Low prevalence of highly sulfadoxine-resistant dihydropteroate synthase alleles in Plasmodium falciparum isolates in Benin

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Abstract

Background: In 2004, in response to high levels of treatment failure associated with sulfadoxine-pyrimethamine (SP) resistance, Benin changed its first-line malaria treatment from SP to artemisinin-based combination therapy for treatment of uncomplicated Plasmodium falciparum malaria. Resistance to SP is conferred by accumulation of single nucleotide polymorphisms (SNPs) in P. falciparum genes involved in folate metabolism, dihydrofolate reductase (Pfdhfr) and dihydropteroate synthase (Pfdhps), targeted by pyrimethamine and sulfadoxine, respectively. Because SP is still used for intermittent preventive treatment in pregnant women (IPTp) and seasonal malaria chemoprevention (SMCP) in Benin, the prevalence of Pfdhfr and Pfdhps SNPs in P. falciparum isolates collected in 2017 were investigated.

Methods: This study was carried out in two sites where the transmission of P. falciparum malaria is hyper-endemic: Klouékanmey and Djougou. Blood samples were collected from 178 febrile children 6–59 months old with confirmed uncomplicated P. falciparum malaria and were genotyped for SNPs associated with SP resistance.

Results: The Pfdhfr triple mutant IRN (N51I, C59R, and S108N) was the most prevalent (84.6%) haplotype and was commonly found with the Pfdhps single mutant A437G (50.5%) or with the Pfdhps double mutant S436A and A437G (33.7%). The quintuple mutant, Pfdhfr IRN/Pfdhps GE (A437G and K540E), was rarely observed (0.8%). The A581G and A613S mutant alleles were found in 2.6 and 3.9% of isolates, respectively. Six isolates (3.9%) were shown to harbour a mutation at codon I431V, recently identified in West African parasites.

Conclusions: This study showed that Pfdhfr triple IRN mutants are near fixation in this population and that the highly sulfadoxine-resistant Pfdhps alleles are not widespread in Benin. These data support the continued use of SP for chemoprevention in these study sites, which should be complemented by periodic nationwide molecular surveillance to detect emergence of resistant genotypes.

Keywords: Drug resistance, Sulfadoxine Pyrimethamine, Pfdhfr, Pfdhps, Intermittent Preventive Treatment in Pregnant, Seasonal Malaria Chemoprevention, Pregnant women, Malaria

Background

Malaria is a major health problem in Benin and is the leading cause of mortality among children under 5 years of age and morbidity among adults. In 2019, the World Health Organization (WHO) reported an estimated four million malaria cases and over 7000 deaths in the country [1]. In 2004, Benin joined many other countries in
Africa in changing their recommended first-line treatment of uncomplicated malaria to artemisinin-based combination therapy (ACT) [2] due to reported high treatment failure rates in children treated with sulfadoxine-pyrimethamine (SP) for uncomplicated malaria [3–5]. However, the WHO recommends the continued use of SP for intermittent preventive treatment in pregnant women (IPTp) [6], as well as for seasonal malaria chemoprevention (SMC), used in combination with amodiaquine (SP-AQ) for the latter indication, in countries with highly seasonal malaria transmission such as the Sahel region of sub-Saharan Africa [7].

Resistance to SP is conferred by accumulation of single nucleotide polymorphisms (SNPs) in two genes that code for enzymes involved in *Plasmodium falciparum* folate metabolism: *P. falciparum* dihydrofolate reductase (*Pfdhfr*) and *P. falciparum* dihydropteroate synthase (*Pfdhps*), which are targeted by pyrimethamine and sulfadoxine, respectively. At least five mutations in *Pfdhfr* confer resistance to pyrimethamine: C50R, N51I, C59R, S108N, and I164L (amino acid substitutions in bold face). Similarly, at least five SNPs in *Pfdhps* are involved in resistance to sulfadoxine: S436A/F, A437G, K540E, A581G, and A613S/T [8–12].

The combination of *Pfdhfr* triple mutant IRN with the *Pfdhps* double mutant GE results in a quintuple mutant, which has been shown to lead to clinical treatment failure of SP [13–15]. In general, these quintuple mutants are commonly found throughout East Africa, but rarely in West and Central Africa [16–18]. In contrast, the *Pfdhfr* triple mutant IRN with the *Pfdhps* A437G is often found in West Africa and is also associated with treatment failure, but to a lesser degree than the IRN plus GE quintuple mutants [16–21]. Studies have demonstrated that the efficacy of SP for IPTp is still acceptable even when a high prevalence of SP resistance markers exists, including the IRN plus GE quintuple mutants and the IRN plus G quadruple mutants [6, 22–25], justifying the continued use of SP for IPTp and SMC. However, the occurrence of additional *Pfdhps* mutations at codons A581G [19, 26–28] and A613S/T [27] or *Pfdhfr* I164L [29, 30] to the quintuple IRN plus GE mutant genotype was shown to lead to declines in SP’s IPTp efficacy [28] and protection in infants [31].

Studies of molecular markers of SP resistance in Benin carried out between 2003 and 2012 in the north, south [32–34], and the coast [4, 35, 36] showed that the majority (>90%) of parasites carry IRN *Pfdhfr* mutations. The most common mutation in *Pfdhps* was A437G (71.4%) [34]. A low prevalence of K540E (8.3%) was found [36] and no mutation was found at codon S436A in a study conducted between 2008 and 2010 [37]. However, recent data on molecular markers associated with SP resistance is lacking. This study investigated the prevalence of SNPs in *Pfdhfr* and *Pfdhps* in *P. falciparum* isolates collected from Benin in 2017.

**Methods**

**Study population and sample collection**

The samples utilized in this study were obtained from a therapeutic efficacy study (TES) of artemether-lumefantrine conducted by the National Malaria Control Programme (NMCP) in Benin in 2017 (results unpublished). The study was carried out in two NMCP sentinel sites, Klouékanmey and Djougou, where the transmission of *P. falciparum* malaria is hyper-endemic (Fig. 1). Criteria for inclusion included children 6–59 months old with monoinfection of *P. falciparum*, measured by microscopy, a parasite density between 2000 and 200,000 parasites/µl, axillary temperature of 37.5 °C or higher, and ability to take oral medication. Children with signs of severe illness and malnutrition were excluded. Enrolled patients were treated with a supervised 3-day course of artemether-lumefantrine and monitored for 28 days with weekly scheduled visits on days 7, 14, 21, and 28. Patients

![Fig. 1](image-url) Location of study sites used for the therapeutic efficacy study, Benin, 2017. Benin map (shaded in gray) indicating the location of the two sentinel sites, Klouékanmey and Djougou (purple dots), in which samples were collected and used to determine the prevalence of sulfadoxine-pyrimethamine resistance markers in *Plasmodium falciparum* isolates.
were also asked to return to the clinic if they became ill any other day during the follow-up period (unscheduled visits). Dried blood spots (DBS) were collected on Whatman grade 3 filter paper (GE Healthcare Life Sciences, Marlborough, USA) from enrolled patients on the day of enrollment (pre-treatment) and on the scheduled and unscheduled visits. Only samples from the day of enrollment were utilized in this study.

**Sample processing and Pf dhfr and Pf dhps molecular analysis**

Genomic DNA was isolated from the DBS using the QIAamp® blood mini kit (Qiagen Inc., CA, USA) per the manufacturer’s recommendations. The Pf dhfr and Pf dhps gene fragments were amplified by polymerase chain reaction (PCR) using previously published primers [38]. SNPs at Pf dhfr codons 50, 51, 59, 108, and 164 and Pf dhps codons 431, 436, 437, 540, 581, and 613 were investigated using Sanger sequencing as previously described [38]. The PCR products were precipitated in 70% ethanol to clean up dye terminators, rehydrated in 10 µl Hi-Di Formamide™, and sequenced using the Applied Biosystems 3130xl sequencer (Life Technologies, Grand Island, NY). Sequences were analysed using Geneious software (Biomatters, San Francisco, CA, USA). The 3D7 P. falciparum Pf dhps (Gene ID: 2,655,294) and Pf dhfr (Gene ID: 9,221,804) were used as reference sequences in the analysis.

**Data management and analysis**

Data were entered into a Microsoft Excel database and descriptive statistics such as percentage, mean, and range were reported as appropriate. The prevalence of different alleles and haplotypes in the Pf dhfr and Pf dhps genes were reported per site. The prevalence of the different alleles was reported as wild type (having only the wild type allele), mutant (having only the mutant allele), or mixed infection (having both wild type and mutant alleles).

**Results**

A total of 178 pre-treatment samples (85 from Klouékanmey and 93 from Djougou) were evaluated for molecular markers of resistance in the Pf dhfr and Pf dhps genes. The mean age of the patients was 33 (SD = 14) months and 62.1% were male. The geometric mean P. falciparum parasite density was 24,154 (95% CI 16,600–31,700); range: 2,081–200,000) parasites/µl.

**Prevalence of Pf dhfr and Pf dhps alleles (SNPs)**

A total of 169 (94.9%) specimens were successfully sequenced for the Pf dhfr gene. Twelve samples (7.1%) had a mixed infection. No mutations were found at codons 50 or 164. Overall, a high prevalence of mutations was observed in codons N51L (86.4%; 146), C59R (89.9%; 152), and S108N (94.7%; 160); an additional 4.1, 6.5, and 2.4% of samples, respectively, contained both mutant and wild-type alleles as part of a mixed infection, Table 1. For the Pf dhps gene, 153 (86.0%) samples were successfully sequenced and 21 of these (13.7%) had a mixed infection. Overall, a majority of the samples had the A437G mutation (94.8%; 145) followed by the S436A mutation (35.3%; 54), Table 1. All samples from Klouékanmey (100%; 68) had the A437G mutation compared to 95.3% (81) in Djougou, of which four (4.7%) were mixed alleles. The A581G and A613S alleles were observed in 2.6 and 3.9% of isolates, respectively, with an additional sample from Djougou having a 613A/S mixed infection. Six isolates (3.9%) were shown to harbour a mutation at codon I431V, three from Klouékanmey and three from Djougou, of which two were mixed with wildtype parasites, Table 1.

**Observed haplotypes per gene**

Table 2 summarizes the observed haplotypes for the Pf dhfr and Pf dhps genes. Haplotypes were constructed using codons C50R, N51I, C59R, S108N, and I164L in the Pf dhfr gene and I431V, S436A, A437G, K540E, A581G, and A613S in the Pf dhps gene. Mixed infections in the Pf dhfr (12) and Pf dhps (21) genes were excluded for the haplotype construction. The majority of parasites (84.6%, 143) harboured the triple mutant Pf dhfr CIRNI haplotype: 89.8% (71) in Klouékanmey and 79.9% (72) in Djougou. The most common Pf dhps haplotype was ISGKAA (49.6%; 76), followed by the double mutant Pf dhps IAGKAA (29.3%; 45). The I431V mutation was seen in combination with other Pf dhps mutations, with three isolates (2.0%) possessing S436A, A437G, A581G, and A613S, and one isolate (0.7%) possessing S436A, A437G, and A613S, Table 2.

**Combined Pf dhfr/Pf dhps haplotypes**

Combined Pf dhfr and Pf dhps haplotypes were constructed using 119 samples that were successfully sequenced at each of the codons investigated, Table 3. Mixed infections at any of the codons were excluded. The N51I/C59R/S108N/A437G haplotype was found in 106 (89.1%) of the samples. Of these 106, 45 also contained the S436A mutation (N51I/C59R/S108N/S436A/A437G) and one contained the K540E mutation (N51I/C59R/S108N/A437G/K540E). Another two samples contained neither the A437G nor the K540E mutation but contained the S436A mutation (N51I/C59R/S108N/S436A). In the samples with the N51I/C59R/S108N/S436A/A437G haplotype, the A613S mutation was found...
in five samples, three of which also contained the A581G mutation.

**Discussion**

Results from this study demonstrate that the prevalence of the *Pfdhfr* IRN triple mutant is very high, implying these mutants are well established in this region, similar to observations made previously in Benin and in many African countries [16, 18, 39, 40]. In contrast, the prevalence of multiple mutations in the *Pfdhps* gene was low, with the majority of parasites having only a single mutation at codon A437G and 29.3% of the parasites with a double mutant (S436A/A437G), as commonly observed in West Africa [18, 21, 41, 42]. Only a handful of isolates had mutations at codons K540E (2.6%), A581G (2.6%), and A613S (4.6%). A low prevalence or complete absence of these mutations was also observed in other studies in Benin [36, 37]. Several studies have shown the prevalence of these mutations in West Africa is very low compared to East Africa, (reviewed in [16, 18]). This is especially

| Table 1 Summary of alleles observed in *Pfdhfr* and *Pfdhps* genes |
|-------------------|-------------------|-------------------|
| **Pfdhfr**        | **Klouékanmey** n = 79 (%) | **Djougou** n = 90 (%) | **Overall** n = 169 (%) |
| C50               | 79 (100)           | 90 (100)           | 169 (100)         |
| 50R               | 0                 | 0                 | 0                 |
| 50 C/R            | 0                 | 0                 | 0                 |
| N51               | 3 (3.8)           | 13 (14.4)         | 16 (9.5)          |
| S1I               | 72 (91.1)         | 74 (82.3)         | 146 (86.4)        |
| S1 N/I            | 4 (5.1)           | 3 (3.3)           | 7 (4.1)           |
| C59               | 1 (1.3)           | 5 (5.6)           | 6 (3.6)           |
| S9 R              | 72 (91.1)         | 80 (88.8)         | 152 (89.9)        |
| S9 C/R            | 6 (7.6)           | 5 (5.6)           | 11 (6.5)          |
| S108              | 0                 | 5 (5.6)           | 5 (3.0)           |
| 108 N             | 78 (98.7)         | 82 (91.1)         | 160 (94.7)        |
| 108S/N            | 1 (1.3)           | 3 (3.3)           | 4 (2.4)           |
| I164              | 79 (100)          | 90 (100)          | 169 (100)         |
| 164L              | 0                 | 0                 | 0                 |
| 164I/L            | 0                 | 0                 | 0                 |

| **Pfdhps**        | **Klouékanmey** n = 68 (%) | **Djougou** n = 85 (%) | **Overall** n = 153 (%) |
| I431              | 65 (95.6)         | 82 (96.4)          | 147 (96.1)          |
| 431 V             | 3 (4.4)           | 1 (1.2)            | 4 (2.6)             |
| 431 I/V           | 0                 | 2 (2.4)            | 2 (1.3)             |
| S436              | 41 (60.2)         | 40 (47.1)          | 81 (52.9)           |
| 436 A             | 22 (32.4)         | 32 (37.6)          | 54 (35.3)           |
| 436 S/A           | 5 (7.4)           | 13 (15.3)          | 18 (11.8)           |
| A437              | 0                 | 4 (4.7)            | 4 (2.6)             |
| 437 G             | 68 (100)          | 77 (90.6)          | 145 (94.8)          |
| 437 A/G           | 0                 | 4 (4.7)            | 4 (2.6)             |
| K540              | 66 (97.0)         | 83 (97.6)          | 149 (97.4)          |
| 540 E             | 1 (1.5)           | 1 (1.2)            | 2 (1.3)             |
| 540 K/E           | 1 (1.5)           | 1 (1.2)            | 2 (1.3)             |
| A581              | 65 (95.6)         | 84 (98.8)          | 149 (97.4)          |
| 581 G             | 3 (4.4)           | 1 (1.2)            | 4 (2.6)             |
| 581 A/G           | 0                 | 0                 | 0                 |
| A613              | 65 (95.6)         | 81 (95.3)          | 146 (95.4)          |
| 613 S             | 3 (4.4)           | 3 (3.5)            | 6 (3.9)             |
| 613 A/S           | 0                 | 1 (1.2)            | 1 (0.7)             |

Bold letters denote mutant alleles
the case for the K540E mutation, which has a prevalence greater than 10% in many countries in East Africa but is rarely reported in West Africa [16, 18]. A significant increase in the prevalence of the mutations at codons A581G and A613S was observed in Nigeria [27, 43], demonstrating the emergence of these mutations. However, their prevalence, even in Benin, is well below the WHO thresholds for consideration of changes in the use of IPTp (>95% for K540E and >10% for A581G) [44].

Ultimately, the combination of mutations in the Pfdhfr and Pfdhps genes is one of several factors that determines a parasite’s response to SP. Marked regional differences in the Pfdhfr and Pfdhps genotypes have been observed across Africa [16, 18]. The quadruple (N51I, C59R, S108N plus A437G) mutants are widespread in West Africa, while the quintuple mutants (N51I, C59R, S108N plus A437G, 540E) and sextuple mutants (addition of PfDHPS A581G and A613T/S or PfDHFR 1164L on the quintuple background) predominate in East Africa [16, 18–20, 38, 45, 46]. In keeping with these observations and with a study conducted in Benin between 2008 and 2010 [37], the majority of isolates in this study were quadruple (N51I, C59R, S108N plus A437G) mutants.

Table 2 Summary of haplotypes observed in PfDHFR and PfDHPS genes

| Haplotypes | Klouékanmey n (%) | Djougou n (%) | Overall n (%) |
|------------|------------------|---------------|---------------|
| PfDHFR     |                  |               |               |
| CIRNI      | 71 (89.8)        | 72 (79.9)     | 143 (84.6)    |
| CNrcn      | 1 (1.3)          | 7 (7.8)       | 8 (4.7)       |
| CNcs      | 1 (1.3)          | 0             | 1 (0.6)       |
| CNSCI      | 0                | 5 (5.6)       | 5 (3.0)       |
| Mixed-infection | 6 (7.6) | 6 (6.7) | 12 (7.1) |
| PfDHPS     |                  |               |               |
| ISGKAAs    | 39 (57.4)        | 37 (43.5)     | 76 (49.6)     |
| IAAGKAA    | 19 (27.9)        | 26 (30.6)     | 45 (29.3)     |
| IAaKAA     | 0                | 3 (3.5)       | 3 (2.0)       |
| VAGaG     | 3 (4.4)          | 0             | 3 (2.0)       |
| ISGEAA     | 1 (1.5)          | 1 (1.2)       | 2 (1.3)       |
| VAGkaS    | 0                | 1 (1.2)       | 1 (0.7)       |
| IAAGS     | 0                | 1 (1.2)       | 1 (0.7)       |
| ISAKAA    | 0                | 1 (1.2)       | 1 (0.7)       |
| Mixed-infection | 6 (8.8) | 15 (17.6) | 21 (13.7) |

Bold letters denote mutant alleles

Table 3 Summary of combined PfDHFR and PfDHPS haplotypes. Shaded boxes and bold letters denote mutant alleles. Key haplotypes associated with SP resistance include: the quadruple haplotypes, N51I, C59R, S108N plus S436A or A437G; the quintuple haplotypes, N51I, C59R, S108N plus A437G and K540E; or N51I, C59R, S108N plus S436A and A437G.
The sustained high prevalence of these quadruple mutant parasites is likely due to persistent SP drug pressure from its continual use for IPTp and SMC in Benin. The quintuple (N511, C59R, S108N plus A437G and K540E) mutant was observed only in one isolate in Klouékanmey. The minority of isolates in this study with the A581G and A613S mutations were found in the absence of the K540E mutation. These results demonstrate that genotypes conferring a high level of SP resistance have not fully emerged in these study sites, providing support for the continued use of SP for IPTp and SMC in Benin.

A few isolates (2.6%) in this study possessed the PfDHPS I431V mutation, which was first described in travelers from Nigeria identified in the UK [47]. A recent study conducted in Nigeria demonstrated an increase in the prevalence of this mutation from 0–6.5% between 2003 and 2008, and as high as 46% in 2010 in Enugu, Nigeria [43]. It was also found in pregnant women from other sites in Nigeria such as Epe and Ibeju-Lekki [42]. Furthermore, the I431V mutation was seen in isolates from pregnant women in Cameroon and Ghanaian travelers [48, 49], suggesting this mutation is emerging in the region. Interestingly, to date, the I431V mutation has not been observed in other parts of Africa. In concordance with previous studies [42, 49], this mutation was observed in combination with other PfDHPS mutations (S436A/A437G/A581G/A613S), suggesting this mutation may have occurred only in the presence of the other PfDHPS mutations. The implications of this combination of PfDHPS mutations remain unclear. While some propose these mutations may disrupt the binding of sulfadoxine to the PfDHPS active site [43], additional studies are needed to fully support this notion and to understand the mechanisms involved. Therefore, it is worthwhile to continue monitoring the prevalence of the I431V mutation, along with other mutations, in this region.

Limitations of this study include the fact that the samples were obtained from a TES conducted in only two sites in Benin, and therefore the results obtained may not be generalizable to other regions or sites. Moreover, the sample size used was small; additional study sites are recommended for future studies.

Conclusions

The results from this study indicate that the highly sulfadoxine resistant PfDHPS alleles are not widespread in Benin, supporting the current policy of using SP for IPTp and SMC in Benin. However, given the continued use of this drug and limited alternative options, frequent monitoring of SP resistance markers in order to inform IPTp and SMC policies in Benin remains important.

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Authors’ contributions

AK, ESH, AS, RS, MP and FD designed the study, SS and AA performed all laboratory work; SSS and NWL analysed and presented the data. SSS, ESH, VW and NL drafted the paper, all authors reviewed and approved the final manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The full anonymized clinical dataset will be uploaded to Worldwide Antimalarial Resistance Network and WHO repositories upon request and after publication.

Ethics approval and consent to participate

Written informed consent was obtained from each study participant before study participation. The ethical approvals were granted by the National Ethics Committee for Research (CNERS) in Benin, IRB#068601/20/2018. Laboratory analysis was performed with a Benin laboratory trainee at the Centers for Disease Control and Prevention (CDC) as part of a technical training program [50]. The work performed at CDC did not constitute engagement in human subjects research (CDC protocol #2017 – 141).

Consent for publication

Authors gave their consent for publication.

Competing interests

The authors have no competing interests.

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