fortune Journals

Research Article

FORTUNE JOURNAL OF HEALTH SCIENCES

ISSN: 2644-2906

High Prevalence of *Pfdhps* and *Pfdhfr* Resistance Markers to Sulfadoxine-pyrimethamine During Subpatent Parasitemia in Doneguebougou, Mali

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Abstract

Blood smear-positive asymptomatic infections are an important malaria parasite reservoir because they harbor gametocytes. However, asymptomatic infections are often submicroscopic, can infect mosquitoes and can only be detected by molecular methods. Malaria prevention programs pursue mass treatment of asymptomatic individuals, which may contribute to development and spread of drug resistance. Here, we investigated the prevalence of molecular markers of drug resistance of *Plasmodium falciparum* dihydrofolate reductase (*Pfdhfr*) and *P. falciparum* dihydropteroate synthase (*Pfdhps*) during subpatent parasitemia compared to patent parasitemia episodes in Mali. From July to December 2019, blood smears and filter paper blood spots were collected from children with subpatent parasitemia (BS-) and patent parasitemia (BS+) in Doneguebougou. Point mutations at codons (50, 51, 59, 108, 140 and 164) of the *Pfdhfr* gene and codons (431, 436, 437, 540, 581 and 613) of the *Pfdhps* gene were evaluated by nested PCR amplification followed by direct sequencing. A total of 84 children under five years of age were evaluated (27 BS- and 57 BS+). When assessed as a binary endpoint (any *versus* no detection of resistance allele), we found a significance higher prevalence of *Pfdhps* 581**G** (51.9% vs. 12.3%, p=0.001) and *Pfdhfr* at codons 50**R** (75.0% vs. 22.2%, p=0.001), 140**L** (66.7% vs. 25.0%, p=0.014) and 164**L** (88.9% vs. 21.2%, p=0.0004) in BS- vs. BS+, respectively. In BS- the prevalence of *Pfdhps* at codons (431**V**, 437**G** and 540**E**) and *Pfdhfr* at codon (51**I**) were higher but not significant in BS- vs. BS+. No *Pfdhps* 540**E** was detected in BS+. For all markers except one, mutations were more prevalent in BS- samples in Mali. Asymptomatic subpatent parasitemia can be a reservoir of drug-resistant parasites and should be regularly surveyed (in addition to BS+ episodes) to accurately monitor the spread of molecular markers of resistance.

Keywords: Malaria elimination, *pfdhps*, *pfdhfr*, subpatent parasitemia, Mali

Introduction

Asymptomatic malaria is not often defined rigorously, but generally refers to an individual harboring blood-stage malaria parasites without experiencing fever or other symptoms that would lead the individual to seek treatment [1]. In most malaria-endemic settings, asymptomatic infections outnumber symptomatic infections [2]. The lack of rigorous definitions for asymptomatic malaria has clouded research to support elimination strategies, which rely on the ability to find and treat the asymptomatic reservoir [3]. As resources

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Citation: Hamma Maiga, Robert D. Morrison, Issaka Sagara, Sara Healy, Abdoulaye Katile, Amatigue Zeguime, and Patrick E. Duffy. High Prevalence of *Pfdhps* and *Pfdhfr* Molecular Markers of Sulfadoxine-Pyrimethamine Resistance During Subpatent Parasitemia in Doneguebougou, Mali. Fortune Journal of Health Sciences, 8 (2025): 470-478.

Received: April 15, 2025
Accepted: April 22, 2025
Published: June 02, 2025

Maiga H, et al., Fortune J Health Sci 2025
DOI:10.26502/fjhs.297

are allotted to eradication efforts, further research is critical to better characterize the asymptomatic reservoir. In general, parasitemia in asymptomatic and symptomatic malaria infection can be detected by blood smear as the gold standard. However, asymptomatic infections are often submicroscopic [1, 4] especially in low transmission settings [5-7], and can only be detected by molecular methods such as PCR and loop-mediated isothermal amplification (LAMP) method [8]. Most studies investigating the reservoir potential of asymptomatic infection have assessed microscopically patent parasitemia, but the role of submicroscopic parasitemia in transmission is unclear. There is some evidence that individuals with submicroscopic malaria can infect mosquitoes. The relative contribution to malaria transmission has been shown to be similar for carriers with submicroscopic and microscopic gametocytemia; transmission occurs efficiently at submicroscopic gametocyte densities and those carriers harboring submicroscopic gametocytemia constitute a considerable proportion of the human infectious reservoir [9].

In Tanzania, submicroscopic gametocytemia is likely to be responsible for maintaining malarial transmission, and molecular detection techniques revealed that carriage of submicroscopic asexual parasites and gametocytes is relatively common in this low transmission area [10]. In Western Kenya, a large proportion of submicroscopic parasites and gametocytes may contribute to stagnation in reducing malaria prevalence, suggesting additional interventions targeting the infectious reservoir are needed [11]. In Ghana, children and pregnant women are particularly important as submicroscopic gametocyte reservoirs and represent important focus groups for control interventions [12]. Seasonal malaria chemoprevention (SMC) programs pursue the mass treatment of asymptomatic individuals with antimalarials during the malaria transmission season. Since 2012, SMC has been recommended by the World Health Organization (WHO) for children aged 3–59 months living in areas of highly seasonal malaria transmission in the Sahel sub-region of Africa [13]. SMC consists of full treatment courses of sulfadoxine–pyrimethamine plus amodiaquine (SP + AQ) given to children 3–59 months of age, at monthly intervals during the malaria transmission season to maintain therapeutic anti-malarial drug concentrations in the blood throughout the period of greatest malaria risk [13].

A major concern associated with SMC is that mass administration of SP may contribute to development of parasite drug resistance [14-17]. Resistance to pyrimethamine and sulfadoxine increases as mutations accumulate in the parasite genes encoding dihydrofolate reductase (dhfr) and dihydropteroate synthase (dhps), respectively. Monitoring the prevalence of these molecular markers of parasite drug resistance is essential for effective malaria control, however, no study has characterized the presence of these markers within submicroscopic parasitemia detected in the context of SMC. Here, we investigate levels of molecular markers of drug resistance in submicroscopic malaria in Doneguebougou, Mali, an area where SMC was implemented four years ago. Outcomes from this study could be useful in understanding and improving interventions against parasite drug resistance to sulfadoxine-pyrimethamine and will provide information to develop new strategies for malaria elimination.

Materials and Methods

Study area

This study was performed in Doneguebougou (latitude 12.6 and longitude -7.9), a village of approximately 2500 inhabitants located 30 km northwest of Bamako (capital of Mali). Malaria transmission is highly seasonal and intense during the rainy season from July to December. The prevalence of *P. falciparum* infection in children under five years of age varies from approximately 30–50% during the dry season to 75% during the rainy season. The Annual Entomologic Inoculation Rate is >100 infective bites per person as the nearby Koba River contributes heavily to persistent *Anopheles* breeding. The climate is hot with daily temperatures ranging from 19 to 40°C, and the rainy season (a period of intense malaria transmission) occurs from June to October/November.

Sample collection

From July to December 2019, samples for molecular analysis were collected from children under 5 years of age who received one cure of artemether-lumefantrine in July. The study clinic was staffed 24 hours a day, 7 days a week during the study follow-up period, serving as the only medical facility in the village. Children presenting with fever (axillary temperature ≥ 37.5 °C) or history of fever during the previous 24 hours were tested for malaria by a rapid diagnostic test (HRP2 SD Bioline®) and blood smears when they presented at clinical hospital. Blood smears were read by two independent certified readers. Conflicting results entered arbitration and were read by a third certified reader. This was considered the final read. The presence of *P. falciparum* was initially confirmed and quantified by microscopy on Giemsa-stained blood smears. Samples were classified into two categories: subpatent parasitemia (BS-) when blood smear is negative who not having any parasite per 100 fields on microscopy and patent parasitemia (BS+) when blood smear is positive. Finger prick blood from each participant was applied to filter paper (Whatmann 3MM) for molecular biology study. Each filter paper was dried and stored in a plastic bag containing silica gel. All dried blood spots (DBS) were subsequently transported to LMIV/NIAID/NIH for molecular analysis.

DNA extraction

A QIAamp DNA blood kit was used for genomic DNA

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extraction, and the concentration and purity of DNA were measured using a NanoDrop2000 UV. The final elute was 50 µl and the extracted DNA was stored at -20°C until use. 3D7, Dd2, and VI/S DNA obtained from the Malaria Research Reagent Resource (MR4) were used as sequence-specific positive controls.

PCR and sequencing of *Pfdhfr* and *Pfdhps*

All parasite-positive and parasite-negative samples by PCR were available for genotyping of *P. falciparum dhfr* and *Pfdhps*. *Pfdhfr* sequences were amplified by PCR at one time [18]. For *Pfdhfr* sequences, Dhfr_Fwd (5TTTATATTTTCTCCTTTTTA 3) and Dhfr_Rev for (5'CATTTTATTATTCGTT-TTCT3') oligonucleotides were used for single amplification producing a 681 bp fragment containing key mutations C50R, N51I, C59R, S108N/T, V140L and I164L. Detailed PCR conditions and volume for amplification of *Pfdhfr* were: water 22 µl, Master mix hot-Taq 25 µl, dhfr Fwd 0.5 µl, dhfr Rev 0.5 µl and 2 µl of DNA in a total volume of 50 µl was run under the following cycling conditions: 95 °C for 5 min then 30 cycles at 92 °C for 30 s, 45 °C for 30 s and extension at 65 °C for 45 s and final extension at 72 °C for 15 min. For *Pfdhps* sequences, genes containing mutations I431V, S436A/F/H, A437G, K540E, A581G, and A613S/T (previously associated with parasite responses to sulfonamide) were amplified by nested PCR using previously published primers [17]. For the first round of PCR, 0.5 for each primer, Master mix hot-Taq 12.5 µl and 5 µl of DNA in a total volume of 25 µl completed by nuclease-free water was run under the following cycling conditions: 94 °C for 3 min then 30 cycles at 94 °C for 30 s, 55 °C for 30 s and extension at 65 °C for 1 min and final extension at 65 °C for 5 min. For the second round of PCR, a total volume of 50 µl made of 19 µl nuclease-free water, 0.5 for each primer, Master mix hot-Taq 25 µl and 5 µl of DNA was run under the following cycling conditions: 94 °C for 5 min then 30 cycles at 94 °C for 30 s, 60 °C for 30 s and extension at 65 °C for 1 min and final extension at 65 °C for 5 min. PCR products for both genes were purified by Qiagen kit and concentration was measured using a NanoDrop2000 UV. The same primers were sent for cycle-sequencing. Cycle sequencing followed the standard BigDye3.1 dye terminator protocol (AppliedBiosystems) on an MJ-Thermocyler. Sequencing reactions were cleaned on Sephadex G10 columns and analysed on an ABI3130xl Genetic Analyser. Sanger sequencing of resistance gene fragments and deconvolution of chromatograms to quantify molecular marker variants were used to genotype *Pfdhfr* and *Pfdhps* [19].

Statistical analysis

This was an exhaustive study of all confirmed and unconfirmed malaria cases detected during the study period. Prevalence and mean fraction of *Pfdhfr* mutations (codons 50R, 51I, 59R, 108N/T, 140L and 164L) and *Pfdhps* mutations (codons 431V, 436F/A/Y, 437G, 540E, 581G and 613S/T) were estimated for BS- versus BS+ samples. Samples containing both mutant and wild type were added to mutant for prevalence estimation by binary call. A Chi-square test was used to compare the prevalence of molecular markers in BS- versus BS+. A Wilcoxon Rank Sum test with a continuity correction was used to compare the mean fraction of *Pfdhfr* and *Pfdhps* mutations between BS- vs. BS+ samples. Statistical significance of differences between the BS- vs. BS+ was assessed using all tests and a p value <0.05 was considered significant.

Ethics

Trial protocol (19-I-N086/N°2019/10/CE/FMPOS; 21 March 2019) was approved by ethics committees of the US National Institutes of Health (Bethesda, MD, USA), Faculte de Medecine et d'Odonto-stomatologie, and Mali Ministry of Health (Bamako, Mali). Studies were conducted with the US Food and Drug Administration (FDA) Investigational New Drug Application. This study was registered with ClinicalTrials.gov (NCT03917654). Overall community and local authorities' permission were also obtained in addition to parent or guardian informed consent.

Results

Parasite prevalence estimation using PCR:

DNA was extracted from 93 samples collected of children under five years of age. A total of 59 was BS+ and 34 of BS- (Figure 1). Of these samples, 57/59 (96.6%) BS+ and 27/34 (79.4%) BS- were detected as positive during sequencing.

Prevalence of *Plasmodium falciparum dhfr* and *Pfdhps* variants by binary call

Mutations in the *Pfdhfr* gene at codons 51 (N/I), 59 (C/R) and 108 (S/N), associated with pyrimethamine resistance, were saturated in both subpatent parasitemia (BS-) and patent parasitemia (BS+) Table 1. Overall frequencies of (97.2% vs. 100%), (100% vs. 100%) and (100% vs. 100%) in subpatent parasitemia (BS-) versus patent parasitemia (BS+) (p>0,05), respectively. The other mutant-types at *Pfdhfr* at codons 50R, 140L and 164L were ranging in high frequency from BS- (75.0%, 66.4% and 88.9%) against BS+ (22.2%, 25.0% and 21.2%), p=0.001, p=0.015 and p=0.0004, respectively in Table 1. Ten amino acid variants were commonly found encoded in the *Pfdhps* gene at the six codons of interest (AI431V, S436A/F/C, A437G, K540E, A581G, A613T/S). High frequency level mutants were present in BS- than the BS+ for 431V (22.73% vs 14%, p=0.49 CI0.13-7.55), 436A/F/C (61.54% vs. 55.77%, p=0.81 CI0.37-2.27), 437G (92.69% vs. 84.21, p=0.04-2.31), 540E (3.70% vs. 0.0% p=0.32 CI0.04-18.47), A581G (51.85% vs. 12.28%, p=0.0002 CI0.04-0.44).

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Pfdhps 613S/T prevalence was lower in BS- vs. BS+ (22.22 % vs. 24.56%, p=1, CI0.35-4.15). The *Pfdhps* 540E mutation was not observed in BS+ in this study (Table 1).

Results were generated using Sanger sequencing of *Pfdhfr* and *Pfdhps* loci of parasites in negative vs. positive parasitemia. Prevalence of the mutants include mutant clones in mixed infections was calculated by chi2 with p-value as 0.05 significant at 95% confidence interval (CI). n is number of mutant and N the total sample.

Mean fraction of *Plasmodium falciparum* dihydrofolate reductase (*Pfdhfr*)

Based in the deconvolution method, the mean fraction is higher in BS- versus BS+ in dhfr codon 50R (30% vs. 5%, p=0.0001), 140L (23% vs. 7%, p=0.004) and 164L (50% vs. 9%, p=0.0001), respectively (Figure 2). In contrast the mean fraction is higher but not significative in BS+ vs BS- in dhfr at codons 16V (2% vs 4%, p=0.77), 51I (87% vs. 89%; p=0.24), 59R (92% vs. 96%; p=0.42), and significative in 108T/N (75% vs. 90%; p=0.03), respectively.

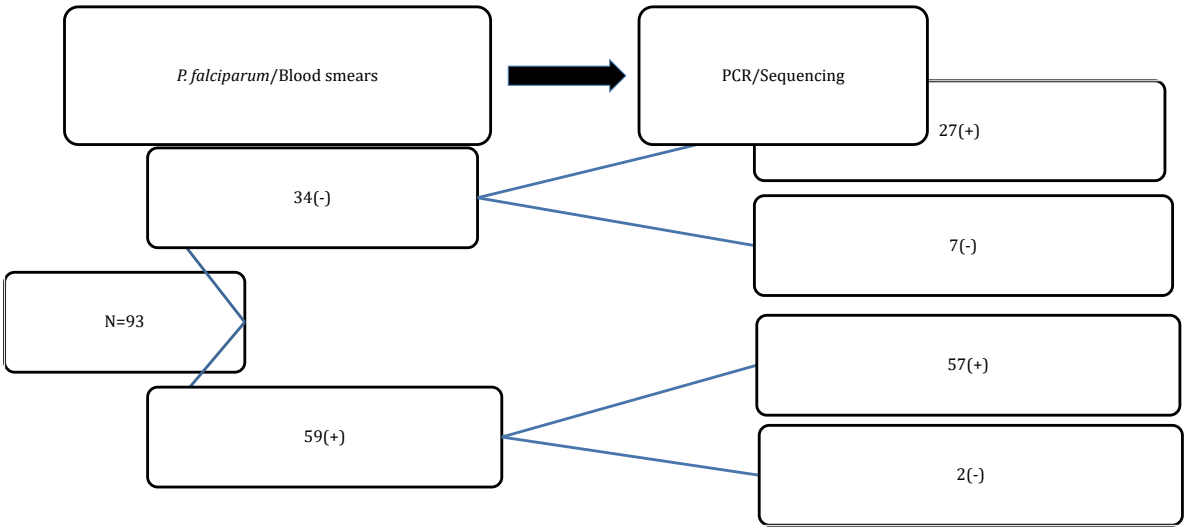

Figure 1: Study profile

Table 1: Prevalence of *P. falciparum dhfr* and *dhps* variants that are associated with pyrimethamine and sulfadoxine resistance in subpatent parasitemia (BS-) versus patent parasitemia (BS+)

No. of samples sequenced	Subpatent parasitemia (BS-)	Patent parasitemia (BS+)	p-value (95% CI)
% of *pfdhfr* mutants including mix (n/N)			
50R	75.0 (9/12)	22.2 (8/36)	0.001 (0.01-0.53)
51I	100 (13/13)	97.2 (35/36)	1 (0.00-107)
59R	100 (13/13)	100 (36/36)	-
108N/T	100 (10/10)	100 (33/33)	-
140L	66.7 (8/12)	25.0 (9/36)	0.014 (0.03-0.83)
164L	88.9 (8/9)	21.2 (7/33)	0.0004 (0.00-0.34)
% of *pfdhps* mutants including mix (n/N)			
431V	22.73 (5/22)	14.00 (7/50)	0.49 (0.13-2.55)
436A/F/C	61.54 (16/26)	55.77 (29/52)	0.81 (0.27-2.27)
437G	92.59 (25/27)	84.2 (48/57)	0.49 (0.04-2.31)
540E	3.70 (1/27)	0.00 (0/57)	0.32 (0.00-18.47)
581G	51.85 (14/27)	12.28 (7/57)	0.0002 (0.04-0.44)
613S/T	22.22 (6/27)	24.56 (14/57)	1 (0.35-4.15)

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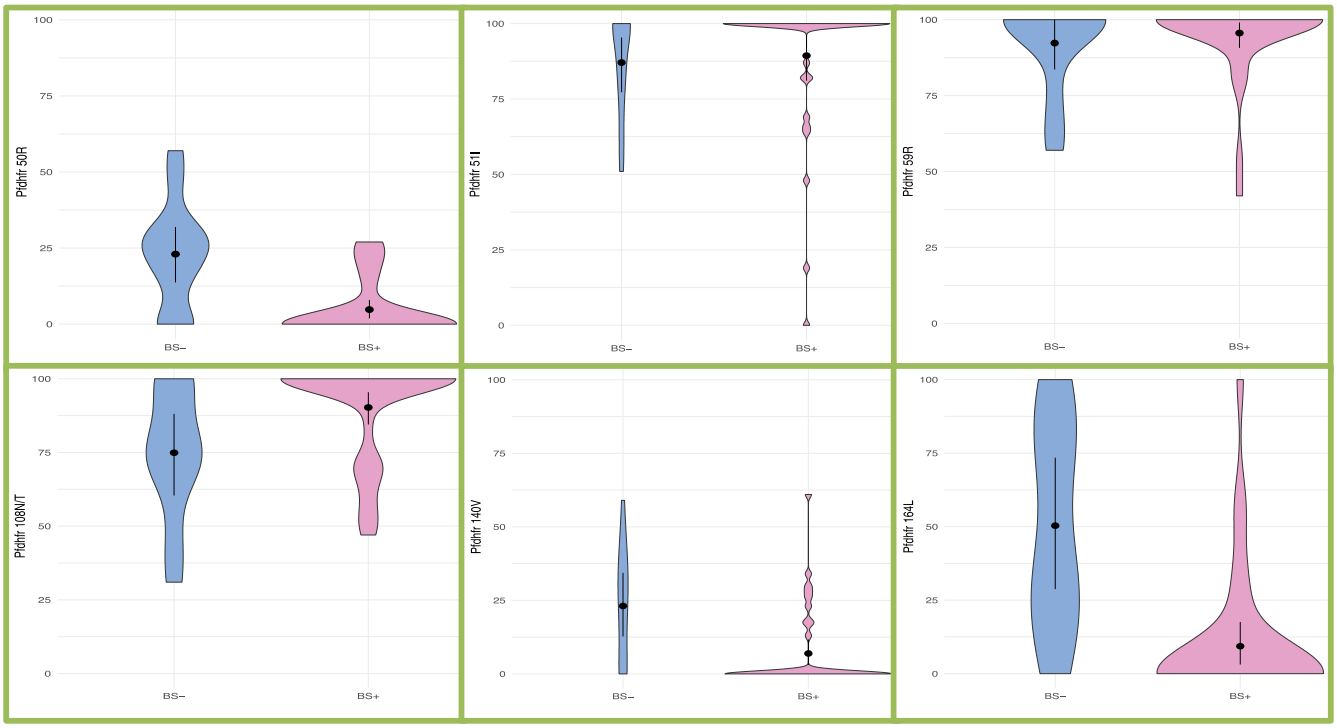

Figure 2: Mean fraction of *Plasmodium falciparum* dihydrofolate reductase (*Pfdhfr)* in subpatent parasitemia (BS-) versus patent parasitemia (BS+)

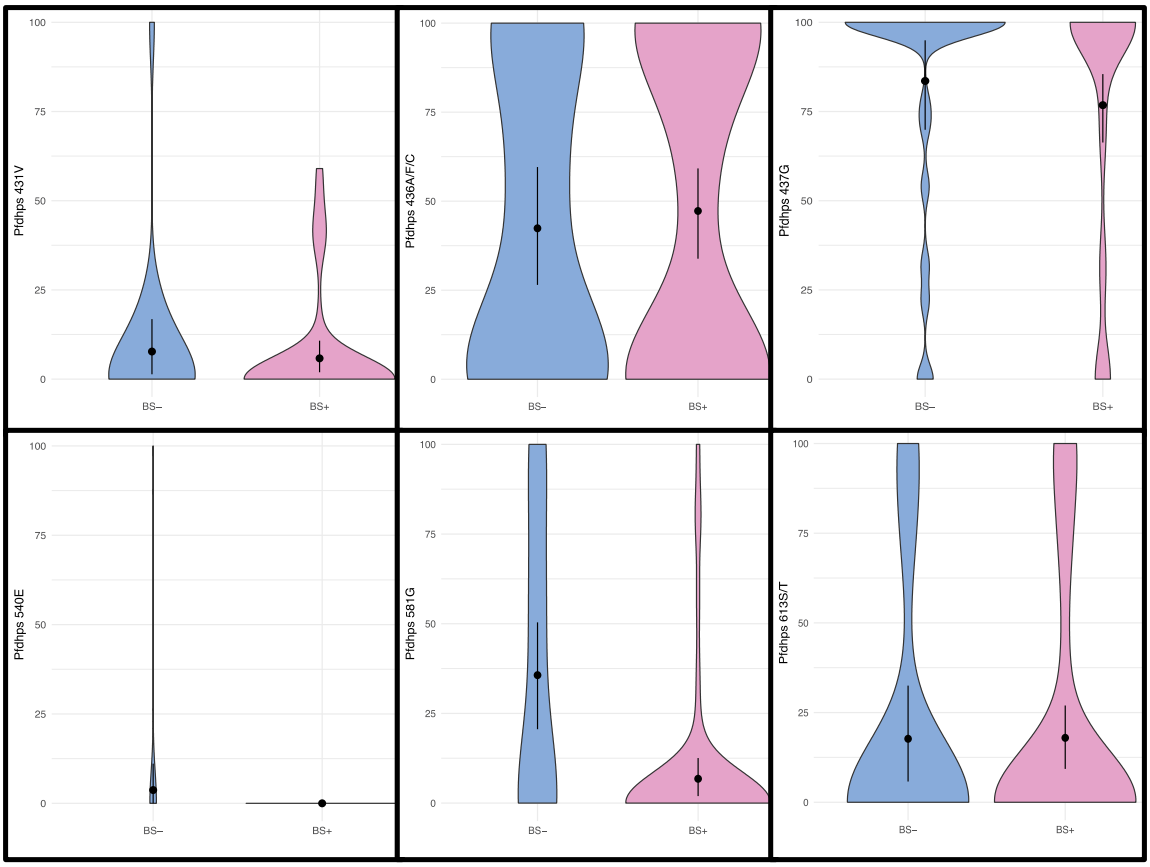

Figure 3: Mean fraction of *Plasmodium falciparum* dihydropteroate synthase (*Pfdhps*) in subpatent parasitemia (BS-) versus patent parasitemia (BS+)

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Mean fraction of *Plasmodium falciparum* dihydropteroate synthase (*Pfdhps*)

In Figure 3, the mean fraction mutation was higher but similar in BS- and BS+ in *Pfdhps* at codons 431V (8% vs. 6%, p=0.45); 437 (84% vs. 77%, p=0.45), 540E (4% vs. 0%, p=0.15), and significant in 581G (36% vs. 7%, p=0.0001), but lower in 436F/A/Y (42% vs. 47%, p=0.86) and same in 613T/S (18% vs. 18%, p=0.87), respectively.

Discussion

Achieving malaria elimination requires targeting the human reservoir of infection, including those with asymptomatic infection. Smear-positive asymptomatic infections detectable by microscopy are an important reservoir because they often persist for months and harbor gametocytes, the parasite stage infectious to mosquitoes. The submicroscopic malaria parasitemia is common in both high- and low-transmission settings, but the drug resistance implications of low-level parasitemia remain unknow. We investigated mutations on *Pfdhfr* and *Pfdhps* genes considered to be confirmed markers of resistance to sulfadoxine-pyrimethamine (SP) and appear associated with treatment failure in Africa [20]. The number of mutations at the *Pfdhfr* and *Pfdhps* loci have been strongly associated with pyrimethamine and sulfadoxine treatment responses, respectively. This study is the first to compare prevalence of these molecular markers of drug resistance to SP in subpatent parasitemia (BS-) and patent parasitemia (BS+) in the seasonal malaria chemoprevention (SMC) context. It is also the first study to report the presence of the *Pfdhfr* 140L and 164L mutant genotype relatively uncommon in West Africa, after five years of SMC implementation in Mali and both mutations were higher in BS- than BS+. These results suggested that current estimates of drug-resistance markers assessed only from BS+ will need correction. Recently, a study in Gabon showed similar results that while *Pfmdr*1 N86 was significantly higher in symptomatic malaria than asymptomatic [21], the mutant 86Y and mixed 86N/Y genotypes were observed only in asymptomatic infections. The *Pfdhfr* triple mutant (51I, 59R and 108N) seemed saturate by binary call but not by deconvolution method which showed the importance of this method. Until here the *Pfdhfr* at codons 140L and 164L mutations were not observed and will compromise the efficacy of sulfadoxine-pyrimethamine which implemented in Intermittent Preventive Treatment in pregnancy (IPTg) and SMC in Mali.

Previous studies have suggested that a high prevalence of submicroscopic gametocytemia may contribute to malaria transmission [22, 23], although others argue that transmission was much less likely to occur at submicroscopic gametocyte levels [1]. Indirectly, if the parasites were more resistant in submicroscopic infections, they maintain and promote, contributing to malaria transmission. Submicroscopic parasite densities were found to be common in adults in settings of low endemicity and chronic infections [5], and low-transmission settings had proportionately greater submicroscopic carriage rates. Submicroscopic carriage rates also suggested that, as malaria prevalence decreases, submicroscopic carriers were responsible for a higher proportion of transmission, and submicroscopic infections may be responsible for 20% to 50% of transmission in pre-elimination and elimination settings [5]. Synchronized sequestration also contributed to submicroscopic carriage (in *P falciparum* only) [23]; large parasite-density fluctuations attributed to synchronized sequestration of infected erythrocytes out of peripheral circulation and into the microvasculature of various organs have been documented for subclinical children and adults [24]. Also, submicroscopic carriage represented an important hidden reservoir of infection [10, 25]. Resistance to anti-malarial drugs has often threatened malaria elimination efforts and historically has led to the resurgence of malaria incidence and death particularly in children under 5 years of age in sub-Saharan Africa [26, 27]. Many studies from Africa have reported substantial proportion of individual treated with artemisinin-based combination, with residual submicroscopic parasitemia between days 3 and 7 [4, 28-30].

Several submicroscopic parasite subpopulations have also been observed in Tanzania with no known *Pfk*-13 resistance-associated mutations, however, they cleared as slowly as parasites in Cambodia, which were labeled drug resistant and do harbor the mutations [31]. Molecular markers of drug resistance were among the tools widely used for surveillance of drug sensitivity [32]. Both *Pfmdr*1 and *Pfcr*t resistant haplotypes were saturated in the parasite population following years of wide-scale use of artemisinin-based combination therapy (ACT) and were being detected both at baseline and in residual submicroscopic parasites detected on day 3 following ACT [33]. These tolerant or resistant asexual parasites that survived the anti-malarial drug pressure may also differentiate into gametocytes and be transmitted to the mosquito vector, where they replicated and expanded the tolerant/resistant parasite population. However, the recent observation of residual submicroscopic parasitemia 3 days after initiation of a full course treatment with an artemisinin-based drug combination leaded to question whether these residual parasites represented viable resistant asexual parasites, which could contributed to the spread of anti-malarial drug resistance [29, 34]. It is worth noting that the markers of drug resistance were established when drug resistance has already developed [35]. Therefore, while molecular markers of drug resistance were very important for the surveillance of anti-malarial resistance, it should be understood that they were not often predictive of clinical resistance [36]. he potential to detect emerging trends of drug resistance before outright clinical failure made molecular

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markers useful tools. PCR-based detection techniques were more sensitive than microscopy [37], being able to detect as little as two parasites per microliter of blood [38].

Malaria parasite nuclear materials were removed from the circulation by the circulating and reticuloendothelial phagocytes and DNA derived from dead parasites circulates for less than 48 h [34, 39, 40]; and it was thought that non-viable cells were unlikely to give PCR positive signals. Therefore, PCR analysis of malaria-infected blood accurately reflected the presence of live parasites [41]. Presence of more than molecular marker drug resistance of SP in submicroscopic parasitemia than microscopic parasitemia after one year SMC treatment initiation leaved question to redefine the politic of resistance of antimalarial. There was a need to determine the potential public health implication of the PCR-determined submicroscopic parasitemia observed on this study. Robust techniques, such as *in vitro* cultivation, should be used to evaluate if the submicroscopic parasites, were viable asexual parasites, or gametocytes, or the DNA of the dead parasites waiting to be cleared from the circulation. A virulence related to the genotype of the parasite, previous antimalarial therapy, or host immune status were factors that may explain these differences. We underlined the study participants have received four years SMC treatment to prevent malaria from 2015 to 2018. Our results suggested that the fitness benefits of parasites harboring more drug-resistance genotypes may promote progression to the asymptomatic and symptomatic stage. In addition, the high presence of parasites with these genotypes resistant in subpatent among asymptomatic infections, which were left untreated, favors their survival and transmission within the community. This showed the importance to include the subpatent parasitemia molecular evaluation in the continued monitoring and characterization of molecular markers of resistance in parasites circulating in symptomatic and asymptomatic infections malaria. These data would provide an overview of the spread of these genetic markers in infected populations to adjust intervention strategies to better fight against drugs resistance in Africa.

Conclusions

Asymptomatic subpatent parasitemia can be a reservoir of drug-resistant parasites and should be regularly surveyed (in addition to BS+ episodes) to accurately monitor the spread of molecular markers of resistance. However, these results should be confirmed and further investigated to understand the mechanism in larger sampling size, including other epidemiological settings and resistance-mediated genes of submicroscopic parasitemia after antimalarial used/ implemented.

Author Contributions

H.M conducted the laboratory study, performed statistical data analysis, and wrote the first draft of manuscript, which was revised by P.E.D for initial submission. R.D.M. designed and implemented the chromatogram deconvolution algorithms and edited the manuscript. A.K and A.Z performed the data collection on the field. I.S and S.H supervised the study and edited the manuscript. All authors accessed and verified the underlying data. All authors reviewed, edited, and approved the final manuscript version after review, and agreed to the published version of the manuscript.

Funding

This work was supported by the Division of Intramural Research of the National Institute of Allergy and Infectious Diseases, National Institutes of Health (NIH). HM was supported by the African Postdoctoral Training Initiative (APTI) Fellowship program jointly managed by the US NIH, The African Academy of Sciences (AAS) and Bill & Melinda Gates Foundation (BMGF) "Grant Reference Number: APTI-18-01".

Acknowledgments

We thank all study participants. We also thank Mr. J. Patrick Gorres of the National Institute of Health (NIH) and NIH Fellows Editorial Board for assistance in reviewing and editing the manuscript. We thank Ms. Holly Torano and Ms. Jillian Neal for technical support.

Conflicts of Interest

The authors declare no competing interests.

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