A countrywide survey of hrp2/3 deletions and kelch13 mutation co-occurrence in Ethiopia

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Malaria elimination relies on detection of Plasmodium falciparum Histidine-Rich Proteins 2/3 (HRP2/3) through rapid diagnostic tests (RDTs) and treatment with artemisinin-combination therapies (ACTs). Data from the Horn of Africa suggest increasing hrp2/3 gene deletions and ACT partial resistance kelch13 (k13) mutations. To assess this, 233 samples collected during a national survey from 7 regions of Ethiopia were studied for hrp2/3 deletions by droplet digital dPCR and k13 mutations by DNA sequencing. Approximately 22% of the study population harbored complete hrp2/3 deletions by ddPCR. Thirty-two of 42 of k13 SNPs identified were R622I associated with ACT partial resistance. Both hrp2/3 deletions and k13 mutations associated with ACT partial resistance appear to be co-occurring especially in Northwest Ethiopia. Ongoing.
national surveillance relying on accurate laboratory methods are required to fully elaborate the genetic diversity of \textit{P. falciparum} to inform public health policy makers.

\textbf{Keywords} malaria, histidine-rich protein 2, rapid diagnostic tests, polyclonal infections, artemisinin, kelch13, drug resistance

\section*{INTRODUCTION}

\textit{Plasmodium falciparum} causes most human malaria cases and 94\% of total malaria-related deaths in the WHO African Region” in 2022 [1, 2]. Current malaria diagnostic approaches rely either on direct identification of the parasite in blood samples using light microscopy or on rapid diagnostic tests (RDTs) [3]. The ease of use and availability of RDTs through subsidies has led to a broad implementation of RDTs. It is now estimated that more than 300 million RDTs are used in malaria control programs each year [1]. RDTs work by detecting the \textit{P. falciparum} histidine-rich protein-2 (HRP2) protein (which is specific to \textit{P. falciparum}) or by detecting pan-\textit{Plasmodium} proteins (Lactate Dehydrogenase or aldolase, or their species-specific variant). Of these, HRP2 based RDTs are the most broadly deployed [4]. Initially, a report in 2010 revealed parasites with the \textit{hrp2} gene deleted, resulting in the escape of diagnosis from HRP2 RDTs [3]. Since then, \textit{hrp2}/3 deleted parasites have also been reported in [would be more specific and add a reference]. Systematic reviews suggest an increase in prevalence of \textit{hrp2}/3 gene deletions between 2018 (17\%), and 2022 (21.3\%) [5]. In their 2021 report, the WHO emphasized that \textit{hrp2}/3 deletions are one of the major threats to malaria control and eliminations programs [1]. The gene, \textit{hrp2}, is located on chromosome 13 with exon 1 being 69 base pairs long, while exon 2 is 848 base pairs long. The gene, \textit{hrp3}, is located on chromosome 8, with exon 1 being 69 base pairs long as well, while exon 2 is 758 base pairs long [6]. The gene, \textit{k13}, is located on chromosome 13. The nested PCR region of the sequenced \textit{k13} propellor domain is 848 base pairs long [7].

\textit{P. falciparum} confirmed cases are mostly treated by artemisinin combination therapies (ACTs). Delayed clearance to artemisinin was first observed in 2008 in Western Cambodia [8]. Single nucleotide polymorphisms (SNPs) in the \textit{pfkelch13} gene (\textit{k13}) coding for the K13 protein propellor domain of \textit{P. falciparum} were identified as the key mutations for delayed parasite clearance following ACT [11]. In recent years, reports have emerged that ACT treatment failure is occurring in Uganda and Rwanda [9, 10]. Slow clearance rates are associated with recrudescence which ultimately reflects the loss of efficacy of ACTs. ACT therapeutic efficacy studies are required to continually assess the clinical utility of this treatment regimen. Additionally molecular epidemiology surveys for the prevalence of \textit{k13} mutations are required.

Infections with \textit{Plasmodium}, and particularly \textit{P. falciparum}, are polyclonal; that is, multiple parasite clones are infecting the human host [12]. These polyclonal infections can harbour different \textit{hrp2} genotypes (deleted/non deleted, or different portion of the \textit{hrp2} gene deleted), which might lead to under or overestimation of \textit{hrp2} deletion prevalence with conventional screening
approaches such as PCR which only amplifies the most abundant clone. Consequently, a limitation to many hrp2/3 deletion studies is the presence of multiple clones within a clinical sample. PCR based methods can lead to either false positives (hrp2 presence observed, while the isolate might contain hrp2 deleted clones), or to false negatives (hrp2 deletion observed when a sample contains wild type parasites). Accurately capturing the prevalence of hrp2/3 deletion within a population is paramount to public health policies, particularly as the WHO recommends that areas with ≥ 5% prevalence of hrp2 deletion should implement non-HRP2 based RDTs for malaria diagnostics [1].

The co-occurrence of diagnostic and treatment resistant parasites is threatening malaria control and elimination programs. The mechanisms and evolutionary forces driving the spread of these mutants are poorly understood. Recent studies conducted in Ethiopia has further emphasized that parasites are evolving to “escape” HRP2 based diagnostics with deletion in the hrp2/3 genes [13-18]. An even more concerning finding from Mihreteab et al., recently published, shows an increased prevalence of artemisinin resistant hrp2 deleted parasites over the past five years in neighbouring Eritrea [19]. Our study provides further evidence that these two phenotypes “drug and diagnostic resistance” are occurring in the region. However, the exact genetic and evolutionary mechanism of this coexistence remains to be elucidated. To this end, in the present countrywide cross-sectional study, we explored the co-occurrence of hrp2/3 deletions and k13 mutations by using a subset of samples from the Ethiopian national malaria survey. Furthermore, we implemented a quantitative assay based on (Droplet digital) (dd)PCR technology to accurately quantify the proportion of hrp2/3 deleted parasites within a sample.

METHODS

Clinical sample selection

This study stems from a national cross-sectional study cohort performed by the Ethiopia Public Health Institute (EPHI) across 7 out of the 9 national regional states in Ethiopia following the WHO protocol [20]. Samples were collected within the malaria peak season (August – December) between 2017 to 2018 for Amhara, Tigray, and Gambella, and between 2020 to 2022 for Afar, Somali, Oromia, and SNNPR. Participants were included through passive enrollment of the population for those presenting symptoms of malaria, such as fever, headache, joint pain, feeling cold, nausea, and/or poor appetite. Whole blood samples from patients testing negative after screening with RDT CareStart Pf/Pv (HRP2/Pv-pDH) (Access Bio, catalog no. RM VM-02571) but positive by SD Bioline Malaria Ag P.f. (HRP2/2/Pf-LDH) RDT (Alere, catalog no. 05FK90) were preserved as dried blood spots (DBS) on Whatman 3MM paper (Cytiva, Malborough, USA) together with 20% concordant positive samples in 2017. In the 2021 survey, RDT CareStart Pf/Pv (HRP2/Pv-pDH) negative and microscopy positive samples were collected using DBS together with 20% concordant samples. A subset of de-identified DBS (representative of 7 regional states of Ethiopia) was sent to the University of Calgary for downstream genetic analysis. Ethical approval was obtained from the Ethiopia Public Health Institute (EPHI) Institutional Review Board.
Board (IRB; protocol EPHI-IRB-033-2017) and WHO Research Ethics Review Committee (protocol ERC.0003174.001). Genetic analysis of de-identified samples was conducted with ethics approval from the Conjoint Health Research Ethics Board at the University of Calgary (REB21-1059).

DNA extraction and preservation

DNA was extracted from preserved DBS. All extractions were performed by systematically hole-punching the DBS and using QIAamp DNA Blood Kits (Qiagen, Hilden, Germany) according to the recommendation of the manufacturer. The obtained DNA was eluted in 50 µL of nuclease-free water (VWR, Radnor, Pennsylvania, USA) and preserved at -80°C until further use.

Genomic DNA controls for molecular assays

The following genomic DNA controls were used in each ddPCR and end point PCR assay: MRA-102 (3D7, wild type), MRA-156 (DD2, Δhrp2), and MRA-155 (HB3, Δhrp3) diluted at 100 genome copy/µL, alongside a mix of MRA-102 and MRA-156 for hrp2 assay, and MRA-102 and MRA-155 for the hrp3 assay. For the ddPCR assay optimization, mixtures of MRA-102, MRA-156, and MRA-155 were analyzed at concentrations of 1000, 500, 100, and 10 gDNA copies per µL at different relative ratios (90:10, 75:25, 50:50, 75:25, and 10:90). Mixtures at each concentration were run in triplicates, and the ratio of hrp2/3 to tRNA ligase was analyzed with their theoretical values to ensure the methods robustness. The following reagents were obtained through BEI Resources: Plasmodium falciparum, Strain 3D7, MRA-102, contributed by Daniel J. Carucci; Strain Dd2, MRA-156G, HB3, and MRA-155G contributed by Thomas E. Wellems.

Detection of hrp2/3 deletions with PCR and digital droplet PCR (ddPCR)

End point hrp2/3 PCR analysis was performed on the selected samples as described previously [13, 21]. Hrp2/3 deletion assays were performed using the ddPCR system from Bio-Rad (Bio-Rad, Hercules, California, USA) by modification of the previously described ddPCR assay [22]. Modifications include that only droplet counts >10,000 per reaction were retained for downstream analysis. Ratios that were above 0.75 hrp2/3 to tRNA ligase were said to have no deletion present. Ratios between 0.75 and 0.25 were labeled as mixed infections, and ratios below 0.25 were said to have a full deletion present. Samples were included if 5 or more tRNA ligase droplets were present to show sufficient parasitic DNA material was present for analysis. Of the 455 selected samples, 322 samples met the 5 or more tRNA ligase droplet threshold requirement for ddPCR analysis and were also evaluated on end-point PCR by screening for the exon 2 for both hrp2 and hrp3 genes. Of the 322 samples that were analyzed using ddPCR and PCR for hrp2 and hrp3, 47 samples lacked sufficient genomic DNA for further analysis. A total of 275 samples underwent k13 nested PCR and sanger sequencing, resulting in successful sequencing outcomes for 233 samples. However, 32 samples failed k13 sequencing.

K13 sequencing and single nucleotide polymorphisms (snps)
The k13 propeller domain was amplified by nested PCR as described previously [11, 23]. PCR products were run on a 2% agarose gel with 1 Kb ladder (New England Biolabs, Ipswich, USA) and the bands at 849 bp were purified using the QIAQuick® Gel Extraction Kit (Qiagen, Hilden, Germany). By-directional Sanger sequencing of the purified PCR products was performed using big dye terminator chemistry on an Applied Biosystems 3730xl instrument at the Centre for Health Genomics and Informatics (University of Calgary). Sequencing results were assessed individually by analysing both strands with MAFFT local pairwise alignment tool on Benchling cloud-based platform (Benchling [Biology Software] 2023) against the P. falciparum 3D7 k13 reference sequence (PlasmoDB: PF3D7_1343700). Supplementary table 3 shows the primers sequences used for nested PCR.

Principal component analysis of the hrp2/3 and k13 status

Across all samples sequenced for k13, a local alignment was performed using MAFFTv.7 with default parameters. A dissimilarity matrix was constructed, and the principal components were depicted to reduce multidimensionality across samples.

Statistical analysis

All p-values below 0.05 were considered statistically significant. All statistical analyses were carried out using R (v. 4.2.2.), RStudio (v.1.3.1056) and Graphpad Prism (v.9.4.1). For distributions with continuous variables a Shapiro-Wilk test and an unpaired two-tailed t-test or Mann-Whitney test was carried out to test for normality. For categorical variables, a 2x2 contingency table was generated followed by a two-tailed Fisher’s exact test. A Kruskal-Wallis test with repeated measures was carried out when more than two distributions were present. A permutational multivariate analysis of variance (PERMANOVA) with 999 permutations was carried out across the Principal component analysis (PCA) to determine if significance between centroids exist.

RESULTS

Samples and study population

A total of 455/757 samples were selected for downstream analysis after statistical evaluation to ensure no bias introduced (data not shown). The population selected for analysis had a sex ratio of 70.63% male and 29.37% female, age range [12-25.75] and median parasite density of 2,144 range [379.1-8419.9]. These 455 samples were analysed with the three molecular tools implemented in this study (hrp2/3 end point PCR, ddPCR quantification of hrp2 and hrp3, and k13 sequencing).

PCR versus ddPCR analysis reveal mixed genotypes

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Of the 233 samples analyzed, hrp2/3 PCR results showed that 16 (6.86%) of samples were positive for hrp2, 71 (30.47%) were negative for hrp2 and 169 (72.53%) were negative for hrp3. 5 (2.15%) samples were positive for both hrp2 and hrp3 and 61 (26.18%) were negative for both hrp2 and hrp3. When the samples were analyzed through ddPCR, 55 (23.61%) showed full hrp2/3 deletions (meaning all the clones detected in these samples were deleted for the targeted gene) in hrp2 exon 1, 51 (21.89%) in hrp2 exon 2, and 161 (69.10%) in hrp3. Furthermore, the fine resolution of ddPCR allowed for the identification of samples that contained mixed genotypes, meaning that multiples clones within the samples present a differing hrp2 deletion status. Mixed genotypes represented 16.74% of samples (39/233) for hrp2. In addition, 4.29% of samples (10/233) had partial deletions in hrp2 (seven in exon 1 and three in exon 2). Only 7.73% of samples (18/233) were mixed hrp3 infections. As the hrp3 status was deemed less significant from a RDT reactivity perspective, downstream analysis grouped hrp3 as not deleted or deleted (with mixed infections being listed as not deleted) (Figure 2A). Details on statistical differences among the techniques used can be seen in the supplementary document (supplementary table 4). A total of 64 samples were negative for HRP2 via RDT, with ddPCR suggesting only 33 were truly negative for the exon that encodes for the HRP2 protein in the RDT. Furthermore, PCR suggested of 36 samples were hrp2 negative.

Detection of snps in the kelch13 propeller domains

A total of 13 unique non-synonymous SNPs were identified amongst 42 total samples containing SNPs (Figure 2B). Of these identified SNPs in the K13 propeller domain, 31 samples encoded for R622I, a validated marker for artemisinin partial resistance. W518C was the only other SNP to be found more than once (n = 2). The region containing the highest proportion of the R662I mutations was found in Amhara (19.44%, 28/144). The Tigray region was the only other region where the R622I mutation was found (8.33%, 3/36). All regions excluding Afar, and Somali contained at least one SNP. Two samples contained two different SNPs, with one located in Amhara (R622I and Y625) and the other in Gambella (Y6230F, A504T). Among the samples presenting the R622I SNP, 12.5% (4/32) were deleted for hrp2 exon 1 and 9.4% for (3/32) hrp2 exon 2 deletion and two samples (6.25%) had deletions in both hrp2 exons. Additionally, 87.5% (28/32) of samples that contained an R622I SNP also had a hrp3 deletion (Figure 3A, B).

PCA of the hrp2/3 and k13 status

The PCA demonstrates that the sequence dissimilarity of the k13 propeller domain is not significantly different by hrp2 status or R622I (Figure 4). In support of this, in separate analysis, no significant association occurred between the presence of hrp2 assessed by ddPCR, regardless of the extent of the deletion (positive, mixed, or partial, negative n = 168) and the presence of any R622I SNPs in the k13 propeller among RDT positive samples (p = 0.146). hrp2 positive/partial/mixed samples had a higher rate of encoding an R622I SNP compared to hrp2 exon 1 and 2 negative samples (p = 0.0330). No significant association was observed between hrp3 deletions (n = 215) and the presence of SNP/R622I in the k13 propeller domain (p = 0.0907).
DISCUSSION

In the current study, hrp2/3 PCR assays suggest that 30.47% of the samples analyzed are HRP2-deleted parasites, while this number decreases to 23.61% for combined hrp2 exon 1 and 21.89% for exon 2 when analyzed with ddPCR. The reason for this difference in deletion estimation (6.86-8.58%) may be attributed to 16.74% of samples retaining polyclonal infections, and 4.29% of samples elaborating partial deletions. Furthermore, since most hrp2 PCR assays exclude the detection of exon 1 [20] the combination of polyclonal infections, partial and full deletions, and a lower sensitivity can cause an overestimation of hrp2 deletions. These differences can also be attributed to the higher sensitivity of ddPCR and its ability to detect partial deletions and mixed infections. The combination of polyclonal infections, partial and full deletions, and a lower sensitivity can cause an overestimation of hrp2 deletions. These differences can also be attributed to the higher sensitivity of ddPCR and its ability to detect partial deletions and mixed infections. We also analyzed hrp3 deletions with ddPCR and determined that almost 69.10% of samples contained HRP3-deleted parasites. A trend in high amounts of HRP3-deleted parasites in the horn of Africa has been reported before by hrp3 PCR [13, 26]. ddPCR analysis also showed that 16.74% of the samples were double-deleted parasites. However, of these 16.74% double-deleted parasites, 69.32% were negative for HRP2 via RDT. This shows an increase compared to previous studies [15-17, 27]. Taken together, our study and that of others suggest that deletion rates exceed the 5% WHO recommendation for HRP2-deleted infections in certain regions of Ethiopia. One note of caution is that PCR assays for hrp2/3 deletions are qualitative in nature and can lead to an overestimation of hrp2/3 deletions when addressing partial deletions and/or polyclonal malaria infections.

As suggested by recent studies in Ethiopia and its neighbouring country, Eritrea [14, 19], the presence of partial artemisinin resistance in Ethiopia is increasing due to occurrence of SNPs in the k13 gene. A worrying trend is increase in the prevalence of the R622I k13 mutation, that was first reported in Ethiopia in 2015 by our group [28]. In 2022, the WHO classified R622I as a validated marker for partial resistance to artemisinin [1, 29]. Other than R622I, no other SNP detected in this study has been validated by the WHO for artemisinin resistance. Only one other SNP identified in this study, W518C (previously reported in Kenya) [30], was identified more than once (2/42, 4.76%). All but one SNP, Y625 (1.9%, 1/42), were previously reported in earlier studies, with two being previously reported in Ethiopia; N599I (1.9%, 1/42), S550F (1.9%, 1/42). All but two SNPs, T535A (1.9%, 1/42) and Y616H (1.9%, 1/42) had been previously identified in Africa.

As reported by Fola et al., [14], and Mihreteab et al., [19], the interplay of hrp2/3 deletions and k13 SNPs co-occurring to resist both detection against HRP2 RDTs and ACT treatment may be increasing due to selective pressure. This was noted in our study, in which 10.26% of double-deleted hrp2/3 samples contained a k13 SNP, with 5.13% being R622I. Moreover, 13.73% of samples that contained an hrp2 exon 2 deletion, the gene that expresses the antigen used for detection in HRP2 RDTs, also encoded a k13 SNP, with 5.88% encoding R622I. Interestingly, it
has been reported that hrp2/3 deletions may be associated with drug resistance [31, 32], as HRP2/3 plays a role in the digestion/metabolism of hemoglobin to hemozoin in *P. falciparum*. By deleting HRP2/3, the metabolism of heme is slowed [31, 32]. Notably, hemoglobin by-products activate artemisinin, so by slowing hemoglobin digestion, the susceptibility of the parasite to ACT is reduced [33-35]. However, the associated fitness cost of these deletions might slow the rate of spread of these mutant genotypes [36, 37]. Additionally, the role of the *k13* gene in drug resistance has also been associated with hemoglobin digestion [33, 38]. Thus, both hrp2/3 deletions and *k13* mutations may arguably have similar roles in providing *P. falciparum* resistance to ACT. Nevertheless, our findings indicate that these deletion and mutations are co-occurring without a clear statistical association. However, further studies that incorporate population genetic signatures are warranted to confirm the exact relationship between the two genotypes.

There are some limitations to our study. Our dataset was predominantly samples from the Amhara region which is the largest population, but having more samples analyzed from the six other regions in the future could provide a better national assessment of the hrp2/3 and *k13* status. Furthermore, the inclusion of next generation sequencing of *k13* SNPs such as amplicon deep sequencing (ADS) may further elucidate the genetic complexity of ACT resistance as recently demonstrated by our studies on atovaquone-proguanil resistance [39].

**CONCLUSION**

To conclude, data presented here demonstrate ddPCR provides a better estimate on the true prevalence of hrp2/3 deletions and how factors such as partial deletions and mixed infections may impact to determine the percentage of parasites that do not encode hrp2/3. We have also highlighted that the *k13* propellor domain SNP that provides partial resistance to artemisinin, R622I, is becoming more prevalent in Ethiopia. Most importantly, we have indicated that co-occurrence of hrp2/3 deletions with *k13* SNPs, may be more complex than initially described. Factors such as polyclonal infections and the subsequent difficulties in identifying hrp2/3 deletions may impact the prevalence of co-occurrence between these genes. Future research is warranted to establish the true relationship between hrp2/3 deletions and *k13* mutations.

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**Author contributions:** SF and AA collected clinical data, coordinated national survey, and performed routine lab analysis. JBG, CK and AW managed the samples. JBG and CK adapted the
ddPCR assay. JBG, CK, AW, and EP performed the ddPCR assay. JBG, SA, and AW performed the PCR analysis. JBG and SA performed the k13 sequencing laboratory work and JBG and DCM conducted bioinformatic analysis. JBG, CK, and SA prepared the initial draft. JBG and DCM performed the statistical analysis, and DCM performed the PCA. DP and SF supervised, designed, and obtained funding for the different elements of this study. All authors critically reviewed the paper and agreed on the final form.

Conflicts of Interest: All authors report no conflicts of interest arising from this work.

Table 1. Demographics and select clinical characteristics of the study population.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Study Population (n=233)</th>
</tr>
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<tbody>
<tr>
<td><strong>Patient distribution</strong></td>
<td></td>
</tr>
<tr>
<td>Afar</td>
<td>7 (3.00%)</td>
</tr>
<tr>
<td>Amhara</td>
<td>163 (69.95%)</td>
</tr>
<tr>
<td>Gambella</td>
<td>5 (2.14%)</td>
</tr>
<tr>
<td>Oromia</td>
<td>24 (10.30%)</td>
</tr>
<tr>
<td>Somali</td>
<td>7 (3.00%)</td>
</tr>
<tr>
<td>SNNPR</td>
<td>10 (4.29%)</td>
</tr>
<tr>
<td>Tigray</td>
<td>36 (15.45%)</td>
</tr>
<tr>
<td><strong>Patient data</strong></td>
<td></td>
</tr>
<tr>
<td>Median age (IQR) - years</td>
<td>19 (12-25.75)</td>
</tr>
<tr>
<td><strong>Sex</strong></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>178 (70.63%)</td>
</tr>
<tr>
<td>Female</td>
<td>74 (29.37%)</td>
</tr>
<tr>
<td><strong>Location of residence</strong>a</td>
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</tr>
<tr>
<td>Urban</td>
<td>63 (27.03%)</td>
</tr>
<tr>
<td>Rural</td>
<td>162 (69.52%)</td>
</tr>
<tr>
<td><strong>Median parasite density</strong></td>
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</tr>
<tr>
<td>(IQR) - µL</td>
<td>2,144 (379.1-8419.9)</td>
</tr>
<tr>
<td><strong>Symptoms</strong></td>
<td></td>
</tr>
<tr>
<td>Fever (&gt;38.5°C)</td>
<td>225 (96.56%)</td>
</tr>
<tr>
<td>Headache</td>
<td>223 (95.70%)</td>
</tr>
<tr>
<td>Nausea b</td>
<td>136 (58.36%)</td>
</tr>
<tr>
<td>Joint pain</td>
<td>203 (87.12%)</td>
</tr>
<tr>
<td>Chills c</td>
<td>155 (66.52%)</td>
</tr>
<tr>
<td>Treatment d</td>
<td>49* (21.03%)</td>
</tr>
<tr>
<td>ACT</td>
<td>36 (15.45%)</td>
</tr>
<tr>
<td>Other e</td>
<td>13 (5.57%)</td>
</tr>
</tbody>
</table>

a: No residential data was obtained from eight.
b: One patient’s data for Nausea was not taken.

c: Treatment was recorded as ‘present’ if the patient received an antimalarial in the two weeks before the study enrollment.

d: No patient history of treatment was recorded for one patient.

h: No patient history of treatment was recorded for two patients.

i: Other types of antimalarial include chloroquine, co-artem, Panadol, and quinine.

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Figure 1. Study workflow and sample selection. A total of 757 dried blood spot samples were received from the Ethiopian National Malaria surveys form 2017 and 2021 across Afar, Gambella, Oromia, SNNPR and Somali regions. 233 samples were analyzed with the three molecular tools.
implemented in this study (hrp2/3 end point PCR, ddPCR quantification of hrp2 and hrp3, and k13 sequencing).

**Figure 2.** Prevalence of hrp2/3 deletions in relation to PCR and ddPCR and k13 SNP sequencing results. A) describes the differences in hrp2/3 status between PCR and ddPCR. PCR estimates that 30.47% (71/233) samples contained hrp2 deletions while ddPCR estimates that 23.61% (55/233).
While PCR can only distinguish hrp2 status by only full deletion or no deletion, ddPCR can distinguish hrp2 by no deletion, full deletion, mixed (16.74%) or partial (4.29%). B) Of the 233 samples analyzed, a total of 13 unique k13 SNPs were identified across 42 samples (all nonsynonymous). The most prevalent k13 SNP detected was R622I (73.81%, 31/42). Only one other SNP, W518C, was detected in more than one sample (4.76%, 2/42).

**Figure 3.** Regional distribution of hrp2/3 and k13 genotypes. The plots demonstrate distribution of samples by region in Ethiopia and their associated k13 and hrp2/3 status. A) shows the presence of k13 SNPs in the propellor domain, with red highlighting the presence of the R622I mutation. B) shows the hrp2/3 status of each region obtained with digital droplet PCR with red highlighting samples that contain both hrp2 and hrp3 deletions representing complete loss of hrp 2/3.

**Figure 4.** Principal component analysis (PCA) of the hrp2/3 and k13 status. The constructed dissimilarity matrix and the principal components in the graph displays the 233 samples analyzed for k13 SNP (wild type in blue, R622I in green, and other SNPs in red). hrp2 status assessed by ddPCR is represented by shapes. The green circles represent samples that were negative for hrp2
and encoded a R622I SNP in the k13 propellor domain. The PCA explains that the sequence dissimilarity of the k13 propellor domain is not significantly different by hrp2 status or R622I.