J Antimicrob Chemother 2023; **78**: 788–791
https://doi.org/10.1093/jac/dkad011 Advance Access publication 21 January 2023

Journal of Antimicrobial Chemotherapy

Baseline prevalence of molecular marker of sulfadoxine/pyrimethamine resistance in Ebonyi and Osun states, Nigeria: amplicon deep sequencing of *dhps*-540

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Received 6 October 2022; accepted 30 December 2022

Background: Chemoprevention plays an important role in malaria control strategy. Perennial malaria chemoprevention (PMC) using sulfadoxine/pyrimethamine (SP) is a WHO-approved strategy to combat malaria in young children and may lead to drug pressure. Introducing SP-PMC may therefore be compromised due to the emergence of *Plasmodium falciparum* resistant to SP, particularly mutation at K540E of the dihydropteroate synthase (*dhps*) gene. Molecular surveillance of resistance markers can support assessment of antimalarial efficacy and effectiveness. High prevalence of 540E is associated with reduced effectiveness of SP, and areas with more than 50% prevalence are considered unsuitable for intermittent preventative treatment in pregnancy (IPTp) implementation. Assessing 540E prevalence is an important undertaking before implementation of SP-PMC.

Methods: We conducted a rapid surveillance of *dhps*-540E to assess the suitability of SP as PMC in field studies from Ebonyi and Osun states in Nigeria. We used an in-house developed amplicon deep-sequencing method targeting part of the *dhps* gene.

Results: Our data reveal that 18.56% of individuals evaluated carried the 540E mutation mixed with the WT K540. Mutant variant 540E alone was not found, and 80% of isolates harboured only WT (K540). Clonal analysis of the sequencing data shows a very low proportion of 540E circulating in both states.

Conclusions: Our data show that both states are suitable for SP-PMC implementation and, based on this finding, SP-PMC was implemented in Osun in 2022. Continuous monitoring of 540E will be required to ensure the chemoprevention effectiveness of SP in Nigeria.

Introduction

Chemoprevention, in addition to early treatment and vector control measures, plays an important role in malaria control. There are four main malaria chemoprevention strategies: intermittent preventive treatment in pregnancy (IPTp); intermittent preventive treatment in infants (IPTi); seasonal malaria chemoprevention (SMC); and mass drug administration (MDA).[1] The WHO prevention strategy has recently been reviewed and a new guideline was published.[2] The new guideline replaces IPTi with perennial malaria chemoprevention (PMC) and removes age and dose restrictions as well as the parasite resistance-marker threshold. Most chemoprevention relies on the antifolate combination sulfadoxine/pyrimethamine (SP) as recommended by the WHO. IPTp and SMC are currently implemented in Nigeria, while PMC is in the process of being

Table 1. *dhps*-540E prevalence by LGA in the two states

LGA	Total samples sequenced	*dhps*-540E	*dhps*-540E prevalence (%)
Ebonyi (total)	413	87	21.07
Abakaliki	71	9	12.68
Afikpo North	29	5	17.24
Ezza North	59	4	6.78
Ezza South	85	24	28.24
Izzi	93	31	33.33
Ohaozara	76	14	18.42
Osun (total)	535	89	16.64
Ife North	55	10	18.18
Ila	57	9	13.43
Iwo	96	14	14.58
Oriade	97	13	13.40
Orolu	172	40	23.26
Total	948	176	18.56

implemented.[3] The strategy includes initiation of PMC through a pilot implementation study for possible scale-up in states not targeted for SMC and malaria vaccine.[4] It is therefore necessary to keep track of the efficacy of SP, the chemopreventive agent central to these programmes.

In the absence of phenotypic data such as parasite clearance, molecular data could provide insight into understanding SP efficacy.[5] Surveillance of molecular markers serves as an early warning signal for emergence of drug resistance during large-scale implementation of SP use and can inform public health policy.[6,7] Mutations in the *Plasmodium falciparum* dihydrofolate reductase gene (*dhfr*) such as S108N, N51I, C59R and I164L are associated with reduced efficacy to pyrimethamine, while mutations in the dihydropteroate synthase gene (*dhps*) such as S436A/F, A437G, K540E, A581G and A613S/T of *P. falciparum* are associated with reduced efficacy to sulfadoxine. The degree of resistance is reported to be proportional to the number of accumulated mutations in a progressive, stepwise manner.[8] Mutations in all of these locations usually correlate with mutation at codon *dhps*-540 (*dhps*-540E) and this serves as a representation of accumulated mutation.[7] Hence the previous recommendation by WHO, that prevalence of *dhps*-540E should be less than 50% to implement SP-PMC in areas with moderate to high malaria transmission.[7] There is paucity of data on the frequency and prevalence of genotypes associated with SP resistance in Nigeria, although recent evidence suggests the emergence of new variants in some areas.[9,10]

In this study, we describe the baseline prevalence and frequency of the *dhps*-K540E mutation in Ebonyi or Osun states in Nigeria before the introduction of SP-PMC. Specifically, a *dhps*-540E mutation prevalence of under 50% in the parasite population is a necessary prerequisite for a PMC effectiveness–implementation hybrid study, prior to its implementation as prescribed by the National Strategic Plan for 2021–2025 in Nigeria.[3]

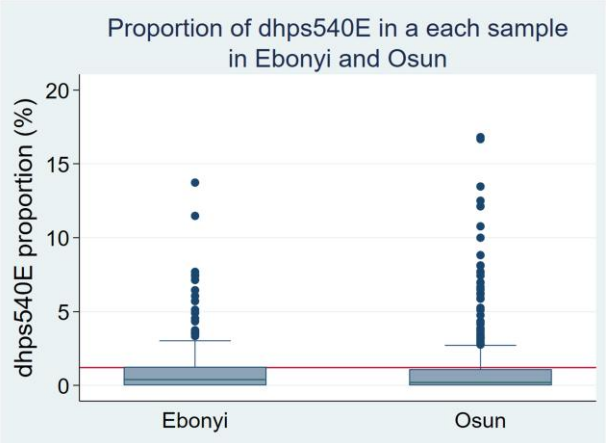

Figure 1. Proportion of dhps540E in each sample in Ebonyi and Osun. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

Methods

The study was conducted in six local government areas (LGAs) in each of Ebonyi and Osun states of Nigeria. The samples were collected from symptomatic patients of all ages attending the selected health facilities. Study sites, survey design and methods are described in the Supplementary Method, available as Supplementary data at *JAC* Online.

DNA extraction

DNA was extracted from all samples using a previously published protocol using a robotic extraction system.[11] Details of methods for the DNA quality check and confirmation of *P. falciparum* infection are described in the Supplementary Methods.

Details of all the laboratory methods for PCR amplification of the targeted *dhps* region, visualization of amplicons,[12] amplicon sequencing using NGS,[13] and data analysis are described in the Supplementary Methods.

Ethics

The study was approved by NIMR IRB (IRB-21-059) and Ethics Committee of the LSHTM (26374). Written informed consent was obtained before the participants were registered.

Results

We screened a total of 1248 samples and 1161 (93%) were *P. falciparum* positive. The *P. falciparum* prevalence in Ebonyi and Osun was 93.06% (536/576) and 93.01% (625/672), respectively. The prevalence of *dhps*-540E by local government authorities is shown in Table 1. No samples carried the *dhps*-540E alone, but this mutation was detected as a minority variant in 180 samples (18.58%). The *dhps*-540K/E was more common in Ebonyi compared with Osun though the difference was not statistically significant (Ebonyi, 21.11%; Osun, 16.54%; $P=0.069$; 95% CI 0.52–1.04).

Our sequence analysis by LGA showed marked differences in the prevalence of *dhps*-K540E in Ebonyi State (mean 17.18; SD 9.82), and a slight difference in Osun State (mean 13.81; SD

5.66). In Ebonyi, the highest prevalence of the *dhps*-K540E variant was observed in Izzi (33.33%) while in Osun, the highest was in Orolu (23.26%). The lowest prevalence was observed in Ezza North (6.78%) and Ede South (6.25%).

Using the amplicon sequencing data, we evaluated the relative abundance of the *dhps*-540E variant in mixed infections. The mean proportion of *dhps*-540E (number of 540E variants per sample) circulating in the two states was very low, ranging from 1% to 16.8% (mean 2.75%) (Figure 1). Mean proportion of mutants per sample was slightly higher in Osun compared with Ebonyi, though the difference was not statistically significant. We also sought to know whether the mutant proportion per sample was different across LGAs, and the highest proportion was observed in Ohaozara (mean 1.09%) and Ife North (mean 1.35%) in Ebonyi and Osun, respectively (Figure S1).

Discussion

We have found the *dhps*-K540E variant at a prevalence of 21.11% and 16.54% in Ebonyi and Osun states, respectively. This is well below the 50% prevalence threshold at which PMC effectiveness might be threatened. We estimate that the mutant *dhps*-540E variant is circulating at a low population frequency, one mutant variant in every 100 WTs circulating in both states. To the best of our knowledge, this is the first time the population abundance of a mutant variant has been estimated from deep-sequencing data in Nigeria.

Mutations at *dhfr* (codons 51, 59 and 108) and *dhps* (codons 437, 540 and 581) are strongly associated with SP resistance. Our study focused on rapid baseline assessment of the *dhps*-540E mutation alone, as this has been shown to reliably predict the presence of the other four or five mutations.[7] SP use in vulnerable populations is known to provide protection despite the presence of the mutations, though the effectiveness is compromised when *dhps*-540E reaches 50%.[14] In this study, we report that the prevalence of *dhps*-540E is lower than 50% in Ebonyi and Osun, which affirms the suitability of both states for chemoprevention with SP.

A recent ACCESS-SMC study in samples collected in 2016 in Sokoto State in Nigeria found no *dhps*-540E but 2 years after implementation of SMC the prevalence was found to be 0.23%.[9] The study sequenced more than 5000 samples in the Sahel and sub-Sahel regions of Africa with seasonal transmission, and found only two samples harbouring parasites predicted to encode full resistance to SMC (SP/amodiaquine). Similarly, *dhps*-540E was also previously reported in Ibadan (0.5%) and Lagos (Kosofe) (1.2%), though the sample size in Ibadan was small.[10,15]

A relatively higher prevalence of *dhps*-540E (22.5%) was reported in Lagos (Victoria Island) in samples collected in 2011.[16] The highest prevalence (94%) was reported in the Ikorodu part of Lagos (Ijede and Agbowa).[17] However, previous studies of samples collected in 2014–15 in northern and southern regions of Nigeria, including those close to the current study sites, found no evidence of *dhps*-540E mutants.[18] The discrepancies might be due to differences in genotyping methods used and the performance in sensitivity of detecting mutant variants as well as the sampling period. (Figure S2).

The relative intra-infection abundance of the mutant variant was found to be low in the two states of Nigeria. This information is useful when monitoring the impact of SP for chemoprevention as it illuminates the dynamics of the variant change within each sample during the intervention to a greater depth. This is particularly useful when the impact on variant change is smaller and slower. This greater-depth analysis was possible because of the new technology of amplicon deep sequencing, and was not possible using previous methods. Most studies in Nigeria previously reported the mix of variants qualitatively as simply WT, mixed or mutant variants. In fact, measures of *dhps*-540E prevalence have been reported to cluster below 20% and above 50%.[7] One possible reason could be the clonal expansion of the mutants, which remained 'invisible' by the old genotyping methods and might have led to a sudden increase of the prevalence from 20% to 50% once the *dhps*-540E become dominant. Further systematic investigation is required to test this hypothesis.

The low prevalence of *dhps*-540E in Ebonyi and Osun is reassuring, and both states are suitable for the implementation of PMC in Nigeria. The data from the current study are valuable information on the suitability of SP for chemoprevention, and it is anticipated that PMC will be implemented in Osun in 2022. The parasite prevalence and molecular marker frequency data reported here provide a well-defined baseline against which the impact of scale-up and sustained use of SP as PMC in Nigeria can be assessed. Careful monitoring of SP resistance markers using the same field protocol and molecular genotyping approaches will be required during the large-scale implementation of SP as chemoprevention strategy.

Funding

The laboratory work reported here was funded by the Bill and Melinda Gates Foundation to the Malaria Consortium. No funding bodies had any role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Transparency declarations

All authors report no conflicts. The corresponding author had full access to all the data in the study and final responsibility for the decision to submit for publication.

Supplementary data

Supplementary Methods, Tables S1 and S2 and Figures S1 and S2 are available as Supplementary data at *JAC* Online.

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