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Comprehensive analysis of molecular markers linked to antimalarial drug resistance in *Plasmodium falciparum* in Northern, Northeastern and Eastern Uganda

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Abstract

Background In Uganda, antimalarial resistance in *Plasmodium falciparum* poses serious public health and treatment challenges. Globally, recent data have highlighted the roles of following genes in malaria resistance: *Plasmodium falciparum dihydrofolate reductase* (*Pfdhfr*), *Plasmodium falciparum dihydropteroate synthetase* (*Pfdhps*), *Plasmodium falciparum chloroquine resistance transporter* (*Pfcrt*), *Plasmodium falciparum multidrug resistance gene 1* (*Pfmdr1*), and *Plasmodium falciparum K13* propeller domain (*Pfk13*). This study investigated the prevalence and characteristics of *P. falciparum* molecular markers linked to antimalarial resistance in Northern, Northeastern, and Eastern Uganda.

Methods This cross-sectional study collected 200 dried blood samples from children (2 months to 12 years) in Northern, Eastern, and Northeastern Uganda. Samples were from malaria-positive cases confirmed by rapid diagnostic tests and microscopy. Genomic DNA was extracted from these samples and analysed using Molecular Inversion Probes to detect *Plasmodium falciparum* genetic mutations. The sequencing was performed on the Illumina MiSeq platform, and raw data was organized and analysed with MIPTools software.

Results The study sequenced over 50% of the samples at each site as follows: Apac 87.7% (43/49), Moroto 68.0% (34/50), Soroti 65.0% (13/20) and Mbale 53.1% (43/81). The *Pfk13* A675V and C469Y mutations varied from 0 to 23.3% and 8.3–14.3%, in four sites, with consistently low prevalence in Apac. The *Pfdhfr* N51I and S108N mutations were fixed in all districts, while C59R was fixed in Moroto and nearing fixation (92–97%) in other regions. The emerging I164L mutation ranged from 1 to 10% in all sites. The *Pfdhps* A437G and K540E mutations were fixed in Soroti, with 3–5% wild-type prevalence in other sites. The A581G mutation showed 2.3% mixed genotypes in Mbale only. The *Pfcrt* K76T was predominantly wild type, except for 5% mutants in Mbale and Moroto. The *pfmdr*1 N86Y were wild type across all districts, except for 15% mixed genotypes in Soroti.

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Conclusion This study reveal rising partial artemisinin resistance and widespread antifolate resistance surpassing WHO thresholds in Northern, Northeastern, and Eastern Uganda. Emerging super-resistant parasites pose a serious threat to malaria control, necessitating urgent enhanced surveillance and alternative treatment strategies.

Keywords Antimalarial resistance, Uganda, *Pfk13*, *Pfdhfr*, *Pfdhps Pfmdr1*, *Pfcrt*

Background

Globally, antimalarial drug resistance is a developing obstacle in the fight against malaria, particularly in the sub-Saharan Africa (SSA) and Southeast Asia (SEA) regions where *Plasmodium falciparum* is prevalent [1]. Emergence of resistance reverses the global gains in malaria control attained in the over two decades of use of artemisinin-based combination therapy (ACT) [2, 3]. Uganda, an East African country in SSA, remains highly burdened with malaria and was ranked 5th top contributor of cases globally in 2021 [4]. Recent reports of the emergence of independent malaria resistance in the northern part of the country [5], have raised concerns that the phenomenon may be widely spread, but has not been systematically established. Some reports on resistance to antimalarial drugs in Uganda have emerged with indication of the spread of artemisinin resistance-associated molecular markers of *P. falciparum* [6, 7], but few data if at all any have comprehensively studied altogether known and emerging markers. Understanding the prevalence, distribution, and characteristics of these molecular markers through detailed analyses are critical for informing malaria control strategies and the continued effectiveness of antimalarial therapies.

Molecular markers of antimalarial resistance of *Plasmodium falciparum*, such as mutations in the K13 propeller domain (*Pfk13*), *Plasmodium falciparum dihydrofolate reductase (Pfdhfr), Plasmodium falciparum dihydropteroate synthetase (Pfdhps), Plasmodium falciparum chloroquine resistance transporter (Pfcrt)* and *Plasmodium falciparum multidrug resistance gene 1 (Pfmdr1) genes* have been extensively studied in SEA [8, 9], and some reports in Uganda are emergent [6, 10–12]. Mutations in the *Pfk13* gene are indicators of emerging resistance to artemisinin derivatives [6], while *Pfdhfr* and *Pfdhps* genes are associated with resistance to antifolate drugs like pyrimethamine and sulfadoxine, respectively [12]. Furthermore, *Pfcrt* and *Pfmdr1* mutations are linked to quinoline and arylaminoalcohol drugs [13].

Previous studies in Uganda point to varying levels of resistance in different parts of the country. Early evidence indicates population and geographic distribution of these molecular markers of antimalarial resistance [12, 14–18], but have a potential for homogeneous diffusion across the country with time, travel and immigration of populations. Data from studies conducted between 2016 and 2023 show emergence of partial resistance to artemisinins in multiple geographic locations, with increasing prevalence and regional spread over time [6]. Therefore, regular updates on district and region-specific data are urgently needed to guide targeted interventions. Such data would update whether partial resistance has progressed to saturation or not. In addition, they are important in determining on geographical variations in the levels of resistance. Comprehensive analysis of resistance markers are paramount in assessing the prevalence of other molecular markers of antimalarial resistance beyond *Pfk13* in heterogeneous malaria transmission intensity settings. Therefore, this study assessed the prevalence of key molecular markers of antimalarial resistance in *P. falciparum* isolates among patients attending care in four settings: Eastern (Soroti and Mbale), Northern (Apac), and Northeastern (Moroto) in Uganda.

Methods

Study setting

The study was conducted as part of the "Development of Geospatio-Temporal Surveillance and Characterisation of Malaria in the Changing Climatic conditions in Eastern Uganda study (GTS-MACS) in four sites in Uganda: Eastern (Soroti and Mbale), Northern (Apac), and Northeastern (Moroto) from February 7, 2024, to August 7, 2024. These sites were selected purposively due to the reported high prevalence of malaria in the locales. Figure 1 below shows the study sites and their access by the community served.

Study design

A cross-sectional study was designed to determine the prevalence of molecular markers of antimalarial resistance in patients from East, North, and Northeastern Uganda.

Participants recruitment and selection

Participants aged two months—twelve years of age and seeking healthcare for non-complicated malaria at any one of the four health facilities in Apac General Hospital, Mbale Regional Referral Hospital, Moroto Regional Referral Hospital, and Soroti Regional Referral Hospital were eligible to participate in the study. Patients who tested malaria positive on rapid diagnostic test (RDT) and confirmed with *P. falciparum* by microscopy were

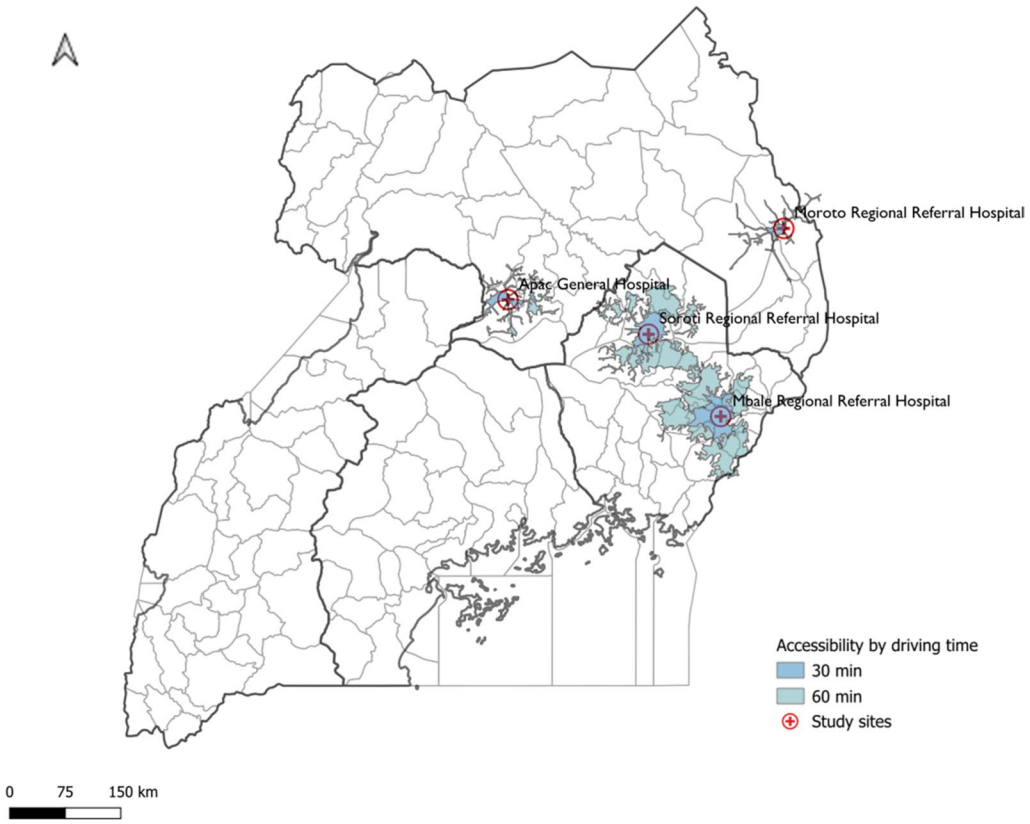

Fig. 1 A map of Uganda showing study sites and their catchment areas

included in the study after consent by a parent or caretaker (and ascent for legally qualifying participants).

A single-tier cluster sampling plan was adopted to obtain a representative sample from each of the four districts in the Northern, Eastern, and Northeastern subregions of Uganda.

Data collection and laboratory procedures

The data used in this analysis was collected between February 7, 2024, and August 7, 2024. A customised questionnaire was developed, pre-tested and administered on one-on-one interview by trained research assistants. Variables such as sex, age, history of fever, and use of antimalarials related to the most recent bout of fever were recorded by trained research assistants.

Study trained laboratory staff were responsible for blood sampling via venipuncture. Blood samples were taken for rapid diagnostic test (RDT) and when positive a confirmatory microscopic examination was done. In addition, a few drops of blood were blotted onto filter papers and left to dry as dried blood spots (DBS). The DBS samples were stored in plastic bags with silica gel to prevent DNA degradation, and transported to the Uganda Ministry of Health's Central Public Health Laboratory (CPHL) in Luzira, Kampala, for further analysis.

The study focused on analyzing resistance markers related to artemisinin and antifolate drugs. Specifically, it examined single nucleotide polymorphisms in the propeller domain of the *Pfk13* gene at codons P441Y, A469Y, C469Y, R561H, C580Y, and A675V, which are associated with artemisinin resistance. Additionally, antifolate resistance markers were investigated by analysing mutations in the *Pfdhfr* gene at codons S108N N51I, C59R, and I164L, as well as in the *Pfdhps* gene at codons S436A, A613S, A437G K540E, and A581G. The study also explored transporter genes, including mutations in the *Pfcrt* gene at codon K76T, and the *Pfmdr1* gene at codons N86Y, Y184F, S1034C,N1042D, and D1246Y.

A total of 200 DBS samples were collected, with the following distribution across the study sites: Apac (49 samples), Mbale (81 samples), Moroto (50 samples), and Soroti (20 samples). Genomic DNA was extracted from the DBS samples using Chelex method as previously described [19]. To detect genetic mutations associated with antimalarial resistance, Molecular Inversion Probes (MIPs) technology protocol was employed as described [14]. This method involves the hybridization of MIPs

with target DNA regions, followed by circularization and amplification of the probes [20]. Briefly, the amplified products were then sequenced using the Illumina MiSeq platform, which allows for high-throughput sequencing and accurate detection of genetic variants. Samples that did not have sufficient coverage after the initial sequencing were either re-pooled or recaptured to improve coverage. For MIP data, MIPTools software (version 0.19.12.13) was used to organize raw sequencing data and perform variant calling (https://github.com/bailey-lab/MIPTools). Individual genotypes were assigned to polymorphic sites covered by a minimum of 5 unique molecular identifiers (UMIs). Variants were required to have a genotype allele count of at least 3 UMI's for alternate alleles and at least 2 UMI's for reference alleles. Downstream analysis was conducted using R, samples that did not work were repeated twice to be declared negative. Negative samples were primarily due to low very parasite count.

Results

The study sequenced over 50% of the samples at each site. Apac had the highest success rate, with 87.7% (43/49) of samples sequenced, followed by Moroto with 68.0% (34/50), Soroti 65.0% (13/20), and Mbale 53.1% (43/81). The main reasons for some samples not yielding genotyping data were very low parasite densities.

Prevalence of molecular markers

Table 1 presents the results of the prevalence of molecular markers in all four study sites.

The *Pfk13* gene mutations revealed varied prevalence across the sites. The A675V mutants were absent in Apac, with 100% of samples showing the wild-type genotypes. In contrast, Mbale exhibited a minor presence of the mutation with 7.0% of samples showing the mutants. Moroto and Soroti had 2.9% and 23.1% of samples with A675V mutant genotypes respectively. Mutations R561H, C469F, and C580Y were not detected in any of the samples from the study sites. The *PfK13* C469Y mutation was observed in approximately 14.3% of samples from Apac, with 14.3% showing mixed genotypes. Mbale, Moroto, and Soroti had 7.0%, 14.7%, and 8.3% of samples with the *Pfk13* C469Y mutation, respectively, with varying proportions of mixed genotypes.

Mutations in the *Pfdhfr* gene demonstrated a significant presence in the samples from all districts. The N51I mutation reached a fixation rate of 100% in all districts. The C59R mutation was widespread, with Apac showing 97.6% of samples with the mutant genotypes and 2.4% mixed. Mbale had 93.0% mutants and 4.7% mixed, while Moroto and Soroti showed 100% and 92.3% mutants, respectively, with the remaining samples showing mixed genotypes. The I164L mutation was present in 11.6% of Apac samples, with the rest being wild-type or mixed. In Mbale, 7.0% of samples had this mutation, and Moroto and Soroti showed 2.9% and 7.7% mixed mutants, respectively. The S108N mutation was fixed in all samples from Apac, Mbale, Moroto, and Soroti.

For the *Pfdhps* gene, the A437G mutation was prevalent across all districts, with Apac showing 88.4% of samples with this mutation and 2.3% mixed. Mbale had 97.6% mutants and 2.4% mixed, Moroto had 97.1% mutants and 2.9% mixed, and Soroti showed 100% mutants. The A581G mutation was not present in Apac, Moroto, and Soroti, while Mbale showed 2.3% mixed genotypes with 97.7% mutants. The K540E mutation was prevalent in Apac with 88.1% of samples showing this mutation and 4.8% mixed. Mbale, Moroto, and Soroti also exhibited high prevalence rates with 97.6%, 97.1%, and 100% mutants, respectively, indicating widespread resistance.

The high prevalence quintuple mutations(*Pfdhfr*;N51I, C59R,S108Nand *Pfdhps:*A437G,G540E) mediating resistance to sulfadoxine-pyrimethamine is widespread in all four districts.

Mutations in the *Pfcrt* gene, specifically K76T, were rare. In Apac, all samples were wild-type with only 2.5% showing the mutant form, Mbale 97.5% were wild-type and 2.5% mutant, Moroto 97.1% wild type, 2.9% mutants, and Soroti all samples had wild-type. This low prevalence of K76T mutation indicates less resistance associated with chloroquine in these regions.

The analysis of the *Pfmdr1* gene mutations showed minimal variation across the study sites. The N1042D, and N86Y mutations was absent in all samples across the study sites. The D1246Y and mutation was present in Soroti with 8.3%, while Apac, Mbale, and Moroto all had wild genotypes. The Y184F mutation was also absent in all samples from the districts.

Figures 2, 3, 4, 5 show the prevalence of different genotypes on the *PfK13, Pfdhfr, Pfdhps, Pfcrt* and *Pfmdr1* genes across sample collection sites in Apac, Mbale, Moroto, and Soroti. The figure displays the frequency of various mutations among genes. The prevalence of mutations is represented as a percentage, with the Y-axis showing the proportion of mutant (red), mixed (yellow), and wild-type (green) genotypes.

Discussion

The study revealed that artemisinin resistance has rapidly emerged in new regions of Uganda. There is a characteristic trend of the spread (Figs. 2–5) that shows that where there is a mutant, the presence of mixed mutant and wild is indicative of the ongoing fitness cost of wild type and mutant genotype strains of *P. falciparum* in the population which gradually mutants genotype will be prominent

Table 1 Shows the prevalence of different mutations per study site

Mutation	Apac			Mbale			Moroto			Soroti		
	Wild	Mixed	Mutant	Wild	Mixed	Mutant	Wild	Mixed	Mutant	Wild	Mixed	Mutant
k13_A675V	43 (100.0%)	0 (0%)	0 (0%)	36 (83.7%)	4 (9.3%)	3 (7.0%)	31 (91.2%)	2 (5.9%)	1 (2.9%)	9 (69.2%)	1 (7.7%)	3 (23.1%)
k13_A561H	43 (100.0%)	0 (0%)	0 (0%)	42 (100.0%)	0 (0%)	0 (0%)	34 (100.0%)	0 (0%)	0 (0%)	12 (100.0%)	0 (0%)	0 (0%)
k13_C469F	36 (100.0%)	0 (0%)	0 (0%)	40 (100.0%)	0 (0%)	0 (0%)	29 (100.0%)	0 (0%)	0 (0%)	11 (100.0%)	0 (0%)	0 (0%)
k13_C469Y	30 (71.4%)	6 (14.3%)	6 (14.3%)	36 (83.7%)	4 (9.3%)	3 (7.0%)	27 (79.4%)	2 (5.9%)	5 (14.7%)	9 (75.0%)	2 (16.7%)	1 (8.3%)
k13_C580Y	43 (100.0%)	0 (0%)	0 (0%)	44 (100.0%)	0 (0%)	0 (0%)	35 (100.0%)	0 (0%)	0 (0%)	13 (100.0%)	0 (0%)	0 (0%)
k13_P441L	42 (100.0%)	0 (0%)	0 (0%)	43 (100.0%)	0 (0%)	0 (0%)	34 (100.0%)	0 (0%)	0 (0%)	12 (100.0%)	0 (0%)	0 (0%)
dhfr_N51I	0 (0.0%)	0 (0%)	42 (10.00%)	0 (0.0%)	0 (0%)	43 (100.0%)	0 (0.0%)	0 (0%)	34 (100.0%)	0 (0.0%)	0 (0%)	13 (100.0%)
dhfr_C59R	0 (0.0%)	1 (2.4%)	41 (97.6%)	1 (2.3%)	2 (4.7%)	40 (93.0%)	0 (0.0%)	0 (0.0%)	34 (100.0%)	1 (7.7%)	0 (0.0%)	12 (92.3%)
dhfr_I164L	33 (76.7%)	5 (11.6%)	5 (11.6%)	36 (83.7%)	4 (9.3%)	3 (7.0%)	32 (94.1%)	1 (2.9%)	1 (2.9%)	12 (92.3%)	1 (7.7%)	0 (0.0%)
dhfr_S108N	0 (0.0%)	0 (0%)	43 (100.0%)	0 (0.0%)	0 (0%)	43 (100.0%)	0 (0.0%)	0 (0%)	34 (100.0%)	0 (0.0%)	0 (0%)	13 (100.0%)
dhps_A437G	4 (9.3%)	1 (2.3%)	38 (88.4%)	0 (0.0%)	1 (2.4%)	41 (97.6%)	0 (0.0%)	1 (2.9%)	33 (97.1%)	0 (0.0%)	0 (0.0%)	13 (100.0%)
dhps_A581G	43 (100.0%)	0 (0.0%)	0 (0.0%)	42 (97.7%)	1 (2.3%)	0 (0.0%)	34 (100.0%)	0 (0%)	0 (0%)	11 (100.0%)	0 (0%)	0 (0%)
dhps_K540E	3 (7.1%)	2 (4.8%)	37 (88.1%)	0 (0.0%)	1 (2.4%)	40 (97.6%)	0 (0.0%)	1 (2.9%)	33 (97.1%)	0 (0.0%)	0 (0.0%)	13 (100.0%)
crt_K76T	43 (100.0%)	0 (0.0%)	0 (0.0%)	39 (97.5%	0 (0%)	1 (2.5%)	33 (97.1%)	0 (0.0)	1 (2.9%)	13 (100.0%)	0 (0%)	0 (0%)
mdr1_N1042D	43 (100.0%)	0 (0.0%)	0 (0.0%)	43 (100.0%)	0 (0.0%)	0 (0.0%)	35 (100.0%)	0 (0%)	0 (0%)	13 (100.0%)	0 (0%)	0 (0%)
mdr1_N86Y	42 (100.0%)	0 (0%)	0 (0%)	39 (97.5%	0 (0%)	0 (0%)	34 (100.0%)	0 (0%)	0 (0%)	12 (100.0%)	0 (0%)	0 (0%)
mdr1_D1246Y	42 (100.0%)	0 (0%)	0 (0%)	43 (100.0%)	0 (0.0%)	0 (0.0%)	35 (100.0%)	0 (0%)	0 (0%)	11 (91.7%)	1 (8.3%)	0 (0%)
mdr1_S1034C	43 (100.0%)	0 (0.0%)	0 (0.0%)	43 (100.0%)	0 (0.0%)	0 (0.0%)	35 (100.0%)	0 (0%)	0 (0%)	13 (100.0%)	0 (0%)	0 (0%)

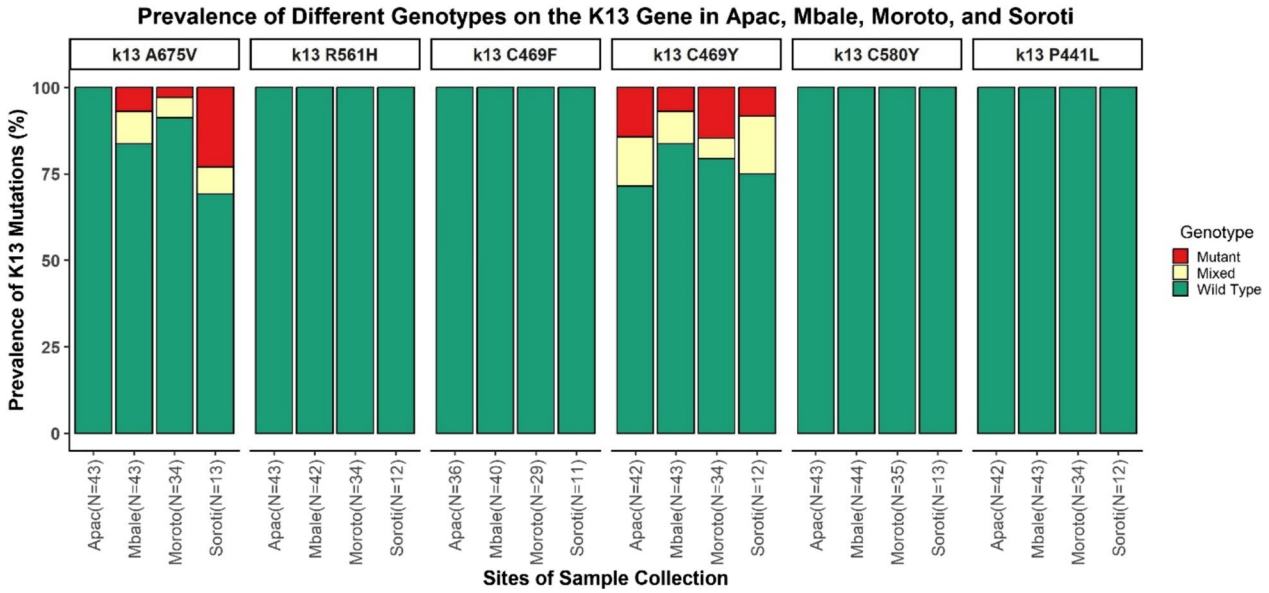

Fig. 2 Prevalence of *Pf*K13 gene mutations

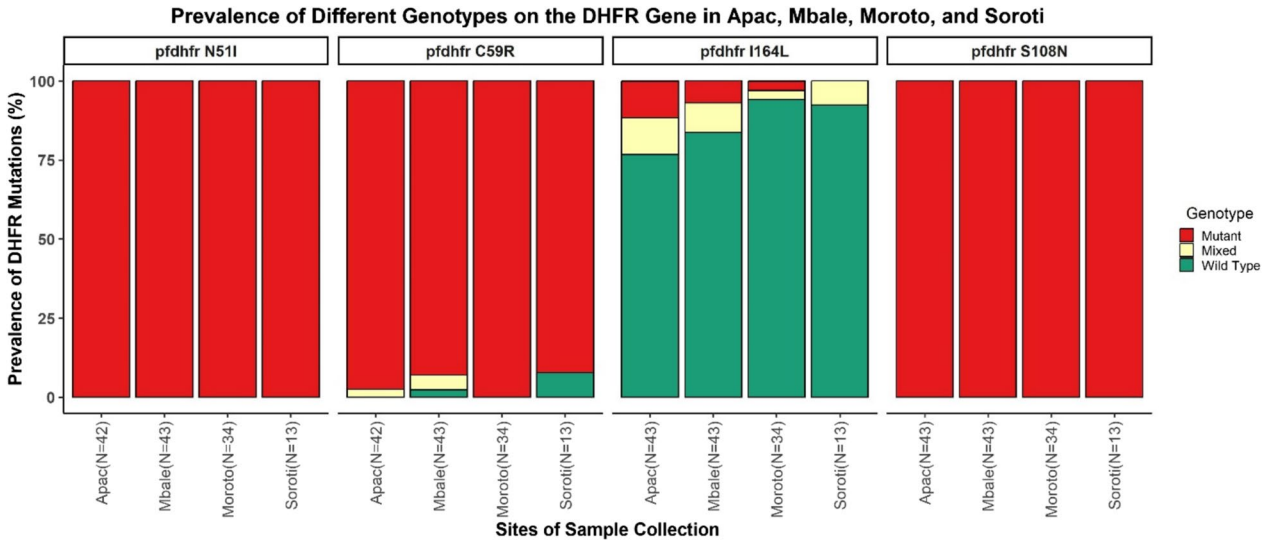

Fig. 3 Prevalence of antifolate mutations in the *Pf*dhfr gene

with increased use of artemisinin. The detection of mutations in the *Pfk13* gene, particularly the A675V and C469Y single nucleotide polymorphisms, suggests the presence of emerging artemisinin resistance in these regions similar to previous reports [5] [6]. To monitor this process requires point in time national surveillance and geospatial mapping to inform targeted interventions [21, 22].

The high prevalence of fixed mutations in the *Pfdhfr* (N51I, S108N, C59R) and *pfdhps* (A437G, K540E) genes across the study sites indicates widespread resistance to antifolate drugs, which are components of the combination therapy sulfadoxine-pyrimethamine (SP). The near-fixation of the C59R and A437G mutations and the emerging I164L mutation highlight the continued selection pressure exerted by SP, despite its limited use as a first-line treatment though used in pregnant women [23], and patients with sickle cell disease [24–26], as prophylaxis or presumptive treatment. The finding of a mixed genotype at the A581G locus in Mbale suggests ongoing selection and potential for further spread of resistance. The results of mutations in antifolate genes mediating

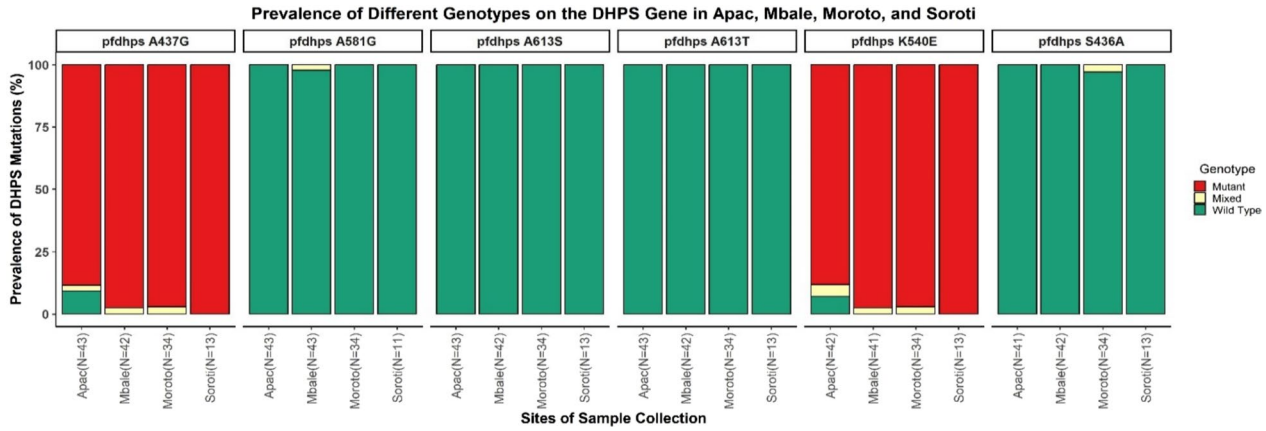

Fig. 4 Prevalence of antifolate mutations in the *Pf*dhps gene

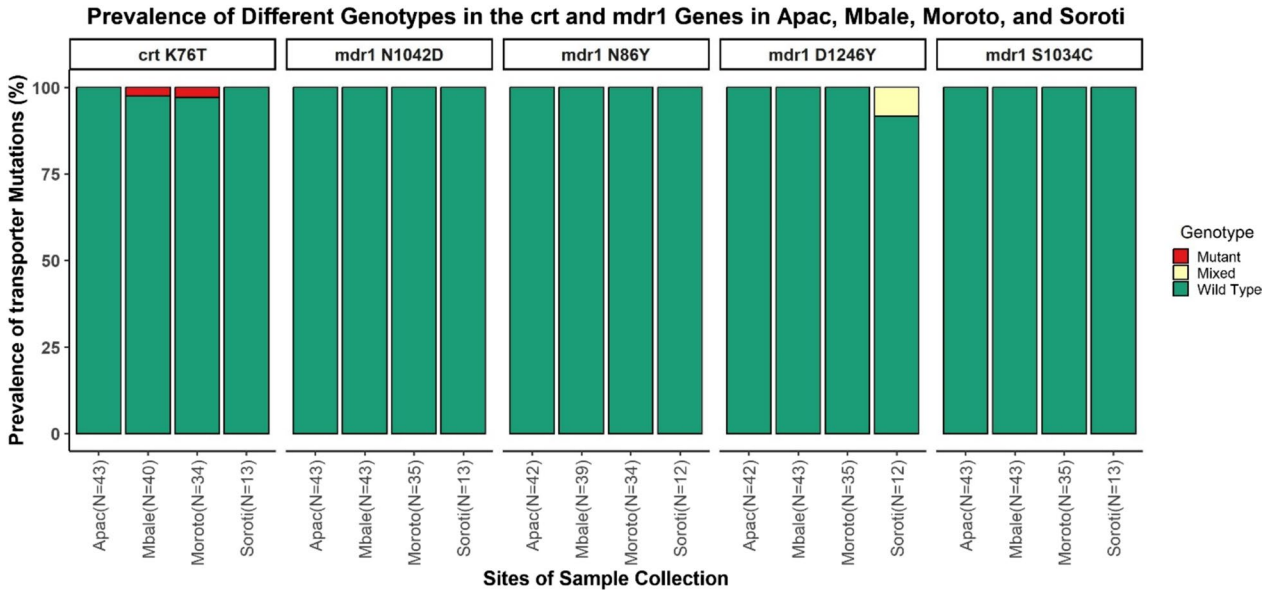

Fig. 5 Prevalence of mutations in the transporter genes

resistance are consistent with reported data from different regions in Uganda [6, 12, 14].

The *Pfcrt* K76T mutation, associated with chloroquine resistance, was predominantly wild type in the four districts, with only a small percentage of mutant alleles detected in Mbale and Moroto. This reflects the successful withdrawal of chloroquine from malaria treatment protocols, leading to a reversion to wild-type alleles and results are consistent with what was reported in other regions of Uganda [6, 12] and also previously reported reversal sensitivity from Malawi [27] and other southern africa countries [28]. However, the persistence of some mutant alleles suggests that chloroquine resistance could re-emerge if the drug were reintroduced without adequate monitoring [29]. This may be one of the major lessons on use of antimalarials. Successful withdrawal of antimalarials potentially paves way for their re-use for therapy in the future. Since this has happened to chloroquine, it is likely to happen to ACT and therefore, artemisinin-based combinations should not be thrown away if the stage of withdrawal reaches.

Finally, the wild-type status of the *Pfmdr1* N86Y allele across most districts, with some mixed genotypes in Soroti, suggests limited selection pressure from drugs targeting this gene, such as mefloquine. Interestingly, the high prevalence of the wild-type N86 and Y184F identified in the four regions are associated with decreased sensitivity to lumefantrine, which is selected by the

current first-line artemisinin combination therapy (AL) in Uganda. This suggests that the same polymorphisms have opposite effects on sensitivity to different drugs [6, 10, 11, 30].

Limitations

A key limitation of this study is the relatively small number of successfully genotyped specimens across all four study areas, with fewer than 50 specimens in total. This limitation was particularly pronounced in Soroti, where only 11 to 13 specimens were successfully analysed. As described in the methodology, low parasitaemia significantly impacted sequencing success. To address this challenge, the study sequenced all collected samples and reattempted sequencing for those with poor DNA yield. In addition, the specimens analysed were not fully representative of the broader parasite population in the study areas, particularly given the low success rate in Soroti. This affects the generalizability of the findings. Despite these efforts, the sample size remains a constraint, and future studies with larger cohorts are planned to strengthen the findings.

Conclusion

The study highlights the complex and evolving landscape of antimalarial resistance in Uganda, with significant implications for malaria control strategies. The findings underscore the need for enhanced surveillance of molecular markers, public awareness campaigns to promote the rational use of antimalarials, and consideration of alternative treatment regimens where resistance is prevalent. Continued monitoring and timely updates of treatment guidelines will be crucial to maintaining the effectiveness of malaria control programmes in Uganda.

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Author contributions

P.OO. conceptualized the study, led the research team, and oversaw the design and execution of the project. G.P. and J.P.A. contributed to the study design, coordinated sample collection, and participated in data analysis and manuscript writing. T.K. provided expertise in molecular analysis, including the use of Molecular Inversion Probes (MIPs), and contributed to data interpretation and manuscript review. A.N. E.O. A.O. G.G.O. S.P. and F.A. contributed to the study overall administration, design, and implementations at the respective study sites. W.O. E.O. A.O. C.B.O. O.P. and D.A. assisted with data collection, fieldwork coordination, and data analysis. S.T. and I.S. provided technical expertise in the use of MIPTools software for data processing and variant calling and contributed to manuscript preparation. S.N.N. and S.L.N. provided overall supervision of the laboratory work, including sequencing and data analysis, and played a critical role in the manuscript review and finalization. All authors reviewed and approved the final manuscript.

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Availability of data and materials

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

A detailed protocol was developed beforehand and approved by the Mbale Regional Referral Hospital Research Ethics Committee [(MRRH-REC), MRRHREC-OUT-011/2020] and Uganda National Council for Science and Technology [(UNCST), HS3725ES]. Ethical considerations were adhered to during the study process. The study conformed to the provisions of ethical standards in Uganda.

Consent for publication

The Mbale Clinical Research Institute (MCRI, www.mcri.ac.ug), a research entity affiliated with the Uganda National Health Research Organization, approved the publication of this manuscript.

Competing interests

The authors declare no competing interests.

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