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Recommended Citation  
https://scholar.dominican.edu/all-faculty/156

DOI  
http://dx.doi.org/https://doi.org/10.1128/AAC.00921-15

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

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c

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 prepared, 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 (IC50%). 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% NaHCO3, 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% O2, 5% CO2, and 92% N2 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-
and 4, respectively (Fig. 1). Ten isolates that grew in culture 15 to with the wild-type sequence) and A578S, in propeller domains 2 isolates revealed two nonsynonymous mutations, S552C (mixed guing against the presence of the artemisinin-resistant phenotype, enhanced growth of DHA-pulsed parasites after 72 h in culture, ar- parasites remained negative at 30 days. Thus, we did not observe en- cultures, beginning 15 to 29 days after culture initiation; 2 cul- lation of the experiment. Twelve of these cultures demonstrated 72 h. These cultures were maintained for up to 30 days after initi- quantifying extremely low parasitemia, we assessed the viability of demonstrated increased parasitemia and healthy-appearing para- survival rates, 0.7% to 1.9%). All 43 untreated control cultures in 2 cultures, and 3 parasites were detected in the third (RSA /H11011 cultures, a single parasite was detected per ex vivo P. falciparum demonstrated to July 2014. Giemsa-stained thin smears from all blood samples /H11349 Coefficients of variation in drug sensitivity data were reported as percentage of parasites in DHA-treated cultures relative to controls 72 h after initiation of a 6-h DHA pulse. /H11021 Table 1 Ex vivo measures of drug sensitivity and molecular features of Ugandan parasitesa

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>CQ IC50 (nM [95% CI])b</th>
<th>DHA IC50 (nM [95% CI])b</th>
<th>RSA at 72 h (%)c</th>
<th>K13</th>
<th>PCRT aa 76d</th>
<th>PfMDR1 aaed</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>15.6 (14.6–16.6)</td>
<td>2.0 (1.7–2.4)</td>
<td>0</td>
<td>WTf</td>
<td>K</td>
<td>N/F S N D</td>
</tr>
<tr>
<td>2</td>
<td>123 (84.4–180)</td>
<td>2.0 (1.5–2.6)</td>
<td>0</td>
<td>WT</td>
<td>T</td>
<td>N/Y F S Y</td>
</tr>
<tr>
<td>3</td>
<td>38.8 (23.5–64.3)</td>
<td>1.1 (1.0–1.3)</td>
<td>0</td>
<td>WT</td>
<td>K/T</td>
<td>N/Y F S N D</td>
</tr>
<tr>
<td>4</td>
<td>11.2 (9.7–13)</td>
<td>1.4 (1.2–1.5)</td>
<td>0</td>
<td>WT</td>
<td>K</td>
<td>N/F S N Y/D</td>
</tr>
<tr>
<td>5</td>
<td>260 (154–440)</td>
<td>2.3 (1.6–3.4)</td>
<td>0</td>
<td>WT</td>
<td>T</td>
<td>N Y S N Y</td>
</tr>
<tr>
<td>6</td>
<td>196 (127–300)</td>
<td>1.8 (1.5–2.0)</td>
<td>0</td>
<td>WT</td>
<td>T</td>
<td>N F S N D</td>
</tr>
<tr>
<td>7</td>
<td>9.8 (9.2–10.5)</td>
<td>1.3 (0.9–1.7)</td>
<td>0</td>
<td>WTf</td>
<td>K</td>
<td>N/F S N D</td>
</tr>
<tr>
<td>8</td>
<td>155 (133–182)</td>
<td>2.3 (2.0–2.6)</td>
<td>0</td>
<td>WT</td>
<td>T</td>
<td>N/Y F S N Y</td>
</tr>
<tr>
<td>9</td>
<td>141 (86.7–228)</td>
<td>1.5 (1.2–1.9)</td>
<td>0</td>
<td>WT</td>
<td>T</td>
<td>N Y F S N D</td>
</tr>
<tr>
<td>10</td>
<td>139 (116–166)</td>
<td>2.0 (1.8–2.2)</td>
<td>0.7</td>
<td>WT</td>
<td>T</td>
<td>N F S N D</td>
</tr>
<tr>
<td>11</td>
<td>1060 (733–1520)</td>
<td>2.2 (2.0–2.5)</td>
<td>0</td>
<td>WT</td>
<td>T</td>
<td>N Y S N D</td>
</tr>
<tr>
<td>12</td>
<td>222 (169–291)</td>
<td>1.6 (1.5–1.7)</td>
<td>0</td>
<td>A578Sf</td>
<td>T</td>
<td>N F S N D</td>
</tr>
<tr>
<td>13</td>
<td>204 (147–283)</td>
<td>2.7 (2.3–3.2)</td>
<td>0</td>
<td>WT</td>
<td>T</td>
<td>N Y/F S N D</td>
</tr>
<tr>
<td>14</td>
<td>240 (183–313)</td>
<td>0.47 (0.45–0.49)</td>
<td>ND</td>
<td>WT</td>
<td>T</td>
<td>N Y S N D</td>
</tr>
<tr>
<td>15</td>
<td>182 (144–231)</td>
<td>0.90 (0.81–0.93)</td>
<td>ND</td>
<td>WT</td>
<td>T</td>
<td>N/Y Y S N D</td>
</tr>
</tbody>
</table>

Data are shown for isolates for which IC50 and K13 sequence data are available. WT, wild type; ND, no data collected. 
Percentage of parasites in DHA-treated cultures relative to controls 72 h after initiation of a 6-h DHA pulse. 
aa, amino acid. Wild-type PCRT is K76, and wild-type PfMDR1 is NYSND. 
Synonymous single nucleotide polymorphism (SNP) 11512c. 
Synonymous SNP c1434g. 
Reported from Tororo, Uganda (10), Democratic Republic of Congo (14), and Bangladesh (23). 

a 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 IC50 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 IC50s 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 Asia are reported to have up to 200-fold lower sensitivity to DHA compared to isolates from Southeast Asia and India (22). As a result, the use of ACTs has generally been rapid (2, 13). Parasites from Southeast
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 generally did not kill all parasites. This appeared that those parasites were more susceptible to a DHA pulse. 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 pfkrt and pfmdr1 alleles that mediate decreased lumefantrine sensitivity (20) and, in ex vivo assays, decreasing sensitivity to lumefantrine (17). Thus, even within 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 K0R55739 to K0R55804.

ACKNOWLEDGMENTS

We thank Jenny Legac, Michelle Verghese, and Hadjiah Nalubega for expert technical assistance.

This work was supported by research grants (AI1R01AI075045 and U19AI089674) and training programs (Training in Malaria Research in Uganda [D43TW000737]) and University of California, Berkeley, Minority Health & Health Disparities International Research Training [T37MD003407], all from the National Institutes of Health. R.A.C. was supported by a travel grant from Dominican University of California.

We have no commercial or other associations that may pose a conflict of interest regarding this research.

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