Novel Plasmodium falciparum K13 Gene Polymorphisms from Kisii County, Kenya during an era of Artemisinin-Based Combination Therapies (ACTs) deployment

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Research Article

Keywords: Artemisinin-based combination therapies, Resistance, Kelch13 propeller gene, Polymorphism.

Posted Date: November 11th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-2252630/v1

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Abstract

Background

Currently, Chemotherapy stands out as the major malaria intervention strategy, however, antimalarial resistance may hamper global elimination programs. Artemisinin Combined Therapies (ACTs) stands as the drug of choice for treatment of *P. falciparum* malaria. *P. falciparum Kelch13* gene mutations are associated with artemisinin resistance. Thus, this study was aimed at evaluating the circulation of *P. falciparum K13* Gene Polymorphisms from Kisii County, Kenya during an era of ACTs deployment.

Methods

Participants suspected to have malaria were recruited. *P. falciparum* was confirmed using microscopy method. Malaria-positive patients were treated with Artemether-Lumefantrine (AL). Blood was withdrawn from participants who tested positive for parasites after day 3 and kept in blood filter papers (ET31CHR; Whatman Limited, Kent, UK). DNA was extracted using chelex-suspension method. A nested polymerase chain reaction (PCR) was conducted and the second-round products were sequenced using Sanger's method. Sequenced products were analyzed using DNAsp 5.10.01 software and then blasted on to the NCBI for *K13* propeller gene sequence identity using the Basic Local Alignment Search Tool (BLAST). To assess the selection pressure in *P. falciparum* parasite population, Tajima’ D statistic and Fu & Li’s D test in DnaSP software 5.10.01 was used.

Results

Out of 275 enrolled participants, 231 completed the follow-up schedule. 13 (5.6%) had parasites on day 28 hence characterized for recrudescence. Out of the 13 samples suspected for recrudescence, 5 (38%) samples were positively amplified as *P. falciparum*, with polymorphisms in the *K13*-propeller gene detected. Polymorphisms at codon 539, 458, 561, 431 and 671 were detected. The sequences have been deposited in NCBI with bio-project number PRJNA885380 and accession numbers SAMN31087430, SAMN31087431, SAMN31087432, SAMN31087433, and SAMN31087434 for Marani, Nyamache, and Bonchari samples, respectively.

Conclusions

Validated WHO resistant Polymorphisms in the *K13*-propeller gene previously reported to be associated with artemisinin resistance were not detected in the *P. falciparum* isolates from Kisii County, Kenya. However, some previously reported un-validated *K13* resistant Single Nucleotide Polymorphisms were reported in this study but with limited occurrences. The study has also reported new SNPs. More studies need to be carried out in the entire country to understand the association of reported mutations if any, with ACTs resistance.

Background

Malaria remains the most prevalent vector-borne tropical disease in the world, causing both mortalities and morbidities especially in pregnant women and infants. According to the World Health Organization, 2021 [1], Kenya registered 6 million malaria cases with 228 million cases reported worldwide, leading to 445,000 deaths globally. This problem is heaviest in sub-Saharan Africa, where approximately 94% of mortalities are registered annually. This situation is predicted to worsen due to the ongoing COVID-19 pandemic, which has greatly compromised malaria treatment and control intervention measures [2]. *P. falciparum* is the most common parasite, causing about 99% of malaria cases in Kenya [3]. Malaria occurrences in Kenya have variations across the country, with the lake endemic zone having the highest prevalence (27%), followed by the coast endemic zone (8%) and the highland epidemic zone (3%). Kisii County where this current study was conducted is located in the Western highland malarial zone. Artemisinin-based combination therapies (ACTs) offers highly successful treatment of malaria. However, the emergence and spread of *P. falciparum (PF)* parasites with decreased susceptibility to ACTs in South-East Asia, South America and some African countries have caused a global concern. Timely detection and subsequent monitoring is vital in anticipating actions to contain malaria resistance to artemisinin-combined therapies in Kenya. Currently, the World Health Organization (WHO) recommends artemisinin-based combination therapies (ACTs) for the treatment of uncomplicated malaria in most countries. In 2002, Kenya recommended the use of Artemether-Lumefantrine (AL) as the drug of choice for treating uncomplicated malaria, however, the actual implementation started in 2006 [4]. The increase of antimalarial resistant *P. falciparum* has increased malaria deaths globally. Given the increasing reports of resistance or poor responses to artemisinin-based combination therapies (ACTs) in other parts of the world, the Sub-Saharan African region affected by the disease may receive a repeat of what happened during the emergence of chloroquine and Sulfadoxine-Pyrimethamine resistance [5]. If such a case arises, malaria control efforts may be compromised. However, in the absence of licensed malaria vaccine, chemotherapy remains the only option for malaria treatment. Bearing in mind that no new antimalarial will be available immediately if ACTs fails, this threatens to reverse the
Materials And Methods

Study area

This study was conducted in Kisii County, Kenya in 2021, during the months of February to June. The county has nine Sub-counties. The county is located approximately 306 kilometers from the capital city, Nairobi. It lies at latitude: (0.41°) South, longitude: (34.46°) East. According to the 2019 Kenya population and housing census, the county population size is 1,266,660 persons [16]. The main economic activity is agriculture. The county is characterized by hilly topography accompanied by ridges and valleys. The county is characterized by seasonal and permanent rivers which flow into Lake Victoria. The county exhibits a highland equatorial climate with an average rainfall of 1500mm/year. The county records two rain seasons namely: long rains season, from March to June; short rain between September and November. The average temperature range is between 21°C -30°C. Most of the population lives in rural areas, residing in local houses. The county health system consists of government and private based health facilities. The government sector has one teaching and referral hospital (KTRH), which serves as a regional reference hospital and a teaching hospital for Kisii University Medical School. This county contains 14 sub-county hospitals. The county also contains 84 dispensaries, 28 health centers and 32 community health units, which serve as centers for minor health cases. The county records three rain seasons namely: April-May, August-September, and November-December, the main killer disease is malaria. The main malaria intervention approaches used to combat malaria in this region include proper case management with antimalarial drugs such as ACTs, intermittent prophylaxis during pregnancy (IPTp) and use of mosquito nets. The current drug of choice for treating uncomplicated malaria is artemether-lumefantrine. Diagnosis and treatment services of malaria are available in all government health facilities and a few private facilities. The current study was conducted in hospitals selected from 4 sub-counties (Kenyenya, Marani, Bonchari,Nyamache) of Kisii County (Fig. 1). Molecular study was conducted at the Molecular Biology and Immunology Laboratory, School of Health Sciences, Makerere University, Kampala, Uganda.

Study design, study population and specimen collection

This was a cross-sectional health point prospective study. We enrolled participants who before the start of the study were presented with malaria clinical characteristics and had resided in Kisii County for at least the last six months. However, we excluded those who were reluctant to give consent to the study. In addition, participants with febrile clinical illnesses initiated by pathogens other than malaria were excluded from the study. Before collecting blood specimen, a complete medical examination and demographic information were obtained. All patients who had been suspected of having malaria infection by having fever (≥ 38°C) or having a history of fever in the past 24 h, were confirmed for the presence of P. falciparum using microscopy (as a confirmatory test). Briefly, thick and thin blood smears were stained with 2% Giemsa for 30 min. A smear was considered negative if no parasites were observed after examination under 100 high-powered fields. For Giemsa stained thin smears, they were fixed in methanol solution before the examination. Blood samples were collected by obtaining 1 ml of venous blood for the participants older than 2 years. 100 µL finger-pricked blood samples were collected in the case of children below 2 years of age. This procedure was repeated during the consequent follow-up visits. The blood spots were made on chromatography filter paper (ET31CHR; Whatman Limited, Kent, UK) and labeled well with the participant identification number. Malaria-positive participants were followed up for a period of 28 days by evaluating clinical and parasitological parameters on days 1, 3, 7, 14, and 28, respectively, after ACTs (A-L) treatment initiation. Finger pricks for follow-up were taken on days 1, 3, 7, 14 and 28 to check for the presence of P. falciparum [17].
Characterization of Merozoite Surface Proteins-2 (MSP-2)

To distinguish between recrudescence and re-infection, 4 drops of blood from malaria-positive patients were collected on filter paper on day zero before treatment, and on any day of recurrent *P. falciparum* malaria. Molecular analysis was conducted following the previously described method [18], with slight modifications. Briefly, blood spotted filter papers were soaked for 24 h in 1 mL of 0.5% saponin-1 phosphate buffered saline. The mixture was washed twice in 1-mL PBS and boiled for 8 min in 100 mL PCR-grade water to release DNA from the cells. To elute the extracted DNA, 150 µL Buffer AE was added to each well using a multichannel pipette and incubated for 1 min at room temperature. This setup was then centrifuged at 2608 RCF for 8 min. DNA was recovered and stored at -80°C. Nested PCR was performed on the extracted DNA for subsequent genotyping of *P. falciparum* polymorphic gene loci encoding Merozoite surface protein 2 (MSP-2) using the method described by [19]. A master mix was prepared according to manufacturer instructions (New England Bio Labs, Massachusetts, USA). 24 µL of the Master Mix was added to the PCR 96 well plate and 25 µL of the master mix was also added to the negative PCR control. The plates were sealed using a thermo seal plate sealer and placed in the PCR thermo-cycler. Amplification was then performed under the following conditions; denaturation (94°C), annealing (55°C), and extension (72°C). Amplification was confirmed by running the nested PCR product together with a DNA ladder on the QIAxcel capillary electrophoresis. The result was classified as recrudescence if at least one identical MSP2 allele was detected in both ACTs pre-treatment and ACTs post-treatment blood samples. Blood samples where MSP2 alleles did not match ACTs pre- and ACTs post-treatment were classified as new infections. Any sample, which failed to amplify was classified as undetermined. Blood samples, which showed recrudescence of parasites on day 28 were further genotyped for *P. falciparum K13* resistance markers. The primers used in this protocol are shown in Table 1.

### Table 1

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5'→3')</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSP-2(1)</td>
<td>ATGAAGGTAATTTAAACATTGTCTATTA</td>
<td>External forward primer</td>
</tr>
<tr>
<td>MSP-2(4)</td>
<td>ATATGGCAAAGATAAAAACAGTTTGGCTG</td>
<td>External reverse primer</td>
</tr>
<tr>
<td>MSP-2(A1)</td>
<td>CAGAAAGTACCTTCTACTGG</td>
<td>Internal forward primer (IC3D7)</td>
</tr>
<tr>
<td>MSP-2(A2)</td>
<td>GATTTGTTTCCGCAATTATTA</td>
<td>Internal reverse primer (IC3D7)</td>
</tr>
<tr>
<td>MSP-2(B1)</td>
<td>CAAATGAAGGTCTACTACTA</td>
<td>External forward primer (FC27)</td>
</tr>
<tr>
<td>MSP-2(B2)</td>
<td>GCTTGGCGTCCTTCCAGTTGATC</td>
<td>Internal reverse primer (FC27)</td>
</tr>
</tbody>
</table>

#### Sequencing of K13-propeller genes

DNA was extracted from blood spotted filter papers by using the chelex suspension method described by [20], with slight modifications. Blood spotted filter papers were soaked for 24 h in 1 mL of 0.5% saponin-1 phosphate buffered saline. The mixture was washed 2 times in 1-mL PBS and boiled for 8 min in 100 mL PCR-grade water. To elute the extracted DNA, 150 µL Buffer AE was added to each well using a multichannel pipette and incubated for 1 min at room temperature. This setup was centrifuged at 2608 RCF for 8 min to recover the DNA and stored at -30°C. *K13*-propeller genes were amplified by the nested PCR protocol described previously (Ocan et al., 2016) by using the primers listed in Table 1. For the first round of PCR, 0.5-mL DNA was amplified with 10 mL PCR Mix (1.25 U/mL, Taq DNA Polymerase, 0.4 mM dNTP Mixture, PCR buffer, and 4 mM Mg2+) 0.5 mL forward primer (10 mM), 0.5 mL reverse primer (10 mM), and sterile ultrapure water to a final volume of 20 mL. For the second round of PCR, 0.5 mL primary PCR products were amplified with a 40 mL reaction system, including 20 mL PCR Mix, 1.0 mL forward primer (10 mM), 1.0 mL reverse primer (10 mM), and H2O. The amplification conditions were maintained at 95°C for 3 min; followed by 30 cycles (95°C for 30 s, 55°C for 30 s, 72°C for 30 s); 72°C. For 5 min; then stored at 12°C. Sequencing was done using Sanger’s method described by (Ocan et al., 2016), with slight modifications. The second round PCR products were purified by using a jet quick PCR product purification kit. 5 µL of the purified second round PCR products were then run on 1.0% (w/v) agarose gel stained with 0.05 µg/mL of Ethidium Bromide (Sigma Aldrich, USA) to counter-check for the presence and concentration of PCR products. This was followed by Bi-directional cycle sequencing of K13 using the second round K133 Forward and K132 Reverse PCR primers (Eurofins Genomics, Germany), using the Big Dye terminator v3.1 cycle sequencing kit (Applied Bio systems, USA). Cycle sequencing PCR was performed in a total reaction volume of 20 µL. 6.0 µL of the Big Dye terminator v3.1 5X sequencing buffer (Applied Bio systems, USA). This was accomplished by mixing the above total reaction volume with; 2.0 µL Big Dye terminator v3.1 (Applied Bio systems, USA), 1.0uL of 10 ng/µL (K133 forward or K132 Reverse) primers and 6.0uL of nuclease-free water. Finally, 5.0 uL of 5.0 ng/µL of the purified second round PCR products were then added to make up the total volume. The following sequencing conditions were used. One cycle of Polymerase activation 96°C for 60s followed by 35 cycles of; denaturation at
96°C for 10s, annealing at 53°C for 30s and extension at 60°C for 300 s (Gene Amp 9700 PCR system, USA). The amplified products were then stored at 4°C until the next step of extension. The extension PCR products were then followed by purification using Dye Ex 2.0 spin Kit (QIAGEN, Maryland, USA). Subsequently 5.0 µL of the purified cycle sequencing PCR products were then mixed with 5.0 µL of De-ionized formamide (Sigma Aldrich, USA) and then loaded in the 310 genetic analyzer (Applied Bio systems, USA). Finally, the products the products were bi-sequenced with POP-7™ (Applied Bio systems, USA) as a sequencing Polymer. The primers used in this protocol are shown in Table 2.

<table>
<thead>
<tr>
<th>Gene fragment</th>
<th>PCR primer sequence</th>
<th>Mutations analyzed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5'_GGGAATCTGGTGGTAACAGC-3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5'_TGATTAAG GTAATTTAAGCTGTCC-3</td>
<td></td>
</tr>
</tbody>
</table>

**Sequence data analysis**

The DNA sequences were analyzed using the sequence analysis software 5 and then blasted on to the NCBI sequence database to confirm the K13 propeller gene sequence identity by using the Basic Local Alignment Search Tool (BLAST) at http://blast.ncbi.nlm.nih.gov/Blast.cgi. The sequences were exported to bio edit sequence alignment editor 7.2.5 for manual editing and then into MEGA 5 software version 5.10 for detection of polymorphism using the PF3D7_1343700 and K13-propeller gene sequences present in the NCBI database were used as the reference sequence. Additional single-nucleotide polymorphism (SNPs) analysis within the K13 propeller gene was performed using the DnaSP software version 5.10.01. To assess the selection pressure in *P. falciparum* parasite population in Kisii County, Tajima’s D statistic and Fu & Li’s D test in DnaSP software 5.10.01 were used. In this analysis, the study evaluated whether the *P. falciparum* K13 propeller domain sequence data show evidence of deviation from the neutrality theory of molecular evolution. The analysis was done using commands in the DnaSP software. In the DnaSP software, the probability of Tajima’s D and Fu & Li’s D are estimated by simulation. The test uses information on the frequency of mutations (allelic variation) [21]. Tajima’s D and Fu & Li’s D test is based on the fact that under the neutral model, estimates of the number of polymorphic sites and the average number of nucleotide differences are correlated. The critical values (Tajima’s D and Fu & Li’s D) obtained were used in interpreting the findings under the neutrality assumption [22, 23].

**Tajima’s D simulation**

\[
D = \frac{n_s}{d_1}
\]

\[
\sqrt{\text{Var}(n_s - a_1)^{1/2} p}
\]

Where, \( n_s \) = Mean pairwise differences

\( S = \) Number of segregating sites

\( p = \) total number of mutations

**Fu and Li’s D simulations**

\[
D = \frac{1}{4} S - a_1 n e - a_1 n e p
\]

Where, \( n e \) = Expected number of derived mutations that are present only once in the sample

\( S = \) Number of segregating sites
Ethical Consideration

Ethical approval was sought from the University of East Africa, Baraton Institutional Review Board (UEAB/REC/4/2/2021, research permit was issued by Kenya National Commission for Science, Technology and Innovation (NACOSTI) License No: NACOSTI/P/21/8974 and Kisii County government (DTR/4/27). The guidelines outlined in the Declaration of Helsinki were followed as follows; written informed consent was obtained from the adult participants. The consent of those below 18 years of age was provided by the parents or the care-givers. Permission for conducting this study was granted by different sub-counties before starting the study. Moreover, participation was voluntary. The participants were coded instead of reflecting their names to maintain confidentiality. Participants who were malaria positive were given antimalarial treatment according to the WHO regulations and they were reimbursed for the travel cost, lost earnings and food expenses. Participants were respected in relation to their right of their cultural beliefs and rights. Participants were allowed to withdraw from the research without any condition. Approval from local leaders was obtained before beginning the study. Since this study was conducted during the COVID-19 Pandemic, standard operating procedures were followed as stipulated by WHO.

Results

Base Line Characteristics Of The Participants

A total of 275 participants were recruited in 2021 during the months of February to June. More female (60.0%) participants were enrolled compared to males (40%). The mean age of the recruited participants was 27.60 ± 0.92 years. 69% (189.75 ± 0.25) of the participants were adults, with a nearly equal proportion between males and females. 84% (231.0 ± 0.84) of the participants completed the efficacy profiling on AL from day 0 to day 28. The temperature recorded at enrollment (day 0) varied across the sites, with Kenyenya recording 37.6°C ± 1.1, Marani recording 37.5°C ± 1.1, Bonchari recording 37.8°C ± 1.1 and Nyamache recording 37.3°C ± 1.1 respectively. However, the temperatures recorded during 28 days of study did not vary across the sites. The geometric mean parasite density (asexual parasites/µL) was significantly higher at Bonchari, having recorded 13,531 (9,242–15,603), (p = 0.047) compared to the other sites; patients enrolled at Nyamache had the lowest parasitemia. The mean weight and median age range varied across all sub-counties (Table 3).

<table>
<thead>
<tr>
<th>Sub-Counties</th>
<th>Variables</th>
<th>Kenyenya n (%) (n = 69)</th>
<th>Marani n (%) (n = 69)</th>
<th>Bonchari n (%) (n = 69)</th>
<th>Nyamache n (%) (n = 68)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (kg), mean (SD)</td>
<td>44.8 ± 7.3</td>
<td>39.9 ± 15.5</td>
<td>36.4 ± 9.7</td>
<td>38.4 ± 8.5</td>
<td>0.15</td>
<td></td>
</tr>
<tr>
<td>Gender (male), n (%)</td>
<td>31 (44.9)</td>
<td>28 (40.5)</td>
<td>31 (44.9)</td>
<td>20 (28.9)</td>
<td>0.38</td>
<td></td>
</tr>
<tr>
<td>Gender (female), n (%)</td>
<td>38 (55.0)</td>
<td>41 (59.4)</td>
<td>38 (55.0)</td>
<td>48 (71.1)</td>
<td>0.51</td>
<td></td>
</tr>
<tr>
<td>Body temperature on day 0, °C, mean (SD)</td>
<td>37.6°C ± 1.1</td>
<td>37.5°C ± 1.1</td>
<td>37.8°C ± 1.1</td>
<td>37.3°C ± 1.1</td>
<td>0.002*</td>
<td></td>
</tr>
<tr>
<td>Parasitemia (µl) on day 0* (95% CI)</td>
<td>11,4301 (7,256–12,785)</td>
<td>12,403 (8,242–13,565)</td>
<td>13,531 (9,242–15,603)</td>
<td>9,221 (7,308–11,252)</td>
<td>0.047*</td>
<td></td>
</tr>
<tr>
<td>Median age in years (males)</td>
<td>34.5</td>
<td>38.5</td>
<td>42.5</td>
<td>41.5</td>
<td>0.10</td>
<td></td>
</tr>
<tr>
<td>Age range in years (males)</td>
<td>(4–62)</td>
<td>(3–82)</td>
<td>(5–69)</td>
<td>(2–75)</td>
<td>0.172</td>
<td></td>
</tr>
<tr>
<td>Median age in years (females)</td>
<td>31.5</td>
<td>28.5</td>
<td>27.5</td>
<td>30.5</td>
<td>0.75</td>
<td></td>
</tr>
<tr>
<td>Age range in years (females)</td>
<td>(5–74)</td>
<td>(3–75)</td>
<td>(5–80)</td>
<td>(2–65)</td>
<td>0.125</td>
<td></td>
</tr>
</tbody>
</table>

*C: degree Celsius; Temperature of ≥ 37.5°C or history of fever during the previous 24 h. Parasitemia*: geometric mean parasite density (asexual parasites/µl); n: number of patients; SD: standard deviation; 95% CI: 95% confidence interval; *p < 0.05, the mean was significantly different.

Recrudescence Molecular Outcome

After a complete follow-up of the participants, 13/231 (5.6%) had *P. falciparum* on day 28. For those samples with the occurrence of parasites on day 28 after treatment had the following PCR; 77% of the samples had bands on both day 0 and day 28, hence were classified as recrudescence. About 23% of the samples had no bands on both days 0 and the respective days of parasite recurrence, hence classified as new
infections (Fig. 2). The 77% of samples showing recrudescence in this study were stored for further *P. falciparum Kelch13* sequencing to evaluate the presence of any mutational polymorphisms conferring ACTs resistance.

**PCR amplification of Pfkelch13 genes**

A total of 13 blood samples confirmed for day 28 recrudescence using microscopy correction were processed for *Pfkelch13* PCR amplifications. Parasites DNA of 5 (38%) samples were successfully amplified as shown in Fig. 3 and Table 4 by using nested PCR reaction for the *K13*-propeller gene mutations and hence were sequenced for mutations.

<table>
<thead>
<tr>
<th>Sub County</th>
<th>No of samples with recrudescence (n / %)</th>
<th><em>PfK13</em> amplified (n/%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Marani</td>
<td>3 (23)</td>
<td>1 (17)</td>
</tr>
<tr>
<td>Bonchari</td>
<td>2 (15)</td>
<td>2 (33)</td>
</tr>
<tr>
<td>Kenyenya</td>
<td>3 (23)</td>
<td>1 (17)</td>
</tr>
<tr>
<td>Nyamache</td>
<td>5 (39)</td>
<td>2 (33)</td>
</tr>
<tr>
<td>Total</td>
<td>13 (100)</td>
<td>6 (100)</td>
</tr>
</tbody>
</table>

**Prevalence of K13 –propeller mutations**

*K-13* propeller single-nucleotide polymorphisms were compared with the 3D7 reference strain (PF3D7-1343700). *K13*-Propeller non-synonymous polymorphisms recorded in the current study include R539T, N458T, R561H, N431S, and A671V. All mutations with an exception of R561H were reported in 1 sample, while R561H mutation was reported in 2 samples, respectively. However, mutation on *K13* propeller gene was not detected at positions 580, 578, 574, 568, 553, 543, 539, 537, 533, 527, 508, 493, 481, 476, and 474, respectively. A detailed analysis of the samples is shown in Table 5. In total, there were six (6) polymorphic sites identified in the 13 samples analyzed.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>No of codon</th>
<th>Wild type A.A</th>
<th>Mutant type A.A</th>
<th>Genetic change</th>
<th>Type of mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>K13-MA-007</td>
<td>R539T</td>
<td>Arg</td>
<td>His</td>
<td>GCT → TCT</td>
<td>Non-synonymous</td>
</tr>
<tr>
<td>K13-BO-011</td>
<td>N458Y</td>
<td>Arg</td>
<td>Val</td>
<td>TCT → TCG</td>
<td>Non-synonymous</td>
</tr>
<tr>
<td>K13-BO-053</td>
<td>R561H</td>
<td>Arg</td>
<td>Ser</td>
<td>GCA → TCA</td>
<td>Non-synonymous</td>
</tr>
<tr>
<td>K13-NYA-040</td>
<td>N431S</td>
<td>Tyr</td>
<td>Glx</td>
<td>ATG → GTG</td>
<td>Non-synonymous</td>
</tr>
<tr>
<td>K13-NYA-030</td>
<td>A671V</td>
<td>Asn</td>
<td>Tyr</td>
<td>TTA → ATA</td>
<td>Non-synonymous</td>
</tr>
</tbody>
</table>

**Haplotype diversity**

The mutations in the samples analyzed were unselectively neutral, as shown by the negative Tajima's D statistic (−1.72305) and Fu & Li's D test (−1.74248). There was no significant haplotype/gene diversity (*P* = 0.305) with a variance in the diversity of 0.00363 and a standard deviation of 0.078 (Table 6).
been reported previously to be associated with ACTs resistance in South East Asia of which nine have been confirmed as resistant candidates.

More than 200 non-synonymous mutations have been recognized in P. falciparum. The current study is the first report on the mutations associated with N431S and A671V, respectively. This is in tandem with other studies which have shown a relationship between mutations in the target points in the parasite and ACTs derivatives resistance. The low frequencies of mutations associated with ACTs resistance reported here in comparison to South East Asia which has reported high mutation has not been validated as a candidate of resistance. However, it is associated with poor drug response by the patients from Mbita district. However, this mutation has not been previously associated with ACTs resistance in South East Asia.

The mutation reported in N458Y locus has also been reported elsewhere in Africa and other South Eastern Asian countries. But previously this mutation has not been detected in one (1) sample, our data emphasize on the threat of the R561H mutation spreading in Kenya. Particularly, by comparing with the past great quantity of R561H mutation across the Myanmar and Thailand human movements. Despite the fact, the mutation was only detected in one (1) sample, our data emphasize on the threat of the R561H mutation in Kenya. The evolution and spread of mutant P. falciparum K13-mediated artemisinin (ART) resistance has led to extensive treatment failures all over Southeast Asia [27]. P. falciparum resistance to ACTs derivatives has been reported across Southeast Asia (SEA), having first confirmed a decade ago in western Cambodia [28, 29, 30].

The present study has reported the presence of Pfk13 polymorphisms at different loci. The mutations detected here includes R561H, R539T, N458Y, N431S and A671V. However, the frequencies of the mutations were low compared to those witnessed in ACTs resistant geographical locations. R561H Single Nucleotide Polymorphism hereby reported in one sample from Bonchari and Marani Sub Counties has previously been associated with reduced parasite clearance in South East Asia [31]. More over this mutation has been previously reported in Rwanda [32] and Tanzania [33], countries located in East Africa, thus raising concern on the probability of importation of ACTs resistant parasites as a result of human movements. Despite the fact, the mutation was only detected in one (1) sample, our data emphasize on the threat of the R561H mutation spreading in Kenya. Particularly, by comparing with the past great quantity of R561H mutation across the Myanmar and Thailand [34], the presence of the R561H variant in the study area points to the risk of ACTs resistance emergence.

Single Nucleotide Polymorphism (SNP) observed in locus R539T in the current study has also been reported in Kenya and Senegal. R539T was reported from a study conducted in Mbiba district, Kenya [35]. This mutation was highly associated with in-vivo delayed parasite clearance among the patients from Mbiba district. However, this mutation has not been previously associated with ACTs resistance in South East Asia.

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More than 200 non-synonymous mutations have been recognized in K13 protein from P. falciparum [38]. However, fifty PF-K13 mutations have been reported previously to be associated with ACTs resistance in South East Asia of which nine have been confirmed as resistant candidates.

### Table 6

<table>
<thead>
<tr>
<th>K13 analyzed Variables</th>
<th>Sub-Counties</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nyamache</td>
</tr>
<tr>
<td>Sample size</td>
<td>5</td>
</tr>
<tr>
<td>Number of haplotypes (h)</td>
<td>2</td>
</tr>
<tr>
<td>Number of nucleotide sites analyzed</td>
<td>671,458, 561</td>
</tr>
<tr>
<td>Haplotype (gene) diversity (hd)</td>
<td>0.312</td>
</tr>
<tr>
<td>Standard deviation of haplotype diversity</td>
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</tr>
<tr>
<td>Nucleotide diversity</td>
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</tr>
<tr>
<td>Variance of haplotype diversity</td>
<td>0.00724</td>
</tr>
<tr>
<td>Tajima's D statistic</td>
<td>-0.78621</td>
</tr>
<tr>
<td>Fu and Li's D test statistic</td>
<td>-0.34123</td>
</tr>
<tr>
<td>No of polymorphic sites</td>
<td>2</td>
</tr>
</tbody>
</table>

### Discussions

Resistance occurs as a result of mutations in the target points in the parasite. Limited countries across South East Asia and malaria-endemic Africa have revealed evidence of low frequency ACTs linked mutations, with an initial indication of indigenous Pfk13 mutations in the East Africa region, speculating that the threat of independent acquisition of resistance should be taken seriously [24]. There is a critical need for augmented, uniform and prospective antimalarial resistance molecular surveillance across Africa. Investigation of the association between P. falciparum mutations and reduced susceptibility to ACTs through genome-wide association studies (GWAS) and gene manipulation studies, have shown a relationship between mutations in K13 and increased parasite survival in the in-vitro conditions [25].

Currently, mutations in Kelch13 propeller gene has been indicated as ACTs resistant molecular marker. Different mutations have been previously reported in Asia, America and Africa continents, with more prevalence recorded in the Asian continent [26]. The evolution and spread of mutant P. falciparum K13-mediated artemisinin (ART) resistance has led to extensive treatment failures all over Southeast Asia [27]. P. falciparum resistance to ACTs derivatives has been reported across Southeast Asia (SEA), having first confirmed a decade ago in western Cambodia [28, 29, 30].

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while eleven are potentials for ACTs resistance. The other thirty K13 mutations have been reported from various locations in South Asia, however, they are not consistent with the clinical findings on ACTs failure. Out of these documented mutations, only nine have been authenticated as candidates for ACTs resistance under the ex-vivo conditions. The validated markers include; C580Y, Y493H F446I, M476IV, N458Y R539T, P553L, I543T, and R561H [39].

Surprisingly, previous studies have reported new non-validated mutations, which were present in patients who had poor recovery after treatment with ACTs [40]. This raises concern whether they have some roles they play in conferring resistance, and should hence be considered in the future as molecular markers of ACTs resistance. A mutation at codon A675V has been reported in Rwanda [41] and Uganda [42]. The same mutation at codon A675V had also previously been reported in SEA [43], an epicenter of the emergence and spread of ACTs resistance.

The circulation of K13 mutations have also been previously reported in Kenya but with limited studies [44]. A previous study conducted in different Malaria Transmission Areas of Kenya viz; Marigati, Kombewa, Kisumu, Kisii, Kericho and Malindi to ascertain the prevalence of K13 mutation during the pre-ACTs and post-ACTs eras, reported different polymorphisms at different locus [45]. The A578S and the V568G mutations reported in SEA were found in both pre-ACTs and post-ACTs parasites. D584Y and R539K mutations were found only in post-ACTs parasites. These mutations were also previously reported from clinical isolates from South East Asia [46], raising the question of the possibility of mediating resistance. The N585K mutation was described for the first time in this previous study among the post-ACTs parasites, and it was the most prevalent mutation at a frequency of 5.2%. However, the prevalence and type of mutations varied across the malaria ecological zones and between the pre- and post-ACT time periods. This study reported A578S in post-ACT parasites in two different study sites, Kombewa (4.3%) and Kisii (2.1%). Kombewa is situated in the holo-endemic lake region and Kisii is located in the highland epidemic region. The N585K allele was reported in only the post-ACTs era in the study areas, with the highest prevalence in Kombewa (10.6%) and Kisumu (9.8%). This mutation witnessed here might be under pressure for evolutionary through antimalarial drugs since the use of Artemether-Lumefantrine is high in Kombewa and Kisumu due to high malaria transmission [47].

Another study conducted at 4 islands in the Lake Victoria basin (Kibuogi, Ngodhe, Takawiri, and Mfangano) and the mainland of Mbita in Kenya reported different mutant alleles in the K13 propeller gene, with C580Y, Y493H and R539T being the most prevalent and significantly associated with in-vivo delayed parasite clearance. However, a new mutation of A578S was detected at Mfangano Island for two consecutive seasons. This mutation is closely related to the single nucleotide polymorphism C580Y detected from Cambodia, indicated to be conferring ACTs resistance [48]. Other mutations reported in this study included M442V, N554S, A569S, C439C, S477S, Y500Y, N531N and G538G. These mutations have not been previously associated with ACTs resistance.

A previous study on the Kenyan coast has reported limited P. falciparum K13 Artemisinin Resistance-Conferring Mutations over a 24-Year Analysis. The K189T mutation was the only polymorphism maintained at frequencies of 10%, while the rest of the observed alleles were rare, including codon A578S, with frequencies barely reaching 2% [49].

A report by the world health organization, 2021 has documented a 30-fold increase in the use of ACTs globally between 2006 and 2013 [50]. Thus, the augmented usage of artemisinin agents is expected to increase drug pressure, leading to resistance development. Consequently, irrational usage of ACTs coupled with the use of substandard drugs in the developing countries such as Kenya, may exacerbate the risk of resistance development. Bearing in mind that previous resistance to antimalarial agents was first detected in South East Asia and then later spread to Africa, it is possible that the artemisinin resistance documented in Cambodia may also spread through Myanmar via India to Africa by following the previous patterns [51]. This is likely to occur due to the increased international travel and migration, especially because Kenya serves as a transition point for travelers from Asia to Africa and South America.

After testing the departures of Nucleotide variability patterns of the sequence products from neutral expectations, the isolates from the current studies showed evidence of positive selection as highlighted by the negative values of the tests (Tajima's D = −1.72305; Fu and Li's D of −1.74248). Previous study has documented that indigenous populations and ecological level courses, such as drug pressure serve as essential mediators of resistance acquisition in the population [52]. The current study was unable to establish if the K13 propeller gene mutations detected in the P. falciparum clinical isolates from Kisii County resulted from local emergence because of ecological and population-level processes or through transfer because of global human travel or local emergence. In contrast to Africa and Kenya, where artemisinin agents are commonly used in the form of combinations, studies have indicated that more than 78% of artemisinin in South East Asia is used as monotherapies [53]. The use of artemisinin agents combined with their short half-lives may still protect the population from wide-spread emergence of resistant parasites.

The accumulation of data from Kenya will increase the understanding of the association between the K13 propeller gene and artemisinin resistance. Unfortunately, most of the molecular drug surveillance conducted in Kenya was performed in western and coastal regions. Thus no clear picture of the molecular data is available.
The current study has reported low circulation of the previously validated $pfK13$-resistant markers, with two new mutations which have not previously been reported globally. However, there were limited polymorphisms for the previously reported $pfK13$ mutations validated as resistance markers. Due to insufficient evidence, the study concludes that artemisinin resistance is yet to be confirmed in Kisii County. Moreover, the new single-nucleotide polymorphism mutations detected in our study need to be characterized further to ascertain their role in conferring ACTs resistance. In addition, molecular surveillance of drug resistance needs to be scaled up in Kenya to provide regular updates on the possibilities of the emergence and spread of ACTs resistance for future malaria containment.

Declarations

Acknowledgements

We hereby wish to appreciate all the participants who took part in this study. We also thank the entire staff of the molecular laboratory of Makerere University, School of Health Sciences, for granting us the permission to use their laboratory for the molecular work and the clinicians of different health facilities used during this study.

Funding

Funding for this work was solely by the authors.

Availability of data and materials

The sequences have been deposited in NCBI with bio-project number PRJNA85380 and accession numbers SAMN31087430, SAMN31087431, SAMN31087432, SAMN31087433, and SAMN31087434 for Marani, Nyamache, and Bonchari samples, respectively.

Authors’ contributions

This study was conducted in collaboration with all the authors. All authors have made substantial contributions to conception and design, acquisition of data, analysis and interpretation of data; took part in drafting the article and revising it critically. All authors have read and approved the final version of this manuscript.

Competing Interest

All authors declare that there are no conflicts of interest existing with regard to this study and publication of the findings.

Ethical Consideration

Ethical approval was sought from the University of East Africa, Baraton Institutional Review Board (UEAB/REC/4/2/2021, research permit was issued by Kenya National Commission for Science, Technology and Innovation (NACOSTI) License No: NACOSTI/P/21/8974 and Kisii County government (DTR/4/27).

References


Plasmodium falciparum


**Figures**

![Map of study area](image_url)

**Figure 1**

A map showing the study area. (A) Shows the country Kenya where Kisii County is located. (B) Shows Nyanza region where Kisii County is located and (C) shows different Sub Counties of Kisii County where Sampling was conducted.
Figure 2

Gel image showing the amplification of *P. falciparum msp2* of recurrent samples. Bands 2,3,4,5,6,7,8,9,10 and 11 show positive msp2 allelic family. Band13 is the negative control, and lanes 1, 12, 14, and 18 show 100 bp Molecular Weight DNA ladder (New England BioLabs, Massachusetts, USA).

Figure 3

Positive PCR products detected using Agarose Gel Electrophoresis. Well 1,2,15, and 16 contain 250 bp DNA ladder, wells 8 and 9 contains a positive control (*PF3D7_1343700*). The rest of the well contained samples. Samples 3,5,6,10,11 and 14 are positive for *Pfkelch13* mutations,
while samples 4, 7, 12 and 13 are negative for *P.falciparum* Pfcytochrome c3 mutations. The expected amplicon size was approximately 800 bp.