage recrudescence kinetics assay (RSKA^{0-24h}) performed on 0-24-hour-old ring stages. DHA = dihydroartemisinin; DMSO = dimethyl sulfoxide; RPMI = Roswell Park Memorial Institute. This figure appears in color at www.ajtmh.org.

per assay by at least two separate microscopists, each of whom examined at least 10,000 RBCs per slide. Parasite growth rate of DMSO-exposed wells ≥ 2 was required to validate the parasite survival data. The artemisinin-resistant parasite line F32-ART and its twin sensitive line F32-TEM were both used as controls.^{16,20}

In vitro ring-stage recrudescence kinetics assay (RSKA^{0-24h}) This assay was close to the recovery assay previously used elsewhere.¹⁷ Recrudescence kinetics assays were first performed in parallel on 0-3-hour-old ring-stage isolates (RSKA^{0-3h}) obtained after Percoll-sorbitol treatments and on parasites at the ring 0-24-hour-old stage synchronized with 5% D-sorbitol (RSKA^{0-24h}) with initial parasitemias ranging from 1.2% to 3.4%. After treatment of the parasites with 700 nM DHA for 6 hours (pharmacologically relevant time and concentration of the active metabolite of artemisinin derivatives^{20,32}), parasitemia was daily measured by Giemsa-stained thin smears to evaluate the kinetics of recrudescence over 7 days (Figure 1D). Both lines F32-ART and F32-TEM were used to determine whether differences of parasite kinetics after DHA treatment could be related to an artemisinin-resistant phenotype. Regarding the results obtained with both categories of parasite ages (0-3 and 0-24 hours), the RSKA^{0-24h} performed on parasites at the ring 0-24-hour-old stage was then preferred.

***Plasmodium falciparum* kelch 13 propeller domain sequencing.** This sequencing concerned both samples before cryopreservation and after isolate adaptation to continuous in vitro culture. *Plasmodium falciparum* kelch 13 propeller domain locus was preamplified by nested polymerase chain reaction (PCR) before sequencing as previously described.³⁸

The quality control of the PCR products was checked by running an electrophoresis agarose gel. The sequencing was performed by Sanger method using the same primers of the nested PCR.³⁸ Sequence quality control was done with Thermo Fisher Scientific® Sequence Scanner 2 (Waltham, MA) (<http://resource.lifetechnologies.com/pages/WE28396/>). The pairwise alignments of the generated sequences to the 3D7 strain sequence (wild-type [WT] reference) were performed in Bioedit® (Tom Hall - Ibis Therapeutics, Carlsbad, CA) (<http://www.mbio.ncsu.edu/bioedit/bioedit.html>). Samples found with any mutations were sequenced for a second time to confirm.

Data analysis. Microsoft Excel (Microsoft® Corporation, Redmond, WA) database was first created for all datasets. Then, these datasets were transferred into GraphPad Prism 6 (GraphPad Software®, La Jolla, CA) for statistical analysis. The mean parasitemia was estimated for each individual isolate studied in the in vitro RSA^{0-3h} and in vitro RSKA^{0-24h} and the errors were estimated. Pearson's χ^2 test was used for the proportions of different artemisinin-tolerant isolates in the ex vivo RSRA. Student's *t* test was used to compare day 7 parasitemias of the isolates in the in vitro RSKA^{0-24h}. Linear regression model was computed to verify any relationship between the variation of initial parasitemias and day 7 parasitemias in the in vitro RSKA^{0-24h}. *P* value ≤ 0.05 was considered as statistically significant for all analyses.

RESULTS

From 182 patients enrolled for the in vivo study, 54 consented to give blood samples at inclusion according to

parasitemia criterion ($N = 46$) and after treatment failures ($N = 8$) during the clinical follow-up (Figure 2). After PCR correction of treatment outcomes, all treatment failures were in fact reinfections (Table 1). Aliquots for all 54 isolate samples were cryopreserved and sequenced at the *pfk13* locus except one sample that failed to yield PCR product for sequencing. A total of 46 fresh isolate samples were treated in recrudescence assay (ex vivo RSRA). After 4-year storage in vapor-phase liquid nitrogen, 60.9% (14/23) of thawed isolates were successfully adapted to continuous in vitro culture. These adapted isolates were then studied using in vitro assays after a random selection from isolates collected under parasitemia criterion and isolates thought to be treatment failures (12 for RSA^{0-3h}, eight for RSKA^{0-24h}, and six for in vitro RSRA, see Figure 2).

Ex vivo sensitivity to artemisinin. The proportions of isolates that survived to artemisinin concentrations in the ex vivo RSRA were estimated according to Giemsa-stained thin smears (Figure 3). Malian isolates showed different sensitivity levels to artemisinin with 91.3% (42/46) of isolates survived to 1 nM artemisinin, 56.5% (26/46) to 10 nM artemisinin, and 26.1% (12/46) to 50 nM artemisinin. Interestingly, 13.0% (6/46)

tolerated up to 100 nM artemisinin, which corresponded to the highest concentration tested in this assay.

In vitro sensitivity to artemisinin. In the in vitro RSRA, from six isolates tested, five survived to 50 nM artemisinin, 4 to 100 nM artemisinin, and 2 to 200 nM artemisinin, which corresponded to the highest concentration tested in this assay. However, there was no correlation between ex vivo and in vitro RSRA results (Table 1). For each of the 12 isolates and the two control lines tested, an average of four RSA^{0-3h} experiments were performed per isolate. Ring-stage survival assay^{0-3h} data showed that the Malian isolates tested are all artemisinin sensitive with survival rates ranged from 0.12% to 1.45% (Figure 4). These values were markedly inferior to that of the artemisinin-resistant control (F32-ART line carrying M476I mutation), which was $10.30\% \pm 0.95$. The most sensitive isolates showed survival rate values very close to that of the artemisinin-sensitive control (F32-TEM line carrying the *pfk13*WT allele). Subsequently, eight out of these 12 isolates were also studied in the in vitro RSKA^{0-24h} to determine their growth kinetics after 6-hour treatment with 700 nM DHA. It is to note that the kinetic assay RSKA was preliminarily conducted in parallel on 0-3- and 0-24-hour-old ring stages. The data obtained with both ring-stage categories led to the same

FIGURE 2. Study flow chart. *Pfk13* = *Plasmodium falciparum* kelch 13 propeller; RSA^{0-3h} = ring-stage survival assay; RSKA^{0-24h} = ring-stage recrudescence kinetics assay; RSRA = ring-stage recrudescence assay.

TABLE 1

Summary of ex vivo/in vitro data and isolates sequencing for 12 randomly chosen isolates from artemisinin-based combination therapies efficacy study in Mali

Isolate ID	Time of sample collection regarding the clinical trial	Treatment outcome	Ex vivo RSRA*	<i>Pfk13</i> genotype	In vitro RSRA*	Survival rate (mean $\pm$ SEM) in vitro RSA ^{0-3 h}	D7 parasitemia (mean $\pm$ SEM) in vitro RSKA ^{0-24 h}	Kinetics profile (in vitro RSKA)
SMT004	Day 0	NA	No survival	WT	Cases of survival to 50 nM (1/3)† and 100 nM (1/3)	0.75 $\pm$ 0.60	0.60 $\pm$ 0.24	Fast
SMT004J28	Day 28	Reinfection	No survival	WT	Cases of survival to 50 nM (2/4), 100 nM (2/4), and 200 nM (1/4)	0.4 $\pm$ 0.80	0.72 $\pm$ 0.32	Fast
SMT010	Day 0	NA	No survival	WT	NP	0.71 $\pm$ 0.04	0.84 $\pm$ 0.08	Fast
KMT001	Day 0	NA	Cases of survival to 50 and 100 nM	WT	Cases of survival to 50 nM (2/5)	0.13 $\pm$ 0.23	0.08 $\pm$ 0.01	Slow
KMT003	Day 0	NA	Cases of survival to 50 and 100 nM	WT	NP	0.81 $\pm$ 0.25	NP	NA
KMT004	Day 21	Reinfection	No survival	WT	No survival	0.39	0.02 $\pm$ 0.02	Slow
KMT006	Day 0	NA	No survival	WT	NP	1.42 $\pm$ 1.05	NP	NA
KMT007	Day 0	NA	Cases of survival to 50 nM	WT	NP	1.28 $\pm$ 0.53	NP	NA
KMT012	Day 21	Reinfection	No survival	WT	Cases of survival to 50 nM (3/5), 100 nM (3/5) and 200 nM (1/5)	0.12 $\pm$ 0.21	0.41 $\pm$ 0.26	Fast
KMT099	Day 0	NA	Cases of survival to 50 and 100 nM	WT	NP	0.54 $\pm$ 0.54	NP	NA
KMT102	Day 0	NA	No survival	WT	NP	1.45 $\pm$ 0.65	1.32 $\pm$ 0.39	Fast
KMT103	Day 0	NA	No survival	WT	Cases of survival to 50 nM (1/2), 100 nM (1/2)	0.87 $\pm$ 0.40	0.09 $\pm$ 0.05	Slow
F32-ART	NA	NA	NA	M476I	NA	10.3 $\pm$ 0.95	5.80 $\pm$ 0.79	–
F32-TEM	NA	NA	NA	WT	NA	0.13 $\pm$ 0.10	0.85 $\pm$ 0.34	–

KMT = Kolle-MRTC-Toulouse; NA = not applicable; NP = not performed; *Pfk13* = *Plasmodium falciparum* kelch 13 propeller domain; RSA^{0-3 h} = ring-stage survival assay; RSKA^{0-24 h} = ring-stage recrudescence kinetics assay; RSRA = ring-stage recrudescence assay; SEM = standard error of the mean; SMT = Sotuba-MRTC-Toulouse; WT = wild type. F32-ART and F32-TEM were the artemisinin-resistant and artemisinin-sensitive control lines, respectively. The parasite IDs represent the studied isolates.

* Artemisinin was used in the ex vivo and in vitro RSRA.

† One case of survival out of three experiments performed.

conclusions, that is, determination of two distinct recovery phenotypes of isolates (Supplemental Table 1). As experiments on 0–24-hour-old ring stages were easier to perform, the RSKA^{0-24 h} was then chosen to discriminate isolate phenotypes and was repeated five times on average for each isolate. Two groups of phenotypes were therefore observed among the isolates. One group, corresponding to three isolates out of eight studied, showed slower recovery with parasitemias at day 7 less than 0.1% (Table 1, Figure 5). The

FIGURE 3. Proportions of isolates that survived to different artemisinin concentrations in the ex vivo ring-stage recrudescence assay.

isolates of the other group grew faster with significantly higher parasitemias at day 7 (more than 0.4%) (Table 1, Figure 5). Differences of recrudescence kinetics can also be observed between the control lines F32-TEM and F32-ART (Supplemental Figure 1). Among the tested isolates, KMT102 showed the highest day 7 parasitemia (1.32 $\pm$ 0.39). To verify whether the initial parasitemias influenced the day 7 parasitemias in the RSKA^{0-24 h}, linear regression analysis was computed for each isolate and demonstrated that the variation of the day 7 parasitemias was independent of the initial parasitemias (Figure 6, Supplemental Table 2). Interestingly, the outcomes of the recrudescence assays (in vitro RSRA) were comparable to those of the kinetic assay (RSKA^{0-24 h}). Indeed, all the isolates that grew rapidly in the in vitro RSKA^{0-24 h} were able, when tested for in vitro RSRA, to tolerate the 48-hour treatment with 100 nM artemisinin in vitro (Table 1). Two of them were even tolerant to 200 nM artemisinin, at least once (Table 1). Similarly, the isolates with slower growth were sensitive to 100 nM artemisinin except for one which was tolerant in just one experiment (Table 1).

***Plasmodium falciparum* kelch 13 propeller domain polymorphisms before and after isolate adaptation.** The sequencing of *pfk13* showed only four synonymous SNPs (Supplemental Table 3). The frequencies of these mutations ranged from 1.9% to 3.8%. No nonsynonymous SNPs were found, even after isolate adaptation to continuous in vitro culture.

Ex vivo and in vitro phenotypes and clinical outcomes. From all patients who received 3-day ACT regimen and followed up for 28 days, nobody was found with positive

FIGURE 4. Ring-stage survival assay^{0–3 h} survival rates of 12 isolates and control lines. The dots represent the mean values of survival rates—the parasitemias themselves were estimated by their mean values. The error bars represent the standard deviation. The horizontal red line represents 1% of survival rate, which is the threshold of the survival rates between *Plasmodium falciparum* kelch 13 propeller domain (*pfk13*) wild-type (WT) isolates and resistant mutants in Southeast Asia.²⁰ F32-ART parasite line was the artemisinin-resistant control that carries *pfk13* M476I mutation. F32-TEM line was the sensitive control with WT *pfk13*. The other parasite IDs represent the study isolates. This figure appears in color at www.ajtmh.org.

parasitemia at days 2 and 3. The isolate phenotypes obtained by different ex vivo and in vitro assays cannot be thus associated with delayed parasite clearance. In addition, after PCR correction of treatment outcomes, no isolate was associated with treatment failure. Two out of three isolates (isolate ID: SMT004 and KMT012) collected after reinfection showed rapid kinetics in the RSA^{0–24 h}, but their survival rates in the RSA^{0–3 h} were only 0.4 ± 0.8 and 0.12 ± 0.21 , respectively (Table 1).

DISCUSSION

The in vitro studies are crucial to determine the intrinsic sensitivity of parasites to drugs. For long, assays based on the

inhibition of trophozoite maturation to schizont stage have been largely used to assess the sensitivity of clinical isolates to artemisinins in sub-Saharan Africa.^{39–44} The IC₅₀ values have been used to determine the in vitro sensitivity level of parasites to artemisinins. These studies mostly presented two significant drawbacks that were considered in the present study. First, the term “in vitro” has been often inappropriately used because most of these studies were performed only on fresh clinical isolates without parasite adaptation into continuous in vitro culture to carry out repeated experiments. Here, we thus performed both ex vivo and in vitro assays even though ex vivo version could not be done for all of the assays because of the limited volume of blood collected from the patients according to the protocol of the in vivo study. In the same vein,

FIGURE 5. Growth levels in the in vitro ring-stage recrudescence kinetics assays^{0–24 h}, after 6-hour treatment with 700 nM dihydroartemisinin. The bars represent the means of day 7 parasitemias. The error bars represent the standard deviation. The numbers of stars indicate the degrees of significance in pairwise comparison of parasite growth levels (* = < 0.01, ** = < 0.001, *** = < 0.0001). The parasite IDs represent the studied isolates.

FIGURE 6. Regression plot of day 7 parasitemia and initial parasitemia in the in vitro ring-stage recrudescence kinetics assay^{0–24 h}. Linear regression analysis (deviation from zero), $P > 0.05$ for all isolates.

46 isolates were used in the ex vivo RSRA whereas only 14 successfully adapted out of 23 thawed isolates could be used in the in vitro assays. However, to ensure the reproducibility of the outcomes, the different in vitro assays reported here were repeated four to five times on average for each sample, after a laborious effort to adapt isolates into continuous culture.

Second, because artemisinin resistance occurs at the parasite ring stage by a quiescence mechanism, the classical IC_{50} values are not significantly modified in artemisinin-resistant parasites and were proven as irrelevant.^{3,16,45,46} That is why all the phenotyping assays used in this study took into account both the ring-stage specificity and the quiescence mechanism of artemisinin resistance.

This study presents the baseline ex vivo and in vitro data on the sensitivity levels of Malian isolates 5 years after ACT implementation. Overall, neither phenotyping assays nor sequencing of *pfk13* locus performed here could evidence artemisinin resistance in Mali. This result is important for the whole sub-Saharan Africa because of the very close relatedness of the parasites from this area based on SNP analysis at a genome-wide scale or *k13* polymorphisms.^{47,48} These data also reassure the national malaria control programs of that area as the findings still confirm the efficacy of the artemisinins. However, even though no *pfk13* nonsynonymous SNPs were found, marked differences in isolate phenotypes were revealed by the assays performed here.

The RSKA^{0–24 h} data seemed to bring complementary in vitro results to RSA^{0–3 h}. Indeed, the survival rates observed with RSA^{0–3 h} demonstrated that all Malian isolates tested were sensitive to artemisinin. All isolates showed RSA^{0–3 h} values $< 1.5\%$, which were correlated with the wild-type K13 genotype as previously reported in Southeast Asia.^{20,28,49} By contrast, the recrudescence kinetic assay, RSKA^{0–24 h}, detected different phenotypes among artemisinin-sensitive isolates evidenced by RSA^{0–3 h}. Indeed, five out of eight studied isolates presented faster kinetics of recrudescence even though this in vitro response to artemisinin treatment does not appear to be associated neither with *pfk13* polymorphisms nor with high survival rates in RSA^{0–3 h}. The RSKA^{0–24 h} monitors during a week the fate of artemisinin-susceptible parasites after drug treatment in vitro. Thus, it allows to study the ability of living parasites detected 66 hours after DHA exposure (like in RSA conditions) to

multiply through three schizogonic cycles (parasitemia monitored during 7 days). As expected, in this assay, the kinetics of recrudescence of the artemisinin-resistant control line (F32-ART) was significantly faster than that of its twin sensitive clone (F32-TEM) after treatment by DHA. This result indicated that the kinetics of parasite recrudescence is related to their response to DHA treatment. Although the difference between both isolate phenotypes (fast and slow recoveries) was weaker than the kinetics difference between both control lines, the RSKA^{0–24 h} results demonstrated that some Malian isolates could be less susceptible to artemisinins. This is also in accordance with the ex vivo and in vitro RSRA data that showed that fast recovering parasites were able to survive the 48-hour treatment with up to 100 nM and 200 nM artemisinin. Therefore, Malian isolates present unclear features that could be early stages of artemisinin tolerance after ACT implementation in the field.

Furthermore, in another study, among isolates from Ratanakiri, Cambodia (collected in artemisinin-sensitive area in 2010–2011), different recovery levels seemed to be also evidenced after DHA treatment¹⁷ (and before any sign of clinical reduced artemisinin efficacy).⁵⁰ Regarding the high level of ACT resistance now reported in the whole Cambodia,⁵¹ further investigations will be useful to characterize the present phenotypes in Africa.

The impact of the parasite genetic background in artemisinin resistance was already evoked²¹ and should be more explored to understand the behavior of African parasites regarding artemisinin treatment. Fortunately, none of the parasite phenotypes described here were associated with treatment failures. They were associated with neither day 3 parasitemia nor treatment failure in patients treated with ACTs and followed up for 28 days. This shows that the artemisinin tolerance levels of the studied isolates are not strong enough to rise clinically and induce delayed parasite clearance or treatment failures. However, such parasites showing less in vitro sensitivity to artemisinin, if at the same time are resistant to the partner drug, may readily lead to treatment failures of ACTs.

The same malaria treatment policy is ongoing in both areas studied. Unlike Sotuba, Kolle is a small village of farmers with very low incomes to access high-quality drugs and where the auto-medication with traditional medicinal plants and use of poor-quality drugs are common. However, to explain different parasite phenotypes, further studies with a larger number of isolates need to be conducted.

In conclusion, the phenotyping tools used in this study shed light on the feature of the in vitro and ex vivo sensitivity of *P. falciparum* isolates to artemisinins in Mali. The data of this study confirm that there is no clinical artemisinin resistance yet to report in Mali. None of the studied isolates carried nonsynonymous SNPs of the gene *pfk13*. However, a significant difference in parasite sensitivity to artemisinin was observed in vitro with some isolates being less susceptible to artemisinin or DHA than others. The presence of such phenotypes needs to be taken into account in the assessment of the efficacy of artemisinins in Africa and suggests that continuous monitoring combining clinical studies and in vitro assays based on the quiescence mechanism and ring-stage specificity of artemisinin resistance should be encouraged.

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REFERENCES

- Walther B, Walther M, 2007. What does it take to control malaria? *Ann Trop Med Parasitol* 101: 657–672.
- WHO, 2016. *World Malaria Report 2016*. Geneva, Switzerland: World Health Organization.
- Dondorp AM et al., 2009. Artemisinin resistance in *Plasmodium falciparum* malaria. *N Engl J Med* 361: 455–467.
- Noedl H, Se Y, Schaefer K, Smith BL, Socheat D, Fukuda MM, 2008. Evidence of artemisinin-resistant malaria in western Cambodia. *N Engl J Med* 359: 2619–2620.
- Phyo AP et al., 2012. Emergence of artemisinin-resistant malaria on the western border of Thailand: a longitudinal study. *Lancet* 379: 1960–1966.
- Kyaw MP et al., 2013. Reduced susceptibility of *Plasmodium falciparum* to artesunate in southern Myanmar. *PLoS One* 8: e57689.
- Thriemer K et al., 2014. Delayed parasite clearance after treatment with dihydroartemisinin-piperaquine in *Plasmodium falciparum* malaria patients in central Vietnam. *Antimicrob Agents Chemother* 58: 7049–7055.
- Na-Bangchang K, Karbwang J, 2013. Emerging artemisinin resistance in the border areas of Thailand. *Expert Rev Clin Pharmacol* 6: 307–322.
- WHO, 2016. *Artemisinin and Artemisinin-based Combination Therapy Resistance*. Geneva, Switzerland: World Health Organization.
- Ménard D et al., KARMA Consortium, 2016. A worldwide map of *Plasmodium falciparum* K13-propeller polymorphisms. *N Engl J Med* 374: 2453–2464.
- Takala-Harrison S et al., 2015. Independent emergence of artemisinin resistance mutations among *Plasmodium falciparum* in Southeast Asia. *J Infect Dis* 211: 670–679.
- Miotto O et al., 2015. Genetic architecture of artemisinin-resistant *Plasmodium falciparum*. *Nat Genet* 47: 226–234.
- Slater HC, Griffin JT, Ghani AC, Okell LC, 2016. Assessing the potential impact of artemisinin and partner drug resistance in sub-Saharan Africa. *Malar J* 15: 10.
- Lubell Y, Dondorp A, Guérin PJ, Drake T, Meek S, Ashley E, Day NP, White NJ, White LJ, 2014. Artemisinin resistance—modelling the potential human and economic costs. *Malar J* 13: 452.
- Saralamba S et al., 2011. Intrahost modeling of artemisinin resistance in *Plasmodium falciparum*. *Proc Natl Acad Sci USA* 108: 397–402.
- Witkowski B, Lelièvre J, Barragán MJL, Laurent V, Su XZ, Berry A, Benoit-Vical F, 2010. Increased tolerance to artemisinin in *Plasmodium falciparum* is mediated by a quiescence mechanism. *Antimicrob Agents Chemother* 54: 1872–1877.
- Witkowski B et al., 2013. Reduced artemisinin susceptibility of *Plasmodium falciparum* ring stages in western Cambodia. *Antimicrob Agents Chemother* 57: 914–923.
- Teuscher F, Chen N, Kyle DE, Gatton ML, Cheng Q, 2012. Phenotypic changes in artemisinin-resistant *Plasmodium falciparum* lines in vitro: evidence for decreased sensitivity to dormancy and growth inhibition. *Antimicrob Agents Chemother* 56: 428–431.
- Teuscher F, Gatton ML, Chen N, Peters J, Kyle DE, Cheng Q, 2010. Artemisinin-induced dormancy in *Plasmodium falciparum*: duration, recovery rates, and implications in treatment failure. *J Infect Dis* 202: 1362–1368.
- Ariey F et al., 2014. A molecular marker of artemisinin-resistant *Plasmodium falciparum* malaria. *Nature* 505: 50–55.
- Strainer J et al., 2015. Drug resistance. K13-propeller mutations confer artemisinin resistance in *Plasmodium falciparum* clinical isolates. *Science* 347: 428–431.
- Ashley EA et al.; Tracking Resistance to Artemisinin Collaboration (TRAC), 2014. Spread of artemisinin resistance in *Plasmodium falciparum* malaria. *N Engl J Med* 371: 411–423.
- Tun KM et al., 2015. Spread of artemisinin-resistant *Plasmodium falciparum* in Myanmar: a cross-sectional survey of the K13 molecular marker. *Lancet Infect Dis* 15: 415–421.
- Taylor SM et al., 2015. Absence of putative artemisinin resistance mutations among *Plasmodium falciparum* in sub-Saharan Africa: a molecular epidemiologic study. *J Infect Dis* 211: 680–688.
- Conrad MD, Bigira V, Kapisi J, Muhindo M, Kamya MR, Havlir DV, Dorsey G, Rosenthal PJ, 2014. Polymorphisms in K13 and falcipain-2 associated with artemisinin resistance are not prevalent in *Plasmodium falciparum* isolated from Ugandan children. *PLoS One* 9: e105690.
- Mvumbi DM, Bobanga TL, Kayembe J-MN, Mvumbi GL, Situakibanza HN-T, Benoit-Vical F, Melin P, De Mol P, Hayette M-P, 2017. Molecular surveillance of *Plasmodium falciparum* resistance to artemisinin-based combination therapies in the Democratic Republic of Congo. *PLoS One* 12: e0179142.
- Maiga AW et al., 2012. No evidence of delayed parasite clearance after oral artesunate treatment of uncomplicated falciparum malaria in Mali. *Am J Trop Med Hyg* 87: 23–28.
- Niaré K et al., 2016. In vivo efficacy and parasite clearance of artesunate + sulfadoxine-pyrimethamine versus artemether-lumefantrine in Mali. *Am J Trop Med Hyg* 94: 634–639.
- Perfect JR, Cox GM, 1999. Drug resistance in *Cryptococcus neoformans*. *Drug Resist Updat* 2: 259–269.
- Witkowski B et al., 2013. Novel phenotypic assays for the detection of artemisinin-resistant *Plasmodium falciparum* malaria in Cambodia: in-vitro and ex-vivo drug-response studies. *Lancet Infect Dis* 13: 1043–1049.
- Chotivanich K, Tripura R, Das D, Yi P, Day NPJ, Pukrittayakamee S, Chuor CM, Socheat D, Dondorp AM, White NJ, 2014. Laboratory detection of artemisinin-resistant *Plasmodium falciparum*. *Antimicrob Agents Chemother* 58: 3157–3161.
- Ye R et al., 2016. Distinctive origin of artemisinin-resistant *Plasmodium falciparum* on the China-Myanmar border. *Sci Rep* 6: 20100.
- Menard S et al., 2016. Insight into k13-propeller gene polymorphism and ex vivo DHA-response profiles from Cameroonian isolates. *Malar J* 15: 572.

34. Dicko A et al., 2007. Year-to-year variation in the age-specific incidence of clinical malaria in two potential vaccine testing sites in Mali with different levels of malaria transmission intensity. *Am J Trop Med Hyg* 77: 1028–1033.
35. Klayman DL, 1985. Qinghaosu (artemisinin): an antimalarial drug from China. *Science* 228: 1049–1055.
36. Kerb R, Fux R, Mörike K, Kremsner PG, Gil JP, Gleiter CH, Schwab M, 2009. Pharmacogenetics of antimalarial drugs: effect on metabolism and transport. *Lancet Infect Dis* 9: 760–774.
37. Moll K, Kaneko A, Scherf A, Wahlgren M, eds., 2013. *Methods in Malaria Research*, 6th edition. Available at: https://www.beiresources.org/portals/2/MR4/Methods_In_Malaria_Research-6th_edition.pdf. Accessed January 7, 2017.
38. Didier M, Menard D, Arieu F, 2013. PCR Sequencing for genotyping SNPs PF3D7_1343700 Kelch protein propeller domain. *Protoc Exch* 2013: doi:10.1038/protex.2013.096.
39. Tinto H et al., 2014. Ex vivo anti-malarial drugs sensitivity profile of *Plasmodium falciparum* field isolates from Burkina Faso five years after the national policy change. *Malar J* 13: 207.
40. Pradines B, Rogier C, Fusai T, Tall A, Trape JF, Doury JC, 1998. In vitro activity of artemether against African isolates (Senegal) of *Plasmodium falciparum* in comparison with standard antimalarial drugs. *Am J Trop Med Hyg* 58: 354–357.
41. Fall B et al., 2011. Ex vivo susceptibility of *Plasmodium falciparum* isolates from Dakar, Senegal, to seven standard anti-malarial drugs. *Malar J* 10: 310.
42. Quashie NB et al., 2013. A SYBR Green 1-based in vitro test of susceptibility of Ghanaian *Plasmodium falciparum* clinical isolates to a panel of anti-malarial drugs. *Malar J* 12: 450.
43. Zatra R, Lekana-douki JB, Lekoulou F, Bisvigou U, Ngoungou EB, Ndouo FST, 2012. In vitro antimalarial susceptibility and molecular markers of drug resistance in Franceville, Gabon. *BMC Infect Dis* 12: 307.
44. Ngalah BS et al., 2015. Analysis of major genome loci underlying artemisinin resistance and pfmdr1 copy number in pre- and post-ACTs in western Kenya. *Sci Rep* 5: 8308.
45. Witkowski B et al., 2013. Reduced artemisinin susceptibility of *Plasmodium falciparum* ring stages in western Cambodia. *Antimicrob Agents Chemother* 57: 914–923.
46. Ménard S et al., 2015. Induction of multidrug tolerance in *Plasmodium falciparum* by extended artemisinin pressure. *Emerg Infect Dis* 21: 1733–1741.
47. Miotto O et al., 2013. Multiple populations of artemisinin-resistant *Plasmodium falciparum* in Cambodia. *Nat Genet* 45: 648–655.
48. MalariaGEN *Plasmodium falciparum* Community Project, 2016. Genomic epidemiology of artemisinin resistant malaria. *eLife* 5: e08714.
49. Amaratunga C, Witkowski B, Dek D, Try V, Khim N, Miotto O, Ménard D, Fairhurst RM, 2014. *Plasmodium falciparum* founder populations in western Cambodia have reduced artemisinin sensitivity in vitro. *Antimicrob Agents Chemother* 58: 4935–4937.
50. Leang R, Barrette A, Bouth DM, Menard D, Abdur R, Duong S, Ringwald P, 2013. Efficacy of dihydroartemisinin-piperaquine for treatment of uncomplicated *Plasmodium falciparum* and *Plasmodium vivax* in Cambodia, 2008 to 2010. *Antimicrob Agents Chemother* 57: 818–826.
51. World Health Organization, 2017. *Artemisinin and Artemisinin-Based Combination Therapy Resistance, April 2017*. Available at: <http://apps.who.int/iris/bitstream/10665/255213/1/WHO-HTM-GMP-2017.9-eng.pdf?ua=1>. Accessed December 6, 2017.