Parasitology Research (2018) 117:801–807
https://doi.org/10.1007/s00436-018-5754-5

ORIGINAL PAPER

# Prevalence of the *Pfdhfr* and *Pfdhps* mutations among asymptomatic pregnant women in Southeast Nigeria

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Received: 1 August 2017 / Accepted: 8 January 2018 / Published online: 13 January 2018


## Abstract

Sulfadoxine-pyrimethamine (SP) is the recommended drug for intermittent preventive treatment of malaria in pregnancy in most of sub-Saharan Africa. Resistance to SP is related to mutations in the *dhfr* and *dhps* gene of *Plasmodium falciparum*. This study determined the prevalence of *Pfdhfr* and *Pfdhps* polymorphisms found in asymptomatic pregnant women attending antenatal care in Calabar, Nigeria. From October 2013 to November 2014, asymptomatic pregnant women attending antenatal care clinics were enrolled after obtaining informed consent. Malaria diagnosis testing was done using thick and thin smears. Dried blood spot filter papers were collected. Parasite DNA was extracted from the filter papers using a chelex extraction. Extraction was followed by nested PCR and restriction enzyme digestion. *P. falciparum* infection was detected by microscopy in 7% (32/459) participants. Twenty-eight *P. falciparum* isolates were successfully genotyped. In the *Pfdhfr* gene, the triple mutation was almost fixed; S108N mutation was (100%), N51I (93%) and C59R mutations (93%), whereas the I164L mutation was absent. The prevalence of *Pfdhps* S436A, A437G, A581G and A613S mutations was 82.1% (23/28), 96.4% (27/28), 71.4% (20/28) and 71.4% (20/28) respectively. The K540E mutation was absent. The prevalence of the *Pfdhfr* triple mutation IRNI was 92.9% (26/28). The efficacy of SP as IPTp in Southeast Nigeria may be severely threatened. The continuous monitoring of SP molecular markers of resistance is required to assess thresholds. The evaluation of alternative preventive treatment strategies and drug options for preventing malaria in pregnancy may be necessary.

**Keywords** Molecular markers · SP resistance · Malaria · Antenatal clinic

## Background

Malaria remains a leading cause of death predominantly in sub-Saharan Africa and East Asia. The latest World Health Organization (WHO) statistics report an estimated 438,000 deaths attributable to malaria in 2015 alone. The Democratic Republic of the Congo and Nigeria together accounted for more than 35% of this global total of estimated malaria deaths

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(World Health Organization 2015). *Plasmodium falciparum* malaria disproportionately affects pregnant women and children aged under 5 years in high transmission areas, particularly in poor and disadvantaged areas. Over 32 million pregnant women are estimated to be at risk of *P. falciparum* malaria in sub-Saharan Africa annually (Dellicour et al. 2010). Malaria infection adversely affects the outcome of pregnancy. The foetus is at increased risk of premature and stillbirth, intrauterine growth retardation, low birth weight, anaemia and congenital malaria (Brabin 1991; Desai et al. 2007; McGready et al. 2012).

In Nigeria, malaria incidence is estimated to have decreased by less than 50% between 2000 and 2015 (World Health Organization 2015). Sadly, malaria remains a significant challenge, as malaria-related deaths still account for up to 11, 25 and 20% maternal, infant and under-five mortality respectively (National Population Commission 2012).

The WHO recommends a package of interventions to prevent the adverse effects of malaria during pregnancy in areas with stable transmission in sub-Saharan Africa including the use of insecticide-treated nets (ITNs), intermittent preventive treatment (IPTp) and effective case management of malaria and anaemia. Sulfadoxine-pyrimethamine (SP) is the recommended drug for IPTp (World Health Organization 2004). The WHO now recommends a dose of SP at each scheduled antenatal care (ANC) visit, beginning as early as possible in the second trimester, and with each dose at least a month apart for areas of moderate-to-high malaria transmission (World Health Organization 2012).

In Nigeria, there has been an overall increase in access to and ownership of ITNs, but geographical variations still exist (National Population Commission 2012; Nigeria Demographic Health Survey 2014). IPTp utilisation remains low with only 13% of pregnant women receiving the recommended preventive treatment (≥ 2 doses of SP) during ANC (National Population Commission 2012).

The development of drug resistance remains one of the most significant challenges of malaria control programmes. While SP is recommended for IPTp, in stable transmission areas, increasing resistance has been reported and is compromising the beneficial effects of SP on birth weight and anaemia (McGready et al. 2011; Cottrell et al. 2015). IPTp with SP causes placental proliferation of resistant parasites in pregnant women (Harrington et al. 2011). Pregnant women with asymptomatic parasitaemia could constitute a reservoir of parasites for the inoculation of mosquitoes (Kern et al. 2011) and may play a role in increasing drug resistance. This growing resistance has enormous implications for malaria control efforts, and thus monitoring of SP resistance remains an important task.

The efficacy of SP is dependent on some mutations which may accumulate in *P. falciparum dhfr* and *dhps* genes which code for the proteins DHFR and DHPS respectively (Kublin et al. 2002). Pyrimethamine and sulfadoxine selectively inhibit dihydrofolate reductase (DHFR) and dihydropteroate synthetase (DHPS) which are both essential for folate biosynthesis in the malaria parasites. *P. falciparum* resistance to pyrimethamine has been associated with specific point mutations (A16V, C50R, N51I, C59R, S108N/T, V140L and I164L) in the *dhfr* gene (Peterson et al. 1990; Plowe et al. 1997) and in the *dhps* gene (I431V, S436A/F, A437G, K540E, A581G and A613S/T) (Triglia and Cowman 1994; Sutherland et al. 2009; Chauvin et al. 2015).

Higher SP resistance is associated with an increasing number of mutations in both the *Pfdhfr* and *Pfdhps* genes. In sub-Saharan Africa, the so-called *Pfdhfr/Pfdhps* quintuple mutation which is a combination of a triple *Pfdhfr* mutation (51I-59R-108N) and the *Pfdhps* double mutation (437G-540E) are predictive of SP treatment failure (Duraisingh et al. 1998; Vinayak et al. 2010; Happi et al. 2005).

Given the central role of SP in the current IPTp policy recommendations, there is the need for continuous molecular surveillance for SP resistance to inform the National Malaria Control Programmes on where SP can still be used effectively and cases where it needs replacing (Naidoo and Roper 2013). In the present study, we investigated the prevalence of *dhfr* and *dhps* point mutations in *P. falciparum* isolates collected from asymptomatic pregnant women who had not received IPTp-SP.

## Methods

### Study area, subjects and sample collection

The study was carried out at the General Hospital situated in Calabar. The General Hospital is the largest government-owned secondary health facility in the city and caters to the health needs of the majority of the inhabitants. Since August 2009, pregnant women and children under 5 years of age receive free medical care as part of a funded welfare program by the Cross River State government. The average annual antenatal clinic attendance and births at the hospital are 16,550 and 3100 respectively (Ekpo A. personal communication). The climate in Calabar is tropical-humid with wet and dry seasons, with average temperatures ranging between 15 and 30 °C and the annual rainfall between 1300 and 3000 mm. The vegetation in Calabar is mangrove swamp forest. Malaria transmission in this area is intense and perennial but with a peak in the rainy season, and *P. falciparum* is the predominant malaria-causing species (World Health Organization 2015; National Population Commission 2012; Oduwole et al. 2011).

Blood samples were collected as part of a clinical trial (PACTR201308000543272) on the effectiveness of intermittent screening and treatment for malaria prevention in

pregnancy. All samples were collected from asymptomatic pregnant women attending ANC. Briefly, all pregnant women attending their first ANC visit for that pregnancy and who had not received any dose of IPTp-SP were invited to participate in the study. After obtaining written informed consent, a finger prick blood sample was obtained. Thick and thin blood smears, as well as dried blood spots (DBS) on filter paper (Whatman® grade 3), were prepared. Molecular genotyping was performed only on microscopy-positive samples (Fig. 1).

## Laboratory procedures

### DNA extraction, genotyping procedures and analysis of *Pfdhfr* and *Pfdhps* genes

Genomic DNA was extracted from DBS filter papers using QIAamp Blood Mini Kit 50 (Qiagen, Krefeld, Germany) according to the manufacturer's instructions.

*P. falciparum* parasites were genotyped for mutations in the *dhfr* and *dhps* genes by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Mutations were investigated at codons 51, 59, 108 and 164 of *Pfdhfr* gene and codons 436, 437, 540, 581 and 613 of *Pfdhps* gene as previously reported (Duraisingh et al. 1998).

## Statistical analysis

Data were entered in Excel version 2013 and analysed using STATA v12 (STATA Corporation, College Station, TX, USA). Mixed genotypes were considered as mutants, and the prevalence of each type of allele (wild or mutant) was calculated.

The frequencies of the mutations were compared based on gravidity and parasite density using chi-square test. Mann-Whitney test was used to compare parasite densities. A $P$ value $< 0.05$ was considered as statistically significant for all tests.

## Ethical considerations

The study proposal and informed consent forms were reviewed and approved by two ethics committees: the Cross River Health Research Ethics Committee, Calabar, Nigeria, and the Ethics Board of the Medical Center of LMU, Munich, Germany. Written informed consent was obtained from all participants before sample collection. Participation was voluntary, and information obtained from all subjects was confidential.

# Results

Out of 459 women screened for malaria parasitaemia, 32 had a microscopically confirmed malaria infection. Twenty-eight pre-treatment samples were successfully genotyped for *Pfdhfr* and *Pfdhps* mutations. Among these 28 women with asymptomatic malaria, the mean age was 27.3 (± 4.3) years. About 46% (13/28) of the women were primigravidae. Ownership of bed nets was 46.4% (13/28), less than half of the women (6/13) slept under a bed net the previous night (Table 1). Women who reportedly slept under a bed net the previous night did not have significantly lower parasite densities compared to those who did not ($P = 0.748$). Most women (67.9%) had haemoglobin concentration between 8 and 10.9 g/dl. Overall, the mean of haemoglobin was 10.1 ± 1.4 g/dl. The median parasite density (interquartile range) was 768.5 (256–2799) asexual parasite/μl.

The most frequent *Pfdhfr* mutation was 108N. *Pfdhfr* mutations were detected in 92.9% (26/28) of *P. falciparum*

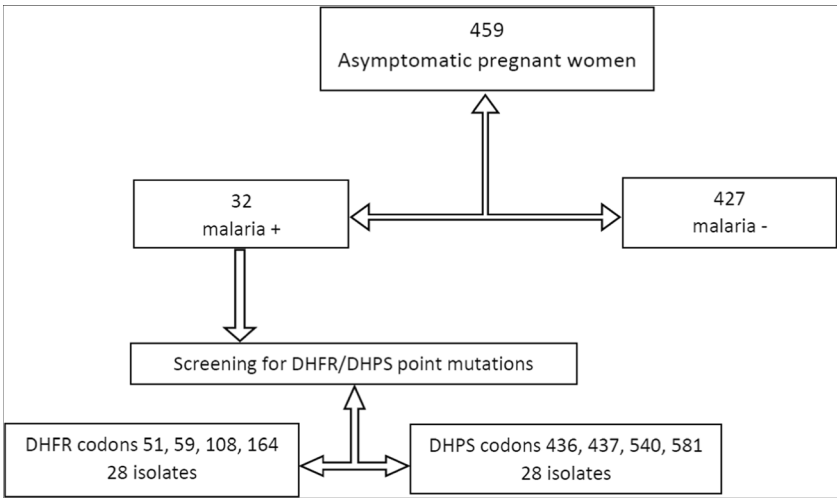


**Fig. 1** Study profile

**Table 1** Characteristics of women with positive malaria microscopy slide at enrolment

| Variables | *n* (%) |
|---|---|
| Educational attainment | |
| Primary | 3 (10.7) |
| Secondary | 9 (32.1) |
| Tertiary | 16 (57.2) |
| Gravidity | |
| Primigravidae | 13 (46.4) |
| Secundigravidae | 9 (32.2) |
| Multigravidae | 6 (21.4) |
| Ownership of bed net | |
| Yes | 13 (46.4) |
| No | 15 (53.6) |
| Slept under bed net (previous night) | |
| Yes | 6/13 (46.2) |
| No | 7/13 (53.8) |
| Haemoglobin (g/dl) level | |
| 11 and above | 6 (21.4) |
| 8–10.9 | 19 (67.9) |
| < 8 | 3 (10.7) |
| Parasite density asexual parasite/μl (median [interquartile range]) | 768.5 [256–2799] |

isolates for codons 51 (51I) and 59 (59R). The 164L mutation was not found in any of the pre-treatment samples (Table 2).

*Pfdhps* mutations were detected in 82.1% (23/28), 96.4% (27/28), 71.4% (20/28) and 71.4% (20/28) of *P. falciparum* isolates for codons 436 (436A), 437 (437G), 581 (581G) and 613 (613S) respectively. None of the samples carried the *Pfdhps* mutation K540E (Table 2).

Table 3 shows the prevalence of mutant alleles and haplotypes. A single mutation in the *Pfdhfr* gene at codon 108 (108N) was detected in only one isolate (3.6%).

A double mutation, made up of single mutations in the *Pfdhfr* and *Pfdhps* genes (108N and 437G), was found in one sample (3.6%). Also, the frequency of quadruple (51I/59R/108N + 437G) and quintuple (51I/59R/108N + 436A/437G) *Pfdhfr-Pfdhps* mutations were both 10.7% (3/28).

The prevalence of *Pf*dhfr and *Pf*dhps mutant alleles and haplotypes were not significantly lower in primigravidae compared to secundi- and multi-gravidae women ($P = 0.436$, $P = 0.144$, $P = 0.11$, $P = 0.871$ for the triple *Pf*dhfr mutation, quadruple, quintuple and septuple *Pfdhfr-Pfdhps* mutations respectively).

Similarly, the prevalence of *Pf*dhfr and *Pf*dhps mutant alleles and haplotypes did not differ significantly between high- and low-density malaria infections (Table 4).

## Discussion

In Calabar, among asymptomatic pregnant women before IPTp-SP, the *Pfdhfr* mutations 51I, 59R and 108N were almost fixed with all three mutations being present in more than 90% of the isolates. There was no I164L mutation in the study samples, a finding similar to a previous study in Nigeria (Happi et al. 2005). However, the frequency of the triple *Pfdhfr* mutation was also very high (93%). The *Pfdhfr* triple mutation is known to confer intense pyrimethamine resistance in vitro (Gregson and

**Table 2** Prevalence of the *dhfr* and *dhps* point mutations associated with SP resistance

| | *dhfr* (*N* = 28) | | | | *dhps* (*N* = 28) | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Codon | 51 | 59 | 108 | 164 | 436 | 437 | 540 | 581 | 613 |
| Mutant, *n* (%) | 26 (92.9) | 26 (92.9) | 28 (100) | 0 (-) | 23 (82.1) | 27 (96.4) | 0 (-) | 20 (71.4) | 20 (71.4) |

**Table 3** Prevalence of *Pf*dhfr and *Pf*dhps mutant alleles and haplotypes

| Mutated codons | | | | | | | | | *n* (%) of isolates (*N* = 28) |
|---|---|---|---|---|---|---|---|---|---|
| *dhfr* | | | | *dhps* | | | | | |
| 51I | 59R | 108N | 164L | 436A | 437G | 540E | 581G | 613S | |
| + | + | + | – | + | + | – | + | + | 20 (71.4) |
| + | + | + | – | + | + | – | – | – | 3 (10.7) |
| + | + | + | – | – | + | – | – | – | 3 (10.7) |
| – | – | + | – | – | + | – | – | – | 1 (3.6) |
| – | – | + | – | – | – | – | – | – | 1 (3.6) |

Plowe 2005). The *Pfdhfr* mutations are known to have emerged about a decade or two before the *Pfdhps* double mutant genotype in Africa (Talisuna et al. 2004) and are now well established across sub-Saharan Africa. The high level of triple *Pfdhfr* mutations found in this study could be explained in part by the fact that pyrimethamine was previously used as weekly chemoprophylaxis to prevent malaria in pregnancy (Fawole and Onyeaso 2008; Yusuf et al. 2008). Also, cotrimoxazole use has been associated with the emergence, spread and intensification of the A437G and K540E mutations in the *Pfdhps* gene (Gesase et al. 2009). In Nigeria, there is a high burden of pneumonia and cotrimoxazole is commonly used as prophylaxis or treatment among HIV patients and children with pneumonia (Onyedum and Chukwuka 2011).

In the *Pfdhps* gene, the frequency of the core mutation, A437G, was over 90%. Although the K540E mutation is very frequently found in association with the A437G mutation (Kublin et al. 2002; Pearce et al. 2009), the K540E mutation was absent from all the *P. falciparum* isolates in this study. Thus, there was no *Pfdhps* double mutation, at codon 437 and 540 which is a predictor of post-treatment SP resistance (Kublin et al. 2002; Plowe et al. 2004). Although *Pfdhfr/dhps* quintuple mutants are rare in West Africa, recent studies have reported emergence of the K540E mutation. This mutation is known to be a reliable marker for parasites carrying the quintuple mutants. Studies from the western part of Nigeria have found an emergence of mutant *P. falciparum* isolates carrying sulfadoxine resistance associated A437G and K540E mutations in the *Pfdhps* gene (Happi et al. 2005; Iwalokun et al. 2015; Olasehinde et al. 2014).

However, the occurrence of A437G combined with A581G mutation confers higher levels of SP resistance (Pearce et al. 2009), and this combination of A581G/A437G mutations was present in 20 of 28 isolates. The prevalence of the S436A mutation, which is an additional mutation that follows the emergence of A437G

**Table 4** Association between *Pf*dhfr-*Pf*dhps haplotypes and malaria parasite density

| Variables | High-density infection (> 500 asexual parasite/μl | Low-density infection (≤ 500 asexual parasite/μl | *P* value |
|---|---|---|---|
| Triple *Pf*dhfr mutation (51I/59R/108N) | | | |
| Yes | 12 (52.2) | 11 (47.8) | 0.254 |
| No | 4 (80) | 1 (20) | |
| Quadruple *Pf*dhfr-*Pf*dhps mutation (51I/59R/108N + 437G) | | | |
| Yes | 2 (66.7) | 1 (23.3) | 0.724 |
| No | 14 (56) | 11 (44) | |
| Quintuple *Pf*dhfr-*Pf*dhps mutation (51I/59R/108N + 436A/437G) | | | |
| Yes | 2 (66.7) | 1 (23.3) | 0.724 |
| No | 14 (56) | 11 (44) | |
| Septuple *Pf*dhfr-*Pf*dhps mutation (51I/59R/108N + 436A/437G/581G/613S) | | | |
| Yes | 10 (50) | 10 (50) | 0.227 |
| No | 6 (75) | 2 (25) | |

mutation, was over 80%. This additional mutation corresponds to an increase in the degree of resistance to SP.

The combination of A437G mutation with the *Pfdhfr* triple mutation (51I/59R/108N) is considered to be associated with SP treatment failure (Mockenhaupt et al. 2005) and was detected in over 70% of the isolates in this study.

## Conclusion

In this study, *Pfdhfr* and *Pfdhps* gene mutations associated with SP resistance were highly prevalent among asymptomatic pregnant women pre-treatment in the study area. The prevalence of the triple *dhfr* mutation and the A437G/A581G mutations were very high; suggesting that the efficacy of SP as IPTp in Southeast Nigeria may be severely threatened. However, the K540E mutation was absent suggesting that SP may still be efficacious when used as IPTp. Nevertheless, screening for *Pfdhps* K540E, a predictor of the quintuple mutant, remains a priority in Nigeria and West Africa. Also, the evaluation of alternative preventive treatment options for preventing malaria in pregnancy may be necessary.

**Acknowledgments** We thank the study participants, the staff of the antenatal clinic and management of the General Hospital Calabar where this study took place. Special thanks to Dr. Olabisi Oduwole, Ms.Obiamaka Okafo and Mr. David Agamse at the Calabar Institute of Tropical Diseases Research and Prevention, University of Calabar Teaching Hospital, Calabar, Nigeria, for their invaluable help. We acknowledge the assistance of Prof Dr. Frank Mockenhaupt at the Institute of Tropical Medicine and International Health, Charité-Universitätsmedizin Berlin, with genotyping of molecular markers. We are also grateful to the Center for International Health (CIH), Ludwig-Maximilians University of Munich, DAAD, BMZ and Exceed for supporting the first author for his PhD program.

**Authors' contributions** EE, MM, NB, MP and TL designed the study. CT and PG were responsible for the laboratory work. EE wrote the first draft of the paper. All authors contributed to the interpretation of the data and the revision of the manuscript.

## Compliance with ethical standards

**Consent for publication** Not applicable.

**Availability of data and materials** The datasets generated during the current study are available from the corresponding author on reasonable request.

**Conflict of interest** The authors declare that they have no competing interests.

**Abbreviations** ANC, antenatal care; DBS, dried blot spot; DHFR, dihydrofolate reductase enzyme; dhfr, dihydrofolate reductase gene; DHPS, dihydropteroate synthetase enzyme; dhps, dihydropteroate synthetase gene; IPTp, intermittent preventive treatment in pregnancy; ITN, insecticide-treated bed net; PCR-RFLP, polymerase chain reaction-restriction fragment length polymorphism; SP, sulfadoxine-pyrimethamine; WHO, World Health Organization

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