Expansion of the Plasmodium falciparum Kelch 13 R622I mutation in Northwest Ethiopia

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Abstract

According to the WHO, almost two thirds of the Ethiopian population are at risk of contracting malaria, where infection with Plasmodium falciparum accounts for approximately 60% of cases today. The risk of artemisinin resistance spreading from SE Asia to Africa is a major concern. We conducted a 28-day in vivo efficacy trial of Artemether-Lumefantrine (Co-Artem) for treatment of uncomplicated malaria (n = 97) in the Gondar Region, North West Ethiopia in 2017–2018. Our results confirmed 100% adequate clinical and parasitological response (ACPR) with no parasites observed at day 3 by microscopy. Further analysis of day 0 samples showed the expansion of a kelch13 mutation R622I to 9.5% from 2.4% of isolates reported three years earlier. Closer examination of the R622I mutation in vitro is warranted.

Introduction

Malaria remains as one of the top leading causes of morbidity and mortality among low-income countries, infecting 229 million people and 409,000 deaths according to the latest World Health Organization (WHO) report (1). Despite global efforts, these numbers have only decreased modestly for the past five years (1). Reasons underlying this include a higher number of hrp2/3 deletions in Plasmodium falciparum that decreases the sensitivity in rapid diagnostic tests (RDTs); high level of detection (LOD) diagnostic tools for mass screen and treatment (MSAT) campaigns; neglect of asymptomatic cases; increased prevalence of insecticide-resistance Anopheles mosquitoes; geographical expansion of artemisinin-resistance clones, among others (1–7). The latter poses a challenge to the effectiveness of artemisinin-based combination therapies (ACT), which is the first-line treatment for uncomplicated Plasmodium falciparum malaria (8–10).

Ethiopia, with a current population of over 110 million is a malaria-endemic country with an estimate of 68% of its population at risk of contracting the disease (1). The main vectors of transmission in the area are A. funestus, A. arabiensis, and A. pharaoensis which are known to be highly seasonal and predominant below an altitude of 2,500 meters (11). Unlike most of Sub-Saharan countries where malaria is almost exclusive to P. falciparum, Ethiopia displays a relatively high number of Plasmodium vivax cases, making it the fourth country with the highest incidence for this strain in 2018 (1). Despite this, Ethiopia has shown a remarkable decrease of malaria cases through public health interventions using prevention and control of malaria among pregnant women by intermittent preventive treatment (IPTp), vector control through indoor residual spraying (IRS), an increase of insecticide treated nets (ITNs) availability, among others (1, 12).

The presence of artemisinin-resistant P. falciparum strains was reported for the first time in 2006(13, 14) at the Thai-Cambodia border, and eventually expanded across southeast Asia Although some cases of artemisinin resistance were found Guyana and Papua New Guinea, there is no clear evidence of ACT failure due to parasite resistance in the African continent. Additionally, Plasmodium falciparum artemisinin-resistant clones have been previously correlated to copy number variation (CNV) of the plasmepsin2/3 genes, along with single nucleotide polymorphisms (SNPs) of the Pfcoronin(15),
pfFd(16), and arps10(16). Nevertheless, the aforementioned genetic polymorphisms could hardly be connected with clinical artemisinin resistance at the field level. Instead, multiple SNPs in the Kelch13 gene including C580Y, F446I, Y493H, R539T, I543T, P553L, R561H, P574L, A675V, R622I, M579I, have been strongly associated with clinical artemisinin resistance (17–19). Previously it was thought that Kelch13 mutants were exclusively found in Viet Nam, Thailand, Cambodia, Guyana, and Papua New Guinea. Nevertheless, recent studies have identified mutants in various African countries, with a considerable prevalence of the A578 mutation (20, 21). Some studies have shown in vitro resistant strains in Rwanda, not affecting the overall efficacy of dihydroartemisinin/piperaquine (DP) or artemether/lumefantrine (AL) (17). According to the Worldwide Antimalarial Resistance Network (WWARN) database, more than 15 countries have reported an artemisinin success rate below 90% across a 28-day drug efficacy trial between 1977 and 2017 (22). While this is not direct evidence of Kelch13 mutants conferring resistance, this data should be taken into consideration when designing public interventions for malaria treatment among these countries. Previous studies have shown how the Kelch13 protein compartment and its interactome play a role in resistance through a decreased rate of hemoglobin endocytosis, which reduces the by-product hemoglobin concentration needed for artemisinin activation (23). Recent studies have shown evidence of in vivo delayed parasite clearance and in vitro artemisinin resistance through ring-stage assays (RSA) of various Kelch13 SNPs.

An in vivo ACT efficacy trial study was conducted in the Gondar region of northwest Ethiopia in 2014 (Fig. 1), where the presence of the R622I SNP in the Kelch13 propeller domain was first documented (19). In this study, we have conducted a follow-up study in the same geographical area in 2018, applying a similar methodology described in 2014.

Methods

This study was reviewed and approved by University of Calgary (Ethics ID 15-3204) and University of Gondar (Ethics ID: CMHS/4571/08). The study was conducted at two health centers (Negade Bahir and Dembia General Hospital) in northwest Ethiopia between 2017 and 2018. A total of 97 patients were enrolled in a 28-day efficacy trial of artemether-lumefantrine (Co-Artem) as per Ethiopian Ministry of Health guidelines. All patients cleared the infection by day 28. Peripheral blood was collected for malaria diagnosis, along with DNA extraction for the Kelch13 gene sequencing. Mono-infected patients were included in the study Finger-pricked samples were collected on a Whatman 903 paper filter (GE Healthcare, Mississauga, Canada) at the time of the diagnosis, air-dried, and stored in zip-lock bags for its transportation to the University of Calgary, Canada. Genomic DNA extraction was performed with the QIAamp DNA Mini kit and by following the manufacturer's protocol of DNA isolation from blood samples (24). The Kelch13 gene was amplified using nested polymerase chain reaction (nPCR) from previous protocols (19). Capillary sequencing was then performed at the Centre of Health Genomics and Informatics (CHGI) at the University of Calgary using Applied Biosystems (Burlington, ON, Canada) 3730 XL 96 capillary DNA analyzer. Multiple sequence alignment (MSA) was done using Clustal Omega software (25) across all of the isolates with the wild type (WT) Kelch13 reference gene (Genbank:
MK877455.1), and edited manually to remove any existing gaps through Jalview (26). Sequenced reads with a low degree of alignment or “Ns” present at the codon of interest were excluded of the analysis.

**Results**

In this study we have identified an increase in prevalence of the R622I mutant in the northwest Ethiopian region close to the Sudan border. This mutation was previously reported in the same region in 2016 (19). This mutant has been recorded by Chinese imported cases between 2016 to 2018 (27), along with isolates from Eritrea (28), and Somalia (29). Capillary sequencing was performed bidirectionally across all the isolates. The MSA file showed a high degree of similarity among the propeller domain gene (supplementary file 1). Five DNA reads were excluded from the analysis due presence of “N” in the codon of interest or due a low degree of alignment with the rest of the sequences.

Demographic and epidemiological variables of interest were collected at the time of diagnosis (Table 1). A total of 86 isolates could be sequenced from 97 dried blood spots collected at day 0 of the diagnosis. Samples prior to treatment were used for this study. Capillary sequencing and MSA was obtained across all of the 86 isolates sequenced, 78 (90.7%) were indicative of the expected wild type arginine at the 622nd position, while the remaining 8 samples (9.3%) showed a consistent isoleucine mutation in both the forward and reverse reads (Table 2). Only one isolate presented a synonymous “CGA” mutation in the forward read and the expected WT “AGA” codon in the reverse read.
Table 1
Epidemiological and clinical features of the study participants. ACPR – adequate clinical and parasitological response.

| Epidemiological variable (n) | n (%)       |
|------------------------------|-------------|
| Age (97)                     |             |
| Under 5                      | 2 (2.06)    |
| Between 6–15                 | 37 (38.14)  |
| Adults                       | 58 (59.79)  |
| Gender (97)                  |             |
| Female                       | 37 (38.14)  |
| Male                         | 60 (61.86)  |
| Fever (97)                   |             |
| No                           | 4 (4.12)    |
| Yes                          | 93 (95.88)  |
| ACPR (97)                    |             |
| Yes                          | 100 (100)   |

Table 2
Non-synonymous and synonymous K13 mutants at two sites in the Gondar region of North West Ethiopia. GH- General Hospital.

| Collection site | Samples collected (n) | Samples sequenced (n) | K13 synonymous mutations (n) | R622I K13 non-synonymous mutations (n) |
|-----------------|-----------------------|-----------------------|------------------------------|----------------------------------------|
| Negade Bahir    | 71                    | 66                    | 2                            | 4                                      |
| Dembia GH       | 26                    | 20                    | 0                            | 4                                      |
The reported R622I mutant prevalence is considerably higher when compared to the 2.4% reported in 2016 (19) (3.88 fold increase), considering the relatively small time frame of four years between sample collection. Besides the R622I, one of the analyzed isolates had the V510V synonymous mutation (“GTG” to “GTA”) that has been previously reported in southern Nigeria in 2018 (30). None of the isolates presented any of the mutations that have been reported in the Greater Mekong Subregion (F446I, Y493H, R539T, I543T, P553L, R561H, P574L, C580Y, A675V) (31, 32)

**Discussion**

In this study we have identified an increase in prevalence of the R622I mutant in the northwest Ethiopian region close to the Sudan border. This mutation was previously reported in the same locale in 2016 but at a lower frequency (19). This mutant has been recorded imported cases to China between 2016 to 2018 (27), along with isolates from Eritrea (28), and Somalia (29). Our data clearly demonstrates an increase of this mutant in northwest Ethiopia, although no association with *in vivo* artemisinin resistance could be established. Several reasons may have contributed to this clonal expansion of this SNP containing parasites in the study area. First, the R662I SNP could potentially provide the parasite with a biological advantage in the presence of artemisinin but has not been correlated to *in vivo* parasite clearance studies potentially due to a higher naturally acquired immunity (NAI) among this study population; a minor proportion of the immunocompromised people may have acted as the reservoir for the spread of the R622I SNP containing parasites. Second, the R662I SNP probably has a role to support elevated *in vivo* fitness of the parasite. Third, these mutant parasites do not possess the necessary background mutations to exert in vivo resistance against ACT.

Some of the limitations of the study include capillary sequencing instead of next-generation sequencing (NGS). By doing this, we were not able to determine if the non-synonymous mutations present were from the dominant *Plasmodium falciparum* clone in each patient. Moreover, the isolates that displayed the wild type arginine may harbor any minority variant clones with mutations as capillary sequencing typically captures the information of the most predominant clone. The small sample size also may skew the true prevalence of this mutation.

While no *in vivo* parasite-delayed clearance has been correlated to *Kelch13* SNPs in Africa, molecular surveillance of artemisinin resistance genes should remain operational in order to identify any changes in treatment phenotypes. Of note, a recent report of the R561H kelch13 mutant in Rwanda suggested clonal expansion of this mutant and correlation to *in vitro* resistance (17). To date, there are no *in vitro* studies correlating the R622I codon to parasite resistance, and while this study does not provide direct evidence of a new treatment-tolerant parasite, CRISPR/Cas9 RSA studies should be conducted to determine the biological significance of this mutant given its clonal expansion and spread to other regions over a relatively short period.

**Abbreviations**
NGS
Next generation sequencing

Declarations

Ethics approval and consent to participate

This study was reviewed and approved by University of Calgary (Ethics ID 15-3204) and University of Gondar (Ethics ID: CMHS/4571/08).

Consent for publication

Not applicable

Availability of data and material

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

DRP is an Associate Editor of Malaria Journal

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Authors’ contributions

Conceptualization: DRP, AGB; Methodology: DCM, AA, HT, ANM, SSG, NB Formal Analysis: DCM, AA, HT, ANM, SSG, NB, DRP, AGB; Investigation: DCM, AA, HT, ANM, SSG, NB, DRP, AGB ; Data Curation: DCM, AA, HT; Writing – Original Draft: DCM, DRP; Writing – Reviewing & Editing: DCM, AA, HT, ANM, SSG, NB, DRP, AGB; Supervision: AGB, DRP; Funding Acquisition: DRP.

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Supplemental Data

Supplementary file 1 not available with this version