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Lack of Artemisinin Resistance in *Plasmodium falciparum* in Uganda Based on Parasitological and Molecular Assays

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We evaluated markers of artemisinin resistance in *Plasmodium falciparum* isolated in Kampala in 2014. By standard *in vitro* assays, all isolates were highly sensitive to dihydroartemisinin (DHA). By the ring-stage survival assay, after a 6-h DHA pulse, parasitemia was undetectable in 40 of 43 cultures at 72 h. Two of 53 isolates had nonsynonymous K13-propeller gene polymorphisms but did not have the mutations associated with resistance in Asia. Thus, we did not see evidence for artemisinin resistance in Uganda.

Artemisinin-based combination therapy is now the standard for treating *P. falciparum* malaria. However, this regimen is threatened by resistance to artemisinins, which manifests as delayed clearance of parasitemia after therapy, in Southeast Asia (1, 2). Recently, resistance has been associated with increased parasitemia in culture, compared to that in sensitive parasites, 72 h after a 6-h pulse with dihydroartemisinin (DHA) and has been associated with polymorphisms in propeller-encoding domains of the *Plasmodium falciparum kelch* (*K13*; UniProt number PF3D7_1343700) gene (3–6). Although artemisinin resistance is evident to date only in Southeast Asia, the bulk of *P. falciparum* malaria occurs in sub-Saharan Africa. Clinical resistance has not been noted in Africa, as rapid parasite clearance has been the rule in many trials, including those in Uganda (7–9). In recent surveys in Uganda (10) and other locations in Africa (11–15), K13-propeller gene polymorphisms were identified, but these were not the mutations clearly associated with artemisinin resistance in Asian isolates. Results for a new correlate of artemisinin resistance, the *ex vivo* ring-stage survival assay (RSA) (3), have not been reported previously for *P. falciparum* isolates from Africa. We characterized artemisinin sensitivity by this assay and assessed *K13* polymorphisms in isolates from Uganda. Parasites were collected from patients diagnosed with malaria from May to August 2014 at Mulago Hospital, Kampala. Samples were delinked from patient information, so clinical data were unavailable. After malaria diagnosis, excess blood collected in a heparinized tube as part of clinical care was promptly delivered to the laboratory. Giemsa-stained thin smears were made, and samples containing only *P. falciparum* isolates with parasitemia of ≥0.1% were placed in culture after washing of erythrocytes, as previously described (16); blood was also spotted onto filter paper. Samples with parasitemia of >1% were diluted with uninfected erythrocytes for a final parasitemia of 1%.

The susceptibility of fresh isolates to DHA and chloroquine was assessed by a standard *ex vivo* histidine-rich protein 2-based enzyme-linked immunosorbent microplate assay, as previously described (17), except that drugs were freshly diluted from frozen stocks prior to each assay. Results from single assays were fitted to a variable-slope sigmoidal function using GraphPad Prism 6.0f to generate 50% inhibitory concentrations (IC_{50}s). All data were visually assessed for parasite growth above the background and integrity of curve fits.

Parasite susceptibility to DHA was also assessed using the new *ex vivo* RSA (3). First, 20-μl erythrocyte pellets were added to 1.0 ml RPMI 1640 medium supplemented with 25 mM HEPES, 0.2% $NaHCO_3$, 0.1 mM hypoxanthine, 100 μg/ml gentamicin, and 0.5% Albumax I (Invitrogen) containing 700 nM DHA or, for controls, 0.1% dimethyl sulfoxide (DMSO) at a hematocrit of 2%. Cultures were incubated at 37°C under 3% O_2, 5% CO_2, and 92% N_2 and, after 6 h, washed 3 times with 10 ml RPMI prewarmed to 37°C; then, they were placed in drug-free culture medium. At 72 h after assay initiation, Giemsa-stained thin smears were prepared. Cultures were considered viable and appropriate for assessment if control parasitemia increased since the culture initiation. Parasitemias in the control cultures were determined by counting parasites per 1,000 to 2,000 erythrocytes. In DHA-pulsed cultures, 10,000 to 20,000 cells were scanned for parasites. RSA survival rates were expressed as the proportion of parasites in the DHA-treated cultures relative to controls at the end of the 72-h assay. For some samples, cultures were maintained for up to 4 weeks after initiation of the experiment, with smears assessed on alternating days. For positive cultures, blood spots were collected on filter paper.

DNA was extracted from filter paper blood spots using Chelex-100, and gene fragments spanning loci of interest were amplified by nested PCR (10, 18). K13-propeller-encoding domains (codons 440 to 726) were dideoxy sequenced, as previously de-

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TABLE 1 *Ex vivo* measures of drug sensitivity and molecular features of Ugandan parasites[a]

Sample no.	CQ IC_{50} (nM [95% CI])[b]	DHA IC_{50} (nM [95% CI])	RSA at 72 h (%)[c]	K13	PfCRT aa 76[d]	PfMDR1 aa[d]				
						86	184	1034	1024	1246
1	15.6 (14.6–16.6)	2.0 (1.7–2.4)	0	WT[e]	K	N	F	S	N	D
2	123 (84.4–180)	2.0 (1.5–2.6)	0	WT	T	N/Y	Y/F	S	N	Y
3	38.8 (23.5–64.3)	1.1 (1.0–1.3)	0	WT	K/T	N/Y	Y/F	S	N	D
4	11.2 (9.7–13)	1.4 (1.2–1.5)	0	WT	K	N	F	S	N	Y/D
5	260 (154–440)	2.3 (1.6–3.4)	0	WT	T	N	Y	S	N	Y
6	196 (127–300)	1.8 (1.5–2.0)	0	WT	T	N	F	S	N	D
7	9.8 (9.2–10.5)	1.3 (0.9–1.7)	0	WT[f]	K	N	F	S	N	D
8	155 (133–182)	2.3 (2.0–2.6)	0	WT	T	N	Y/F	S	N	Y
9	141 (86.7–228)	1.5 (1.2–1.9)	0	WT	T	N	Y/F	S	N	D
10	139 (116–166)	2.0 (1.8–2.2)	0.7	WT	T	N	F	S	N	D
11	1060 (733–1520)	2.2 (2.0–2.5)	0	WT	T	N	Y	S	N	D
12	222 (169–291)	1.6 (1.5–1.7)	0	A578S[g]	T	N	F	S	N	D
13	204 (147–283)	2.7 (2.3–3.2)	0	WT	T	N	Y/F	S	N	D
14	240 (183–313)	0.47 (0.45–0.49)	ND	WT	T	N	Y	S	N	D
15	182 (144–231)	0.90 (0.81–0.93)	ND	WT	T	N/Y	Y	S	N	D

[a] Data are shown for isolates for which IC_{50} and *K13* sequence data are available. WT, wild type; ND, no data collected.
[b] CQ, chloroquine; CI, confidence interval; the 95% CI describes precision of the IC_{50} determined from the curve fit. *Ex vivo* IC_{50} tests are single assays.
[c] Percentage of parasites in DHA-treated cultures relative to controls 72 h after initiation of a 6-h DHA pulse.
[d] aa, amino acid. Wild-type PfCRT is K76, and wild-type PfMDR1 is NYSND.
[e] Synonymous single nucleotide polymorphism (SNP) t1512c.
[f] Synonymous SNP c1434g.
[g] Reported from Tororo, Uganda (10), Democratic Republic of Congo (14), and Bangladesh (23).

scribed (4). To detect polymorphisms in *pfcrt* and *pfmdr1*, multiplex ligase detection reaction-fluorescent microsphere assays were performed, as previously described (19, 20).

We collected 53 isolates from patients with malaria from May to July 2014. Giemsa-stained thin smears from all blood samples demonstrated *P. falciparum* only, at parasitemias of 0.1% to 24.3%. We determined *ex vivo* IC_{50} data for 15 isolates. Values ranged from 0.4 to 2.7 nM (geometric mean, 1.6 nM) for DHA and 9.8 to 1,060 nM for chloroquine (Table 1).

As standard drug sensitivity assays have not identified artemisinin resistance in Asian isolates, we also assessed DHA susceptibility using the *ex vivo* RSA (3). Ten isolates were lost to contamination or failed to grow. For the remaining 43 isolates, assessed 72 h after culture initiation, most DHA-pulsed cultures contained pyknotic forms, and only 3 of 43 demonstrated healthy-appearing parasites, all with parasitemia that was ≤0.025%. Of the 3 positive cultures, a single parasite was detected per ~15,000 erythrocytes in 2 cultures, and 3 parasites were detected in the third (RSA survival rates, 0.7% to 1.9%). All 43 untreated control cultures demonstrated increased parasitemia and healthy-appearing parasites over the course of the assay. Because of the challenges of quantifying extremely low parasitemia, we assessed the viability of 14 DHA-pulsed cultures that did not demonstrate parasites after 72 h. These cultures were maintained for up to 30 days after initiation of the experiment. Twelve of these cultures demonstrated parasites, beginning 15 to 29 days after culture initiation; 2 cultures remained negative at 30 days. Thus, we did not observe enhanced growth of DHA-pulsed parasites after 72 h in culture, arguing against the presence of the artemisinin-resistant phenotype, although most cultures survived the DHA pulse.

Sequencing the K13-propeller-encoding domains from the 53 isolates revealed two nonsynonymous mutations, S552C (mixed with the wild-type sequence) and A578S, in propeller domains 2 and 4, respectively (Fig. 1). Ten isolates that grew in culture 15 to 29 days after the DHA pulse all had wild-type *K13* sequences. Two of these samples were from isolates that were positive by microscopy 72 h after the DHA pulse. Considering the putative drug transporters encoded by *pfcrt* and *pfmdr1*, polymorphism prevalences were similar to those described recently from other regions of Uganda, with an increased prevalence of wild-type sequences at *pfcrt* K76, *pfmdr1* N86, and *pfmdr1* D1246 compared to older studies (Fig. 1) (20, 21). Sensitivity to chloroquine correlated well with the *pfcrt* K76T sequence, with IC_{50}s that were >120 nM for all mutant parasites, <16 nM for all wild-type parasites, and intermediate for one mixed sample (Table 1).

Artemisinin resistance, with delayed clearance of parasites after treatment with artemisinin monotherapy or artemisinin combination therapies (ACTs), is of great concern but not clearly present in Africa, where clearance of parasites after treatment with ACTs has generally been rapid (2, 13). Parasites from Southeast

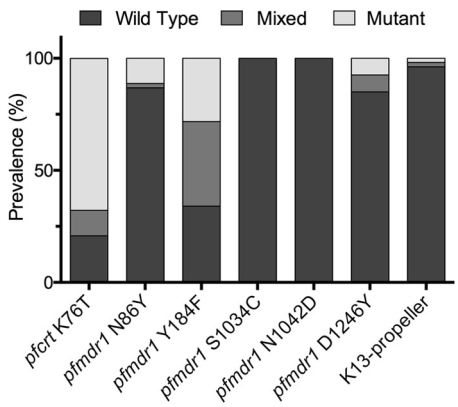

FIG 1 Prevalences of wild-type, mixed, and mutant sequences at the indicated positions.

Asia with delayed clearance typically do not show decreased sensitivity with standard *in vitro* assays but demonstrate decreased sensitivity to a DHA pulse in the RSA (3) and have specific mutations in K13-propeller-encoding domains (4). We screened fresh *P. falciparum* isolates from Uganda for these artemisinin resistance markers. Using the RSA, nearly all freshly isolated Ugandan parasites had undetectable parasitemia 72 h after exposure to a pulse of DHA. In contrast, DHA-treated Southeast Asian parasites had measurable parasitemia at 72 h, with parasitemia greater in those with delayed clearance in clinical trials and the presence of K13-propeller gene polymorphisms (3). A small percentage of Ugandan parasites had K13-propeller gene mutations, but these were not the mutations previously associated with drug resistance. Thus, our results suggest that artemisinin resistance is not yet a problem in Uganda.

Recent evidence suggests spreading of artemisinin resistance beyond the initial reports from Cambodia (1), with the delayed clearance phenotype and *K13* mutations detected in parts of Thailand, Vietnam, Myanmar, and southern China (2, 6, 22). Additional K13-propeller gene mutations have been identified from other regions of Asia (23) and Africa (11-13, 15), but these have not been among the >10 mutations linked with delayed clearance in Southeast Asia (4, 22). The *K13* mutations detected in our study, S522C and A578S, have been found elsewhere in Africa and in Bangladesh, but they were not associated with a resistance phenotype (14, 15, 23). Considering our ring-stage survival assay results in which 40 of 43 DHA-pulsed samples were smear-negative, it appeared that those parasites were more susceptible to a DHA pulse than even susceptible parasites from Southeast Asia (3). With prolonged observation, most samples showed regrowth of parasites, indicating that, although its effect was marked, the 6-h DHA pulse generally did not kill all parasites.

Our results are reassuring concerning artemisinin resistance in Uganda, but they must be considered together with results showing important changes in *P. falciparum* in the country in recent years. With adoption of artemether-lumefantrine as standard therapy for malaria within the last decade, parasites have demonstrated increasing prevalence of *pfcrt* and *pfmdr1* alleles that mediate decreased lumefantrine sensitivity (20) and, in *ex vivo* assays, decreasing sensitivity to lumefantrine (17). Thus, even without the artemisinin resistance phenotype, changes in parasite drug sensitivity may threaten the antimalarial efficacies of ACTs, in particular artemether-lumefantrine, in Uganda.

Our study had some limitations. We studied a convenience sample of isolates, and consideration of host factors that may have impacted on parasite biology or genetics was not possible. The total number of isolates studied was small, and we were able to perform some assays only in subsets of these isolates. We also could not determine how an elevated survival phenotype would manifest in our *ex vivo* assay, since resistance-conferring *K13* mutations were not evident in our study. Further, identification of unusual polymorphisms may have been difficult in samples with high complexity of infection, as is common in Uganda. For these reasons we may have missed uncommon resistance markers, but our results nonetheless argue against significant artemisinin resistance in Uganda at this time.

Taken together, results from our parasitological and molecular assessments and from recent clinical trials suggest that artemisinin resistance is not yet an important problem in Uganda. However, the recent spread of resistance in Asia suggests that the introduction or *de novo* emergence of resistance in Africa, the major world focus of *P. falciparum* malaria, is likely. Further, altered sensitivity to artemisinin partner drugs already threatens the efficacy of ACTs for the treatment of *P. falciparum* malaria in Africa (17, 20). Thus, regular surveillance for markers of artemisinin resistance is needed across Africa.

Nucleotide sequence accession numbers. Nucleotide sequence data are available in the GenBank database under accession numbers KR055739 to KR055804.

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