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SUSCEPTIBILITY

Impact of Short-Term Storage on *Ex Vivo* Antimalarial Susceptibilities of Fresh Ugandan *Plasmodium falciparum* Isolates

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ABSTRACT We measured susceptibilities of Ugandan *Plasmodium falciparum* isolates assayed on the day of collection or after storage at 4°C. Samples were incubated with serial dilutions of 8 antimalarials, and susceptibilities were determined from 72-h growth inhibition assays. Storage was associated with decreased growth and lower 50% inhibitory concentration values, but differences between assays beginning on day 0 or after 1 or 2 days of storage were modest, indicating that short-term storage before drug susceptibility determination is feasible.

KEYWORDS *Plasmodium falciparum*, Uganda, antimalarials, cold storage, drug susceptibility, *ex vivo*, growth, malaria

Determination of the *ex vivo* susceptibility of *Plasmodium falciparum* isolates immediately after collection of samples from individuals with malaria offers an important means of measuring antimalarial drug activity and resistance (1–3). However, facilities for the culture of malaria parasites are limited in settings with high incidence of malaria, and projects often resort to short-term storage of blood during transport to laboratories before measurement of drug susceptibility. Such storage facilitates the analysis of samples from sites distant from established laboratories. However, a systematic consideration of the effects on drug susceptibility measures of storage is lacking. Therefore, we compared susceptibility to a panel of standard antimalarials of isolates collected in eastern Uganda assayed shortly after collection and after 1 or 2 days of storage at 4°C.

From June 2018 to June 2021, 176 *P. falciparum* isolates were collected from patients presenting with malaria in Busia, Tororo, and Mbale districts in eastern Uganda by following methods described previously (1). Participants with falciparum malaria determined by thick blood smear were enrolled after written informed consent for those aged ≥18 years or from parents or guardians for those <18 years, with assent from participants aged 8 to 17 years. For each participant, 2 to 5 mL of venous blood was collected into a heparin-containing vacutainer tube before initiation of antimalarial treatment with artemether-lumefantrine. Patients with danger signs or severe malaria were excluded. Blood samples were stored at 4°C and transported to the laboratory for processing. Parasitemia was determined from Giemsa-stained thin smears by counting parasites per at least 1,000 erythrocytes. Samples for study were limited to those with a minimum parasitemia of 0.3% and *P. falciparum* monoinfection. The study was approved by Makerere University School of Biomedical Sciences Research Ethics Committee and the University of California, San Francisco, Committee on Human Research.

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Within 3 h of sample collection, blood was centrifuged at 2,000 rpm for 10 min at 4°C, plasma and buffy coat were removed, and the pellet was washed three times with RPMI 1640 medium (Thermo Fisher Scientific). The pellet was then resuspended in complete medium consisting of RPMI 1640 with 25 mM HEPES, 24 mM $NaHCO_3$, 0.1 mM hypoxanthine, 10 μg/mL gentamicin, and 0.5% AlbuMAX II (Thermo Fisher Scientific) to produce 50% hematocrit and stored at 4°C; aliquots were assayed immediately after processing (day 0) and after 1 or 2 days of storage.

The median age of the participants was 4.0 years (range, 0.5 to 24 years), and 70.5% (124/176) were $\leq$5 years of age. Median parasitemia was 3.0% (range, 0.5 to 20.0%). Of the 176 total collected isolates, 165 were assayed for *ex vivo* drug susceptibility on day 0 and after 1 day of storage at 4°C; 31 of these 165 isolates were also assayed after 2 days of storage. Six samples (3.6%; 6/165) were not considered in the final analysis due to inability to measure the 50% inhibitory concentration (IC_{50}) on any day of testing, typically due to poor parasite growth in culture. Subsequently, to explore the effect of storage of whole blood rather than storage after processing, 11 additional samples were stored without processing until washing with RPMI 1640 medium, as described above, immediately before assays on days 1 and 2.

The mean time ($\pm$ standard deviation) from sample collection to introducing parasites into drug assays was 5.0 $\pm$ 1.6 h on day 0, 23 $\pm$ 2.0 h on day 1, and 47 $\pm$ 1.7 h on day 2. For all samples, morphologically normal-appearing ring-stage parasites were observed on Giemsa-stained thin smears made at the time that assays were started on days 0, 1, and 2.

Ex vivo drug susceptibility was measured against 8 standard antimalarials using a 72-h growth inhibition microplate assay with SYBR green detection, as previously described (1). Antimalarial drugs were from the Medicines for Malaria Venture. Drugs were solubilized as 10 mM stocks in dimethyl sulfoxide, except for pyrimethamine, which was prepared as a 50 mM stock, and chloroquine, which was dissolved in distilled water. Drugs were serially diluted 3-fold in RPMI complete medium in 96-well microplates at 50 μl/well, with dilutions adjusted to capture full dose-response curves, and inclusion of drug-free and parasite-free control wells. Parasite samples, diluted with uninfected donor erythrocytes, were added for a total volume of 200 μl/well at 0.2% parasitemia and 2% hematocrit. Plates were incubated in a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2 at 37°C in a modular incubator (Billups Rothenberg). After 72 h, cultures were resuspended and 100 μl/well was transferred into a black 96-well microplate containing 100 μl/well lysis buffer (20 mM Tris, 5 mM EDTA, 0.008% saponin, 0.08% Triton X-100, and 0.2 μl/mL SYBR green I [Invitrogen]), followed by mixing. Plates were incubated for 1 h in the dark at room temperature, and fluorescence from each well was measured using a FLUOstar Omega plate reader (BMG LabTech; 485-nm excitation/530-nm emission). IC_{50} values were obtained by plotting fluorescence against log drug concentration, followed by fitting the data by variable-slope, sigmoidal curve fit in GraphPad Prism 8.4.3. Drug susceptibility was summarized as median IC_{50} values based on single measures per drug concentration. The Wilcoxon matched-pairs signed-rank test was used to compare median IC_{50} values and parasite growth in assays with two time points. The Friedman test was used to detect overall differences in IC_{50}s or growth in assays with three time points, and the Wilcoxon signed-rank *post hoc* test with a Bonferroni correction was applied to determine which matched pairs were significantly different from each other. Statistical tests were conducted in R v3.4.4 with the rstatix package (R Core Team 2020) (4). All tests were two-tailed and were considered statistically significant at a P value of <0.05.

In assays performed on day 0, parasites were generally highly susceptible to the drugs tested, except for pyrimethamine, with results for all drugs being similar to those reported previously from eastern Uganda (1, 3). Susceptibility results for assays performed on day 1 and day 2 were similar to those performed on day 0, but median IC_{50} values were generally lower over time (Table 1, Fig. 1), with some differences being statistically significant.

In an attempt to explain differences in measured drug susceptibility after storage, we compared parasite growth in samples assayed on days 0, 1, or 2, using SYBR green fluorescence (mean plate fluorescence in drug-free wells minus mean plate fluorescence at the highest drug concentration) as a proxy for growth (5) (Fig. 2). By this

TABLE 1 *Ex vivo* IC_{50} values for *P. falciparum* isolates assayed on the day of collection (day 0) and after storage for 1 (day 1) or 2 days (day 2) at 4°C

	Median IC_{50} (nM) on day:			IC_{50} change between indicated days (%)			
Drug and collection time	**0**	**1**	**2**	**0, 1**	**0, 2**	**1, 2**	***P* value[a]**
Chloroquine							
Days 0 and 1; early processing[b]	22.4	20.8		−7.1			0.03
Days 0, 1, and 2; early processing[c]	19.1	17.9	17.4	−6.3	−8.9	−2.8	0.38
Days 0, 1, and 2; late processing[d]	13.9	13.4	13.0	−3.6	−6.5	−3.0	0.03
Monodesethylamodiaquine							
Days 0 and 1; early processing[b]	7.2	6.6		−8.3			0.003
Days 0, 1, and 2; early processing[c]	6.2	6.1	4.9	−1.6	−21.0	−19.7	0.03
Days 0, 1, and 2; late processing[d]	8.8	8.1	6.7	−8.0	−23.8	−17.2	0.04
Piperaquine							
Days 0 and 1; early processing[b]	7.2	5.9		−18.1			0.04
Days 0, 1, and 2; early processing[c]	3.4	3.3	2.7	−2.9	−20.6	−18.2	0.13
Days 0, 1, and 2; late processing[d]	3.5	3.5	3.3	0.0	−5.7	−5.7	0.76
Pyronaridine							
Days 0 and 1; early processing[b]	1.2	1.0		−16.7			<0.0001
Days 0, 1, and 2; early processing[c]	0.5	0.6	0.6	20.0	20.0	0.0	0.005
Days 0, 1, and 2; late processing[d]	1.3	1.2	0.7	−6.2	−42.6	−38.8	0.08
Lumefantrine							
Days 0 and 1; early processing[b]	5.2	4.7		−9.6			<0.001
Days 0, 1, and 2; early processing[c]	4.3	4.1	3.7	−4.7	−14.0	−9.8	0.09
Days 0, 1, and 2; late processing[d]	7.3	5.6	5.5	−23.3	−24.7	−1.8	0.08
Mefloquine							
Days 0 and 1; early processing[b]	11.3	9.9		−12.4			0.005
Days 0, 1, and 2; early processing[c]	10.2	9.6	7.7	−5.9	−24.5	−19.8	<0.0001
Days 0, 1, and 2; late processing[d]	9.8	9.4	8.0	−4.1	−18.4	−14.9	0.009
Dihydroartemisinin							
Days 0 and 1; early processing[b]	1.9	1.3		−31.6			<0.0001
Days 0, 1, and 2; early processing[c]	1.9	1.3	1.3	−31.6	−31.6	0.0	<0.0001
Days 0, 1, and 2; late processing[d]	1.5	1.5	1.4	−5.2	−11.8	−6.9	0.01
Pyrimethamine							
Days 0 and 1; early processing[b]	39,400	42,330		7.4			0.91
Days 0, 1, and 2; early processing[c]	38,375	33,175	32,375	−13.6	−15.6	−2.4	0.01
Days 0, 1, and 2; late processing[d]	32,090	25,420	23,062	−20.8	−28.1	−9.3	0.09

[a]Wilcoxon matched-pair test for paired tests (day 0 and day 1); Friedman test for repeated measures (day 0, day 1, and day 2); $P < 0.05$ was considered statistically significant.
[b]Samples processed (3 washes of pellets in RPMI medium) on day 0 before storage. $N = 159$.
[c]Samples processed on day 0 before storage. $N = 31$.
[d]Samples processed on days 0, 1, or 2 immediately before assay. $N = 11$.

measure, relative to assays initiated on day 0, parasite growth decreased significantly after storage (4.7% for samples stored for 1 day; 28% after 2 days of storage) (Fig. 2B).

Our results with 159 isolates were based on samples that were processed soon after collection. To assess the impact of storage without prior removal of buffy coat and plasma, we studied 11 isolates that were stored in whole blood without processing, with processing deferred until the day of assay. Results for these samples were similar to those for samples processed on day 0; median IC_{50} values and parasite growth decreased over time (Table 1 and Fig. 1C and 2C).

Our results indicate that storage of fresh *P. falciparum* isolates for up to 2 days at 4°C before initiation of drug susceptibility assays leads to changes in measures of susceptibility. However, changes were generally modest, with differences in IC_{50} values of <10% for 50% and <25% for 89% of comparisons between samples stored for 0, 1, or 2 days (Table 1). Thus, it is feasible to collect parasite isolates from distant sites and transport them to a parasitology laboratory before assays, although this strategy requires maintenance of a cold chain.

Previous studies of *ex vivo* antimalarial drug susceptibility have assayed parasites either immediately after collection or after refrigerated storage (typically up to 24 h), but

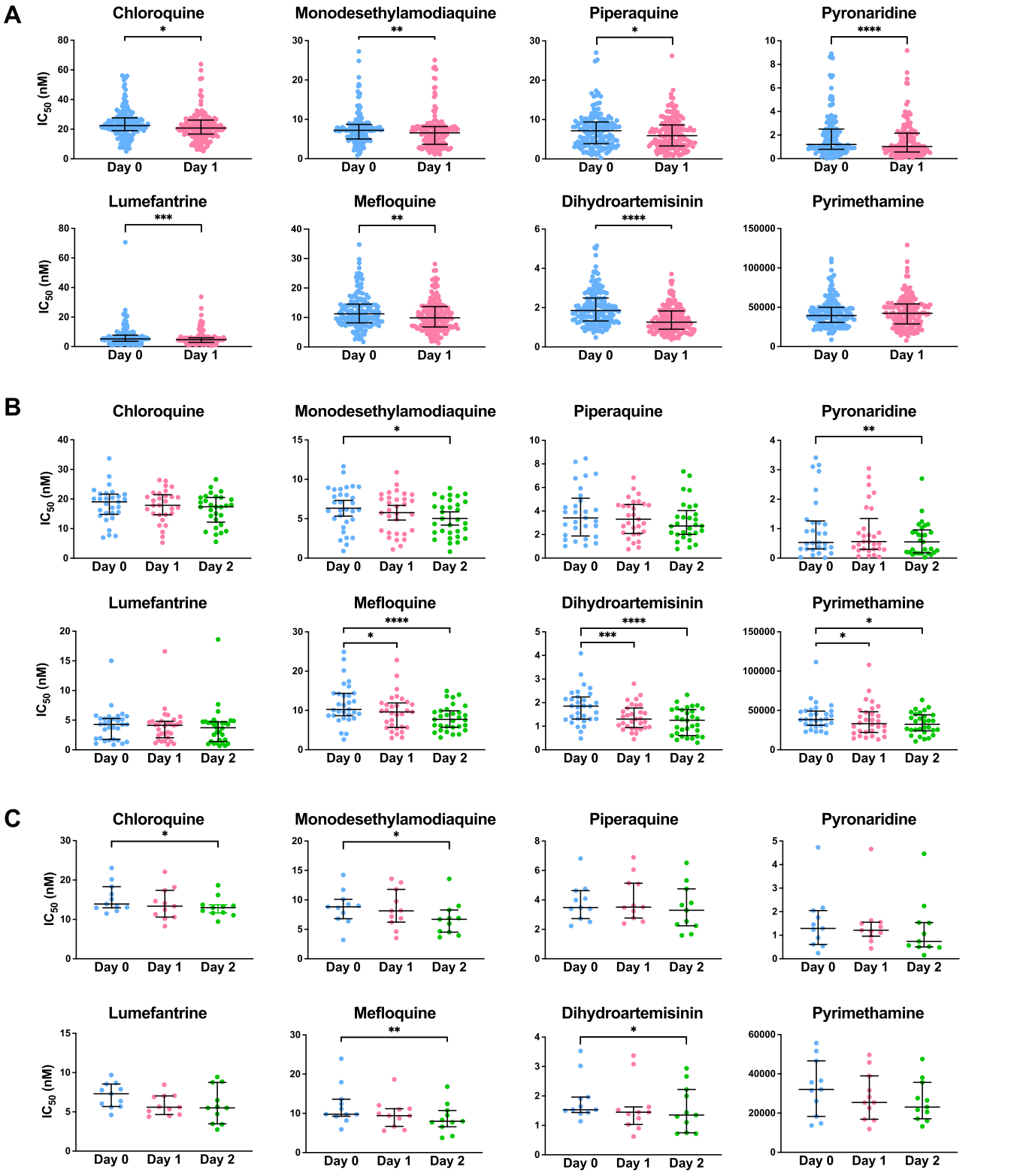

FIG 1 *Ex vivo* growth inhibition assays. Each point represents the result from a single isolate. Median IC_{50} values from 72-h *ex vivo* microplate growth inhibition assays conducted on samples stored at 4°C and assayed on day 0 and day 1 ($N = 159$) (A), samples assayed on days 0, 1, and 2 ($N = 31$) (B), and samples stored at 4°C and processed immediately before assay on days 0, 1, and 2 ($N = 11$) (C). Whiskers depict interquartile ranges. Comparisons of IC_{50}s between groups in panel A were made using the Wilcoxon matched-pairs signed-rank test. Multiple comparisons in panels B and C were made using the Friedman test for repeated measures with *post hoc* analysis using the Wilcoxon signed-rank test with a Bonferroni correction (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

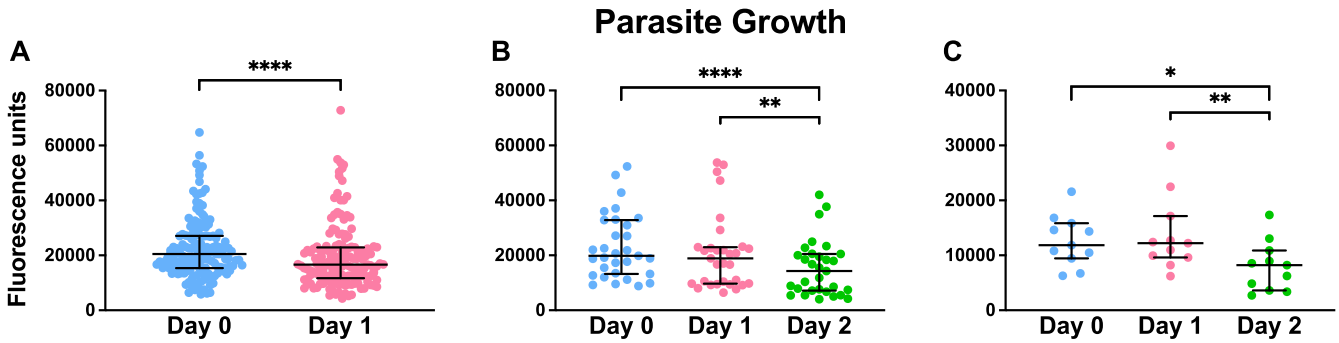

FIG 2 Parasite growth. Each point represents the result from a single isolate. Growth (mean plate fluorescence from untreated controls minus background) in 72-h *ex vivo* drug susceptibility assays from samples stored at 4°C for 0 or 1 day ($N = 159$) (A), for 0, 1, or 2 days ($N = 31$) (B), or for 0, 1, or 2 days but with processing delayed until immediately before assay ($N = 11$) (C). Bars depict medians, whiskers depict interquartile ranges. Groups were compared using the Friedman test for repeated measures, with *post hoc* analysis using the Wilcoxon signed-rank test with a Bonferroni correction (*, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$).

to our knowledge, none have compared susceptibility and parasite growth before and after storage of freshly collected samples. Ring-stage parasites are known to be cold tolerant, with viable parasites recovered from erythrocytes infected with laboratory strains and stored at 4°C for as long as 14 days (6). Field isolates could be successfully cultured after storage at 4°C for 72 h or more (7). Others demonstrated that 24-h storage of laboratory lines and recently adapted field isolates at 4°C eliminated mature parasites, leaving viable ring stages suitable for drug susceptibility studies; in these experiments drug susceptibility and growth did not differ between parasites previously subjected to cold temperatures and those cultured only at 37°C (8). We found that assays conducted after overnight storage were robust, with Z factor values (9) of 0.7 to 0.8, indicating low signal-to-noise ratios in assays of Ugandan isolates (1) and consistent with Z factors obtained from previous studies (7, 10).

Our study had some limitations. First, *ex vivo* dose response assays cannot be repeated, limiting precision of results. However, our large sample size assured us of meaningful results. Second, transport from the clinic to the laboratory and sample processing necessitated delays of some hours before our day 0 analyses. Third, due to protocol changes based on initial results, only 31 isolates were studied 2 days after sample collection and only 11 isolates were studied for the effects of storage in whole blood. However, our sample size was adequate to show similar measures 1 or 2 days after collection and with or without processing on day 0. Fourth, different sites in Africa will vary in terms of malaria transmission intensity, host immunity, and levels of parasitemia, such that our results may not be fully representative of other settings.

Markers of drug resistance may vary between nearby sites, as we recently showed in Uganda (11, 12). Thus, *ex vivo* study of parasites collected over a geographic range will best inform regarding drug susceptibility in a region. Our results offer confidence that it will be possible to survey *ex vivo* susceptibilities of *P. falciparum* from a fairly large geographic area after transporting samples to a central parasitology laboratory.

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REFERENCES

1. Tumwebaze PK, Katairo T, Okitwi M, Byaruhanga O, Orena S, Asua V, Duvalsaint M, Legac J, Chelebieva S, Ceja FG, Rasmussen SA, Conrad MD, Nsobya SL, Aydemir O, Bailey JA, Bayles BR, Rosenthal PJ, Cooper RA. 2021. Drug susceptibility of *Plasmodium falciparum* in eastern Uganda: a longitudinal

phenotypic and genotypic study. Lancet Microbe 2:e441–e449. https://doi.org/10.1016/S2666-5247(21)00085-9.
2. Plowe CV. 2003. Monitoring antimalarial drug resistance: making the most of the tools at hand. J Exp Biol 206:3745–3752. https://doi.org/10.1242/jeb.00658.
3. Rasmussen SA, Ceja FG, Conrad MD, Tumwebaze PK, Byaruhanga O, Katairo T, Nsobya SL, Rosenthal PJ, Cooper RA. 2017. Changing antimalarial drug sensitivities in Uganda. Antimicrobial Agents Chemother 61:e01516-17. https://doi.org/10.1128/AAC.01516-17.
4. Kassambara A. 2019. Practical statistics in R II-comparing groups: numerical variables. https://www.datanovia.com.
5. Smilkstein M, Sriwilaijaroen N, Kelly JX, Wilairat P, Riscoe M. 2004. Simple and inexpensive fluorescence-based technique for high-throughput antimalarial drug screening. Antimicrob Agents Chemother 48:1803–1806. https://doi.org/10.1128/AAC.48.5.1803-1806.2004.
6. Chattopadhyay R, Majam VF, Kumar S. 2011. Survival of *Plasmodium falciparum* in human blood during refrigeration. Transfusion 51:630–635. https://doi.org/10.1111/j.1537-2995.2010.02872.x.
7. Akala HM, Eyase FL, Cheruiyot AC, Omondi AA, Ogutu BR, Waters NC, Johnson JD, Polhemus ME, Schnabel DC, Walsh DS. 2011. Antimalarial drug sensitivity profile of western Kenya *Plasmodium falciparum* field isolates determined by a SYBR Green I in vitro assay and molecular analysis. Am J Trop Med Hyg 85:34–41. https://doi.org/10.4269/ajtmh.2011.10-0674.
8. Yuan L, Hao M, Wu L, Zhao Z, Rosenthal BM, Li X, He Y, Sun L, Feng G, Xiang Z, Cui L, Yang Z. 2014. Refrigeration provides a simple means to synchronize in vitro cultures of *Plasmodium falciparum*. Exp Parasitol 140:18–23. https://doi.org/10.1016/j.exppara.2014.03.010.
9. Zhang J-H, Chung TDY, Oldenburg KR. 1999. A simple statistical parameter for use in evaluation and validation of high throughput screening assays. J Biomol Screen 4:67–73. https://doi.org/10.1177/108705719900400206.
10. Quashie NB, Duah NO, Abuaku B, Quaye L, Ayanful-Torgby R, Akwoviah GA, Kweku M, Johnson JD, Lucchi NW, Udhayakumar V, Duplessis C, Kronmann KC, Koram KA. 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. https://doi.org/10.1186/1475-2875-12-450.
11. Asua V, Conrad MD, Aydemir O, Duvalsaint M, Legac J, Duarte E, Tumwebaze P, Chin DM, Cooper RA, Yeka A, Kamya MR, Dorsey G, Nsobya SL, Bailey J, Rosenthal PJ. 2021. Changing prevalence of potential mediators of aminoquinoline, antifolate, and artemisinin resistance across Uganda. J Infect Dis 223:985–994. https://doi.org/10.1093/infdis/jiaa687.
12. Asua V, Vinden J, Conrad MD, Legac J, Kigozi SP, Kamya MR, Dorsey G, Nsobya SL, Rosenthal PJ. 2019. Changing molecular markers of antimalarial drug sensitivity across Uganda. Antimicrob Agents Chemother 63:e01818-18. https://doi.org/10.1128/AAC.01818-18.