Assessment of Artemisinin Tolerance in Plasmodium Falciparum Field Isolates in Children with Uncomplicated Malaria in a Ghanaian Population

Samuel Yao Ahorhorlu
University of Ghana Medical School, University of Ghana

Neils Ben Quashie
University of Ghana Medical School, University of Ghana

Rasmus Weisel Jensen
University of Copenhagen

William Kudzi
University of Ghana Medical School, University of Ghana

Edmund Tetteh Nardey
University of Ghana Medical School, University of Ghana

Nancy Odurowah Duah-Quashie
University of Ghana

Felix Zoiku
University of Ghana

Bartholomew Dzudzor
University of Ghana, University of Ghana

Christian William Wang
University of Copenhagen

Helle Hansson
University of Copenhagen

Michael Alifrangis
University of Copenhagen

George Obeng Adjei (gadjei@ug.edu.gh)
University of Ghana Medical School, University of Ghana

Research Article

Keywords: Artemisinin tolerance, Plasmodium falciparum, Uncomplicated Malaria, Ex vivo ring-stage survival assay, Pfk13, Pfcoronin, Ghana
Abstract

Background

Artemisinin-based combination therapies (ACTs) are the first-line treatments for uncomplicated malaria in Ghana. Artemisinin (ART) tolerance in *Plasmodium falciparum* has arisen in Southeast Asia (SEA) and recently, in parts of East Africa. This is ascribed to the survival of ring-stage parasites post treatment. We sought to assess and characterize correlates of potential ART tolerance based on post-treatment parasite clearance, *ex vivo* and *in vitro* drug vulnerability, and molecular markers of drug resistance in *P. falciparum* isolates from children with uncomplicated malaria in Ghana.

Methods

Six months to fourteen years old children having uncomplicated malaria (n = 115) were enrolled from two hospitals and a health center in Ghana's Greater Accra region and treated with artemether-lumefantrine (AL) according to body weight. Pre- and post-treatment parasitemia (day 0 and day 3) was confirmed by microscopy. The *ex vivo* ring-stage survival assay (RSA) was used to detect percent ring survival while the 72 hr SYBR Green 1 assay was used to measure the 50% inhibition concentration (IC$_{50}$s) of ART and its derivatives and partner drugs. Genetic markers of drug tolerance /resistance were evaluated using selective whole genome sequencing.

Results

Of the total of 115 participants, 85 were successfully followed up on day 3 post-treatment and had 2/85 (2.4%) parasitemia. The IC$_{50}$ values of ART, artesunate (AS), artemether (AM), dihydroartemisinin (DHA), amodiaquine (AQ), and lumefantrine (LUM) were not indicative of drug tolerance. However, 7/90 (7.8%) pre-treatment isolates had > 10% ring survival rates against DHA. Of the four isolates (2 RSA positive and 2 RSA negative) with high genomic coverage, *P. falciparum* kelch 13 K188* and Pfcoronin V424I mutations were only present in the two RSA positive isolates with > 10% ring survival rates.

Conclusions

The observed low proportion of participants with post-treatment parasitemia suggests AL remains efficacious. However, the increased rates of survival observed in the *ex vivo* RSA against DHA, maybe a pointer of an early start of ART tolerance. Furthermore, the role of two novel mutations in PfK13 and Pfcoronin genes, harbored by the two RSA positive isolates that had high ring survival in our study, remains to be elucidated.
Artemisinin (ART) resistance or more appropriately tolerance, by *Plasmodium falciparum* has been reported mostly in Southeast Asia (SEA) and partly in East Africa (1–3). This is a cause for concern as any widespread resistance to ARTs and artemisinin-based combination therapies (ACTs) in the absence of other potent, safe, and effective antimalarial drugs, could reverse the clock of progress in the fight against global malaria. This will consequently be dire for sub-Saharan Africa (SSA) where the disease is most prevalent and a major public health burden (4).

Taking a cue from the devastating effects of *P. falciparum* resistance to chloroquine, which originated from SEA and spread to East Africa and then to other parts of SSA, monitoring and surveillance of parasite dynamics in drug susceptibilities is important. These strategies remain key in detecting subtle changes in local malaria parasite populations although currently clinically indiscernible, could eventually lead to ART tolerance / resistance. Dondorp et al. described the observation of a subset of surviving ring-stage *P. falciparum* parasites following treatment with artesunate (AS) monotherapy or ACTs as delayed parasite clearance (1). This observation was subsequently made by other studies (5, 6), however “true” artemisinin resistance remains to be confirmed (7). Recent *in vitro* and *in vivo* studies have postulated molecular grounds for delayed parasite clearance by implicating several mutations in the *P. falciparum* kelch 13 (PfK13) propeller domain (1, 8–10). The WHO Global Malaria Programme Status Report on ARTs and ACTs efficacy 2016, defines delayed parasite clearance as partial ART resistance (7); while describing “having 10% or more patients with persistent parasitemia by microscopy at 72 hours (± 2 hours; i.e. day 3); or having 10% or more patients with a half-life of the parasite clearance slope at 5 or more hours after treatment with AS monotherapy or ACTs; or having 5% or more patients harboring K13 resistance confirmed mutations, as suspected endemic ART resistance” (7). Per the same document, having 5% or more patients harboring K13 resistance confirmed mutations, with all found to have either persistent parasitemia by microscopy on day 3 following treatment with AS monotherapy or ACT, or with half-life of the parasite clearance slope of 5 or more hours, is described as confirmed endemic ART resistance (7).

Studies conducted in East African countries, Rwanda, and Uganda, has revealed a recent independent emergence of partial ART resistance with the observation of increased clones of confirmed PfK13 mutations R561H and A675G, respectively for the two countries (11, 12). Though ACTs continue to be effective in these countries with no swift consequence for patients, there is apprehension that this partial ART resistance could mediate ACT partner drug resistance spread in the WHO African Region (4).

The ART-tolerant *P. falciparum* phenotype, however, appears to be evolving and so the above characterization may not be conclusive in describing the emerging tolerance; for instance, there are reports of PfK13-independent ART or ACTs tolerance (13–15). Witkowski et al. established that *ex vivo* RSA threshold of 10% ring survival was closely associated with delayed parasite clearance after drug treatment and considered this a surrogate for the ART-resistant phenotype (16, 17). Presently in Ghana, there is no clinically reported ART resistance, however, the prospect of low-key changes taking place in our local parasite population which could eventually lead to the emergence of ART-tolerance cannot be ruled out. Local evolutionary conditions of ART tolerance may vary between geographical regions (18). Continuous search for potential predictors of early onset of parasite tolerance to ART and its derivatives
in endemic settings such as Ghana is therefore warranted. Thus, we sought to assess and characterize correlates of potential ART tolerance based on posttreatment parasite clearance, ex vivo and in vitro drug sensitivity, and genetic markers of tolerance / resistance in parasite isolates from children with uncomplicated malaria in Ghana's Greater Accra region.

Methods

Study design

This was a longitudinal, single-arm, prospective study to evaluate *P. falciparum* tolerance to ART and its derivatives in children with uncomplicated malaria aged 6 months to 14 years in 3 health facilities in the Greater Accra region of Ghana. The study focused mainly on day 3 post artemether-lumefantrine (AL) treatment parasitemia, 72-hour ex vivo parasite clearance post dihydroartemisinin (DHA) treatment, 72-hour parasite clearance in vitro against a panel of 6 drugs: ART, AS, artemether (AM), DHA, amodiaquine (AQ), lumefantrine (LUM), and molecular markers of drug tolerance. It sought to set up correlates of ART tolerance.

Study Sites

This research was carried out in the Princess Marie Louise Children's Hospital (PMLCH), Accra, the Danfa Health Center (DHC), La Nkwantanang-Madina District, and the Shai Osudoku District Hospital (SODH), Dodowa, in the region of Greater Accra, Ghana. These health facilities are situated in the South-east shoreside expanse of the country with about nine months season of annual transmission of malaria increasing from May through to November. The Greater Accra Region has a malaria prevalence of 5% in children under 5 per the 2016 Ghana Malaria Indicator Survey (19). The PMLCH is a 74-bed capacity government hospital located in central Accra, that offers principal healthcare. It is second to the Korle-Bu Teaching Hospital (KBTH) in terms of specialist pediatric care services in Accra where children (1 day to 17yrs old) are seen (20). Over the last ten years, the Out-patient (OPD) attendance in this facility has increased from 45,000 in 1996 to almost 73,000 per year (20). The SODH, is an ultra-modern hospital facility that has a bed capacity of 125, 6 wards and 2 operating theatres, that serves as a sentinel site for the National Malaria Control Program (NMCP) of Ghana and other tropical diseases. The DHC is a University of Ghana Medical School facility that serves about 11, 000 inhabitants in the La-Nkwantanang-Madina district and neighboring communities. Initially set up for the training of general medical practitioners in the rural areas to offer effective healthcare and services such as family planning through research by the department of community health of the school, the facility also serves as a sentinel site for the NMCP.

Study Population
The study involved 115 Ghanaian children with uncomplicated malaria per WHO criteria (21) aged 6 months to 14 years having fever (>37.5°C) measured at the time of admission, parasitemia in the range of 1000 to 250,000 per microliter of blood, with no known incidence of hypersensitivity reactions to AL, after obtaining informed consent. Children with severe malaria, malnutrition, anemia (haemoglobin < 8 g/dL), or who were RDT positive for malaria but microscopy negative for malaria were excluded from the study. Participant enrolment was carried out between June and November 2018. Socio-demographic and relevant clinical data were collected from each participant and 5 mL of venous blood was obtained (prior to AL treatment) for the assays.

Detection Of Day 3 Post-treatment Parasitemia

Day 3 parasitemia post-treatment with AL was evaluated via light microscopy under oil-immersion. Summarily, both thin and thick blood smears were made and stained with Giemsa-stain after air-drying the methanol-fixed thin smear in accordance with standard guidelines. Detection of parasite load per microliter of blood was achieved by the count of parasites per 200 WBCs or 500 WBCs, times 40 or 16 apiece per standard protocol (22). The slides were read by two microscopists independent of each other and results were recorded as positive when both microscopists recorded a specimen as positive. Provision was made for a third microscopist should the readings of the two be discordant, for quality control purposes.

Detection Of Percent Ring-stage Survival Rates

Percent ring-stage parasite survival was detected by carrying out ex vivo RSA on newly obtained \textit{P. falciparum} field parasites as previously presented by Witkowski \textit{et al}. (23). In brief, parasite density was lowered to 1% and the hematocrit altered to 2% for every sample with uninfected O-positive red blood cells (RBCs). Parasite count was done and noted as ‘initial parasitemia’. The test was performed in a 48-well plate having a well with DHA and another well without DHA (contains dimethyl sulfoxide, DMSO) as control well. To both wells were added \textit{P. falciparum}-infected RBC suspension. The contents were gently mixed, after which the plates were incubated at 37°C under standard conditions for exactly 6 hours. The samples were then washed in Roswell Park Memorial Institute (RPMI) medium for excess drug removal. Complete medium was used to again mix the cells and the mixture was incubated for 66 hours under the same conditions. To differentiate viable and dead parasites, microscopic examination under 100x oil-immersion was carried out on a thin blood smear made 72 hours after drug exposure. Viable parasites were quantified as follows: Growth rate was computed as parasites count from the non-exposed well / initial parasitemia, while Percent ring survival (%) was computed as parasite count from DHA-exposed well / parasitemia from DHA non-exposed well) x 100. Percent survival rates were only explicable for growth rate of 1 or more. Information on how the parasite mix and assay reagents were prepared is provided as Supplementary Information.
Detection Of Molecular Markers Of Art Tolerance

DNA was extracted from malaria positive pre-treatment whole blood samples by use of the QIAamp 96 DNA Blood Kit per the manufacturer's protocol. Selective whole genome sequencing (sWGS) was done for a set of isolates with > 10% ring survival (n = 4) and a few isolates which were sensitive to DHA (n = 3) in the ex vivo RSA to detect molecular markers of ART tolerance in P. falciparum. The molecular markers detected were focusing on identifying single nucleotide polymorphisms (SNPs) in the genes coding for the proteins: P. falciparum kelch 13 (PfK13), Pfcorionin, multidrug resistance protein 1 (Pfmdr1), multidrug resistance protein 2 (Pfmdr2), dihydropteroate synthetase (Pfdhps), dihydrofolate reductase (Pfdhfr), signal peptide peptidase and multidrug resistance-associated protein 2 genes. The 3D7 reference genome (GCA_000002765.3, Sep 01, 2020, Mar 01, 2022) was used for the comparative analysis to detect the SNPs. Briefly, selective PCR was carried out using 10 short oligonucleotide probes of 8–12 mers as primers and Phi29 (Φ29) DNA polymerase (extracted from Bacillus subtilis phage) to increase the genome of selected parasites (without the telomeric regions) for the sWGS following the protocol as already detailed by Oyola et al. (24). Initially, the FastQC was used to quality-assess the Illumina paired-end sequencing reads for the gene variation analysis. Trimming of the reads was done via a Phred ≥ 20 quality score after which comparison of the reads were made to the 3D7 laboratory strain reference genome using the Interactive genome viewer version 2.6.3 (IGV). For each selected gene, the matching location of the genome and gene ID were verifed in the PlasmoDB database (PlasmoDB rel. 9.0, 2012-MAY-18). From the IGV each gene was inspected for SNPs, deletions, or insertions in comparison to the 3D7 reference genome. Only genes that had a adequate coverage got analyzed and each verified SNP was converted into AA sequence and recorded for each RSA “tolerant” or sensitive isolate.

Supplementary Table ST1, 2, & 3 and Supplementary Figure SF1 has information on primer sequences, the PCR reaction mix, the PCR thermal cycling conditions, and an image of the gel of the PCR products of the isolates of interest for the sWGS.

Detection of in vitro ART and its derivatives and Partner drugs Tolerance

The 50% inhibition concentrations of ART, AS, AM, DHA, LUM, and AQ (IC\textsubscript{50}) were detected for isolates of interest by the SYBR Green 1 fluorescence-based in vitro drug vulnerability assay method previously described by Johnson et al. (25). Briefly, parasite mix was added to pre-dosed plates with the test drugs, placed in a hypoxic chamber and incubated at 37°C under standard conditions (2.03% O\textsubscript{2},
5.53% CO₂, and 92.44% N₂) for 72 hours. The 3D7 laboratory reference clone served as internal control. About 100 µL SYBR Green contained in Malaria SYBR Green 1 fluorescent (MSF) lysis buffer was added to each well and the content thoroughly mixed, wrapped in aluminium foil and kept in the dark for 2 hours, after which the microtiter plate reader was used to measure fluorescence. The Worldwide Antimalarial Resistance Network (WWARN) online automated “in vitro analysis and reporting tool (IVART)” (26) and GraphPad Prism version 7.0 were used to calculate the IC₅₀ values. The geometric mean IC₅₀ for the combined data from the study sites was detected for the 6 selected drugs. The IC₅₀ threshold values from literature denoting resistance employed in our study were 20nM for AS; 30Nm for AM; 12nM for DHA; 12nM for ART; 80nM for AQ; and 150nM for LUM (27).

Information on the preparation of the drugs and the parasite mix are included in the Supplementary Information.

Data analysis

Statistical analysis was carried out using GraphPad Prism version 7.0. Data for the IC₅₀ values was first transformed logarithmically, analyzed, and reported as geometric mean of the IC₅₀ together with their 95% confidence interval. Percent ring stage parasite survival was evaluated by parasite-infected cells count in 10,000 RBCs as compared with survival of parasites in the non-DHA exposed well.
The Spearman correlation test was employed to detect any association that may exist between the ex vivo RSA sensitive and tolerant isolates and potential resistance mutations from the sWGS. Association between percent ring survival and the IC\textsubscript{50} values of the test drugs was evaluated by the Pearson correlation analysis. The association is deemed significant at a \( P \)-value below 0.05.

Results

The median age, temperature, hemoglobin, and parasitemia of the 115 study participants were respectively 72 months, 38\(^\circ\)C, 10.3g/dl, and 39.014 parasites/\( \mu \)l (Table 1). About 55 (\( n = 55, 47.8\% \)) of the study participants were females. Children < 5 years (i.e., 6–59 months) constituted most of the study participants (51/115 (44.35%). Children (10 to 14 years) were (45/115 (39.13%)), and those between age 5 and 9 years were 19/115 (16.52%) (Table 1).
Table 1
Descriptive Data of Research Subjects (A) Clinical and Demographic Data; (B) Age Ranges in relation to Sex; (C) Below 5yrs, 5–9yrs and 10–14yrs.

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<th>IQR %</th>
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### Parameters

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*N* denotes number of samples; *IQR* denotes interquartile range; *SD* denotes standard deviation.

Out of the 115 study participants, 85 (73.9%) of them were successfully followed up to day 3. Of the 85 participants, 83 (97.6%) had no parasitemia by microscopy on day 3 while only two (2.4%) participants remained parasite positive.

### Detection Of Percent Ring-stage Survival Rates

*Ex vivo* RSA results for freshly collected malaria-positive pretreatment whole blood samples against DHA were successfully obtained for 78.3% (90/115) of the study participants. About 17.4% (20/115) samples were unsuccessful for the *ex vivo* RSA on account of low blood volumes and low percent parasitemia. Five samples (4.3%) had contamination and were discarded. The control sample was an ART-susceptible 3D7 laboratory strain. At a DHA concentration of 700nM, there was no ring survival in the 3D7 control, and this was similar for 92.2% (83/90) of the clinical isolates. However, about 7.8% (7/90) isolates had percent survival rates > 10% (Fig. 1).

### Detection Of Molecular Markers Of Art Tolerance

Seven *P. falciparum* isolates, four (D10, D30, P11, and P55) of which had >10% ring-stage survival rates and three isolates with no ring survival were selectively whole genome sequenced. Admissible high genomic coverage was obtained for D10 and D30 RSA positive isolates and P32 and P36 RSA negative isolates. P11 and P55 isolates had very low genomic coverage, while P45 had virtually no coverage (data not shown). Sequences from the four isolates; D10, D30, P32, and P36, with high genomic coverage were
then compared to the 3D7 reference genome with focus on known malaria resistance marker genes (Pfk13, Pfcoronin, Pfmdr1, Pfdhfr, and Pfdhps, the latter two with no relationship to artemisinin resistance). The distribution of SNPs, Insertions and Deletions (INDELs) in selected known drug resistance genes for isolates with admissible high genomic coverage is contained in Table 2. V424I missense mutation in the Pfcoronin gene was harbored by the RSA positive isolates D10 and D30. The N86Y, D650N, and N652D mutations in the Pfmdr1 gene, and N245S in the Pfmdr2 gene were carried by the isolate D10. The D30 isolate had D650N and D1951N mutations in the Pfmrp2 gene (Table 2).
Table 2
Distributions of SNPs/MNPs/INDELs of *P. falciparum* kelch 13 (PfK13), Pfcoronin, multidrug resistance protein 1 (Pfmdr1), multidrug resistance protein 2 (Pfmdr2), dihydropteroate synthetase (Pfdhps), dihydrofolate reductase (Pfdhfr), multidrug resistance-associated protein 2 (Pfmrp2), and signal peptide peptidase (PfSPP) genes for D10 and D30 (RSA Positive) and P32 and P36 (RSA Negative) isolates and their frequencies.

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Detection of in vitro ARTs and partner drugs tolerance

*In vitro* drug sensitivity testing to determine the IC$_{50}$ of ART and its derivatives as well as partner drugs was performed for D10 and D30 (RSA positive) and P32, P52, S22, and S19 (RSA negative) field isolates using for a control, the 3D7 laboratory strain (Table 3). The IC50s of ART sensitive isolates for DHA (4.55 nM, 5.18 nM, 3.57 nM, 6.70 nM) were like the IC$_{50}$ values of the 3D7 control (4.08 nM) and that of one of the ART tolerant isolates D30 (5.35 nM). One of the ART-tolerant isolates, D10, however, had an IC$_{50}$ of 11.78 nM. The geometric mean IC$_{50}$ values and their corresponding 95% CI for the drugs of interest were 7.05 nM (4.5–10.9), 6.49 nM (5.3–7.9), 7.11 nM (6.3–7.9), 5.73 nM (3.7–8.8), 11.77 nM (9.1–15.3), and 13.62 nM (10.5–17.7) for ART, AS, AM, DHA, LUM, and AQ respectively (Fig. 2).
Table 3

*In vitro* drug sensitivity of a few *P. falciparum* isolates (D10, D30, P32, P52, S19, S22, and D37) to dihydroartemisinin, DHA, artesunate, AS, artemether, AM, artesinin, ART, amodiaquine, AQ, and lumefantrine, LUM

<table>
<thead>
<tr>
<th>Isolate</th>
<th>ART IC50 nM (95% CI)</th>
<th>AS IC50 nM (95% CI)</th>
<th>DHA IC50 nM (95% CI)</th>
<th>AM IC50 nM (95% CI)</th>
<th>LUM IC50 nM (95% CI)</th>
<th>AQ IC50 nM (95% CI)</th>
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<tr>
<td>3D7</td>
<td>4.58 (4.17–5.00)</td>
<td>2.75 (2.66–2.85)</td>
<td>4.08 (3.76–4.41)</td>
<td>4.6 (4.38–4.82)</td>
<td>7.16 (6.4–7.93)</td>
<td></td>
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<tr>
<td>D30</td>
<td>4.65 (3.19–6.11)</td>
<td>6.17 (5.08–7.25)</td>
<td>5.35 (3.76–6.93)</td>
<td>7.23 (5.92–8.55)</td>
<td>15.71 (8.91–14.69)</td>
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<tr>
<td>P52</td>
<td>12.93 (11.22–14.65)</td>
<td>6.64 (6.19–7.10)</td>
<td>5.18 (4.16–6.2)</td>
<td>5.95 (5.6–9.00)</td>
<td>9.16 (8.79–9.53)</td>
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</tr>
<tr>
<td>P32</td>
<td>6.43 (5.35–7.5)</td>
<td>6.06 (5.37–6.74)</td>
<td>4.55 (3.42–5.68)</td>
<td>7.49 (6.03–8.95)</td>
<td>16.43 (14.44–18.41)</td>
<td></td>
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<tr>
<td>S22</td>
<td>5.75 (4.38–7.12)</td>
<td>6.33 (5.1–7.56)</td>
<td>6.7 (5.93–7.46)</td>
<td>6.71 (5.41–8.01)</td>
<td>11.26 (10.46–12.06)</td>
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<tr>
<td>S19</td>
<td>5.09 (2.95–7.23)</td>
<td>5.13 (3.5–6.77)</td>
<td>3.57 (2.29–4.85)</td>
<td>8.31 (6.11–10.50)</td>
<td>9.73 (7.9–11.57)</td>
<td></td>
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</tbody>
</table>

There was no significant relationship between percent *ex vivo* ring-stage-survival rates and IC50 of DHA for the six selected isolates ($r_s (4) = 0.67, p = 0.200$).

**Discussion**

Ghana remains one of eleven countries with the highest burden of malaria in SSA (28). The emergence of ARTs tolerance in SEA and parts of East Africa poses a new threat to the fight against malaria in Ghana and elsewhere. Early detection and evaluation of tolerant strains in local parasite populations could help
in containment efforts to avert treatment failures. This study was conducted to assess and characterize correlates of potential ART tolerance based on post-treatment parasite clearance, \textit{ex vivo} and \textit{in vitro} drug sensitivity, and molecular markers of parasite isolates from children with uncomplicated malaria in the Greater Accra Region of Ghana. We detected day 3 parasitemia following AL treatment in two participants and this suggests high AL efficacy in the studied parasite population. This largely agrees with findings from under 5 children with uncomplicated malaria in studies from Ghana and Nigeria which all reported day 3 positive parasitemia below 5\% \cite{29, 30}. WWARN earlier proposed a day 3 positive parasitemia of 5\% threshold as more suitable in keeping track of ART resistance in SSA instead of the currently recommended 10\% by WHO due to high acquired immunity against the parasite in African populations and its contribution to faster parasite clearance \cite{31}.

Our study found percent ring stage survival rates to be 7.8\% in the studied population while most of the isolates had no surviving rings. ART-sensitive 3D7 reference strain was used as control and had no ring survival post exposure to 700 nM DHA concentration. Our findings compare with the 2.1\% isolates reported with >10\% survival rates in a Ugandan population in 2018 by Ikeda \textit{et al.} \cite{32}. Earlier, Witkowski \textit{et al.} accurately identified slow-clearing infections foremost in eastern Cambodia by strongly correlating >10\% \textit{ex vivo} ring stage survival rates with \textit{in vivo} parasite clearance half-lives > 5h (where an isolate with 12.2\% ring stage survival had a half-life of 8.17h). They proposed this to be a proxy for ART-resistance \cite{23}. Therefore, the seven clinical isolates with >10\% \textit{ex vivo} ring survival rates in our study, could indicate the potential onset of ART tolerant \textit{P. falciparum} strains in a portion of the studied population.

Prior reports proposed strong correlations between mutations in the PfK13 propeller domain and ring survival rates \cite{10, 33}. For instance, one of the isolates with >10\% survival rates in a Ugandan population reported by Ikeda \textit{et al.} was reported as harboring A675V, a candidate marker of ART resistance \cite{32}. Mutations in the PfK13 >440 to 680 codon region (propeller domain) which has been associated with ART resistance in SEA, was not detected by our study. Nonetheless, D10, (an isolate with high ring survival) harbored a PfK188* nonsense mutation which to the best of our knowledge, is a novel mutation. Previous reports by Cowell \textit{et al} suggest that some nonsense mutations in the \textit{P. falciparum} isolates resulting in stop-gained mutations may play drug detoxification roles \cite{34}. Ariey and colleagues also observed an \textit{in vitro} selection of a cysteine proteinase-2a gene (S69*) mutation in an African isolate after ART selection pressure \cite{35}. However, the role of PfK188* coupled with background mutations in ring survival remains to be verified.

Though we could not establish implicated variations in the PfK13 propeller domain for isolates with high ring survival in this present work, the potential evolution of ART-tolerant \textit{P. falciparum} cannot be ruled out in our studied population. This assertion is validated by accounts of isolates with as high as 19.4\% ring survival rates without K13 mutations in the China-Myanmar border where the researchers proposed the involvement of secondary and/or additional mechanisms in ART tolerance \cite{33}.

A reduction in sensitivity of \textit{P. falciparum} isolates to ARTs was associated with mutations in the gene encoding PfCoronin by Demas et al. \cite{15}. The PfCoronin SNPs (G50E, R100K, and E107V) that were
implicated by Demas and colleagues, were not detected in our study (15). However, our study detected a missense mutation (V424I) which is non-resident within the WD40 domain in D10, and D30 (isolates with high ring survival rates). This mutation, to the best of our knowledge, is new in Ghanaian isolates, though previously reported in a Peruvian population. Its role in the observed high ring survival rates remains to be verified. Parasite genetic background may potentially impact specific polymorphisms in the malaria parasite and influence its response to antimalarial drugs (36). The extent of ARTs and partner drug usage, the background mutations inherent in the parasite, and the selection pressures within our local malaria parasite population may vary from what pertains in SEA (37). Therefore, correlates of ART tolerance in our local malaria parasite population and Africa at large, may vary from that of SEA.

Mutations in codon 86, 184, 1034, 1042, and 1246 of Pfmdr1 gene have been involved in resistance to various antimalarial drugs and the emergence of parasites having multidrug resistance (36). The D650N and N652D mutations in Pfmdr1 borne by one isolate with high ring survival in this study are novel in Ghanaian isolates but have been described by Mernard and colleagues as harbored by both their F32-ART5 (ART resistant) and F32-TEM (ART sensitive) Tanzanian isolates (38), while the Y184F and N86Y Pfmdr1 mutations carried by the same isolate were reported as having reduced sensitivity to ACT partner drugs piperaquine (PPQ) and AQ (39, 40). This same isolate harbored the Pfmdr2 mutation (F423Y) which was earlier associated with in vitro pyrimethamine resistance (41). Also borne by this isolate, was S208N and I492V Pfmdr2 mutations whose roles in ART resistance is not known as has also been previously reported in African isolates (41, 42). Furthermore, this isolate harbored N245S mutation and (ATT/ASN) deletion in the Pfmdr2 gene and as far as we are aware, these are new in a Ghanaian population and their potential effect in drug tolerance remains to be elucidated.

An insertion (T) in the P. falciparum signal peptide peptidase (PfSPP) gene was detected in D10 (an isolate with high ring survival in this study). The PfSPP gene is essential for the survival and growth of the parasite in the erythrocytes of the human host (43, 44). However, the role of this insertion in ring survival remains to be known.

Additionally, we explored mutations in Pfdhps (S436A, A581G, A613S, I431M) and Pfdhfr (N51I, C59R, S108N, D212I) genes which though not related to ART resistance, are still worth reporting due to their potential effect on Sulfadoxine-Pyrimethamine, (SP) therapy in the Ghanaian population. As far as we know, this is the first time D212I and I431M are being reported in Ghanaian clinical isolates.

IC\(_{50}\)s of D10 and D30 (isolates with > 10% ring survival) together with isolates (4) that had no ring survival after exposure to 700nM DHA were measured to evaluate any potential association of ring survival with the IC\(_{50}\)s of ART, AS, AM, DHA, LUM, and AQ. In comparison to literature threshold values for drug resistance (45–49), the geometric mean IC\(_{50}\)s of all the drugs assayed could not establish drug resistance although the IC\(_{50}\) values of these drugs were marginally elevated than that described by Quashie et al for 2012 from three sentinel sites (Cape Coast, Navrongo, and Hohoe) that suggests risen trends in AS IC\(_{50}\)s in Ghana (46). Although IC\(_{50}\)s were measured for only few isolates, the observations
made may be an indicator of a slow onset of drug-tolerance in the studied *P. falciparum* population over time.

There was no significant correlation between percent ring-stage-survival *ex vivo* and the reported IC50s of DHA for the six isolates of interest. Results from our study compare with the report by Ikeda *et al* and Witkowski *et al* proposing the absence of significant association between increased *ex vivo* ring survival rates and the IC50s to DHA in the conventional *ex vivo* drug sensitivity assay (16, 32).

**Limitations**

The study participants were recruited within a geographically limited area (Greater Accra Region of Ghana) therefore, our findings cannot be generalized. However, the outcome of this study may serve as grounds for continuous monitoring of ART tolerance in Ghanaian clinical isolates. Limited samples were used for the whole genome sequencing and IC50 assays, so, our findings may have to be verified with a much larger sample size.

**Conclusion**

The very low proportion of individuals with day 3 post-treatment parasite presence by microscopy indicate that AL is still quite potent in the studied parasite population. However, the endurance / survival of some rings in the 72-hour *ex vivo* ring stage survival test, may be a sign of early development of ART tolerance in a portion of the parasite population under study. The IC50 values of the selected isolates were not indicative of drug tolerance / resistance, and no notable correlation was established between percent ring survival and IC50 values of RSA positive (> 10% survival rates) and those with no ring survival. The genetic backgrounds noted in other drug resistance genes in isolates of > 10% ring survival calls for further inquiry to establish their combined effect on malaria parasite ring stage survival.

**Abbreviations**

ACTs Artemisinin-based combination therapies

AL Artemether Lumefantrine

AM Artemether

AQ Amodiaquine

ART Artemisinin

CMP Centre for Medical Parasitology

DHA Dihydroartemisinin
DHC Danfa Health Centre
Pfdhfr Dihydrofolate reductase
GMIS Ghana Malaria Indicator Survey
IC50 50% Inhibition Concentration
INDELs Insertions and Deletions
IVART *in vitro* analysis and reporting tool
PfK13 Plasmodium falciparum Kelch 13-propeller domain
LUM Lumefantrine
MNPs Multiple nucleotide polymorphisms
MSAT Mass screening and treatment
NMIMR Noguchi Memorial Institute for Medical Research
PCR Polymerase Chain Reaction
Pfmdr1 *P. falciparum* multidrug resistance protein 1
Pfcrt Plasmodium falciparum chloroquine transporter
Pfmrp2 *P. falciparum* multidrug resistance-associated protein 2
Pfmdr2 *P. falciparum* multidrug resistance protein 2
PfSPP *P. falciparum* signal peptide peptidase
PMLCH Princess Marie Louise Children's Hospital
Pfdhps Plasmodium falciparum dihydropteroate synthetase
RBCs Red Blood Cells
RDTs Rapid diagnostic tests
RSA Ring-stage survival assay
SEA Southeast Asia
SNPs Single Nucleotide Polymorphisms
SODH Shai Osudoku District Hospital

sWGS Selective whole genome sequencing

WGS Whole genome-sequencing

WWARN Worldwide Antimalarial Resistance Network

WHO World Health Organization

All authors read and approved the final manuscript

**Declarations**

*Ethical Considerations*

The study was approved by the University of Ghana College of Health Sciences Ethical and Protocol Review Committee (protocol # CHS-Et/M.10-p 4.8/2016-2017) and the Ethics Review Committee of the Ghana Health Service (protocol # GHS-ERC:015/07/17).

*Consent for publication*

Not applicable

*Availability of data and materials*

All data generated or analyzed during this study are included in this published article and in the supplementary information.

*Competing interest*

The authors declare that they have no competing interests

*Funding*

This study was funded with grants offered to Samuel Yao Ahorhorlu by the Building Stronger Universities in Developing Countries (BSU II project), sponsored by the Danish government as part of his PhD Fellowship.

*Authors’ contribution*

SYA, GOA, NBQ, and MA conceived the idea, designed the experiments, and wrote the manuscript. SYA, FZ, carried out the assays and helped with the revision of the manuscript. NBQ, HH, and MA supervised the laboratory experiments, and participated in the overall interpretation of data and revision of the manuscript. NOD-Q provided laboratory space and revised the manuscript. HH, ETN, and RWJ analyzed
the data and revised the manuscript. CWW, and WK were involved in data interpretation and revision of the manuscript.

All authors read and approved the final manuscript

Acknowledgements

Our sincere thanks go to the participants and their guardians who consented to be part of this study. We are also grateful to the staff of Shai Osudoku District hospital, Dodowa, staff of Danfa Health Center, and staff of the Princess Marie Louise Children Hospital (PMLCH), Accra, for their support during the sampling process. We are also thankful to staff of the Centre for Medical Parasitology (CMP), University of Copenhagen, Denmark, and staff of the Epidemiology Department of the Noguchi Memorial Institute for Medical Research (NMIMR), for their support during the laboratory phase of this project.

References


Figure 1

Distribution of isolates with > 10% *Ex vivo* ring-stage Survival Rates and 3D7 against 700nM DHA
Figure 2

Frequency Distribution of IC$_{50}$s of the Test Drugs on Selected *P. falciparum* Isolates (D10, D30, P32, P52, S19, S22)

**Supplementary Files**

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- SupplementaryInformation.docx