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Molecular determinants of sulfadoxine-pyrimethamine resistance in *Plasmodium falciparum* in Nigeria and the regional emergence of *dhps* 431V

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ABSTRACT

There are few published reports of mutations in dihydropteroate synthetase (*dhps*) and dihydrofolate reductase (*dhfr*) genes in *P. falciparum* populations in Nigeria, but one previous study has recorded a novel *dhps* mutation at codon 431 among infections imported to the United Kingdom from Nigeria. To assess how widespread this mutation is among parasites in different parts of the country and consequently fill the gap in sulfadoxine-pyrimethamine (SP) resistance data in Nigeria, we retrospectively analysed 1000 filter paper blood spots collected in surveys of pregnant women and children with uncomplicated falciparum malaria between 2003 and 2015 from four sites in the south and north.

Genomic DNA was extracted from filter paper blood spots and placental impressions. Point mutations at codons 16, 50, 51, 59, 108, 140 and 164 of the *dhfr* gene and codons 431, 436, 437, 540, 581 and 613 of the *dhps* gene were evaluated by nested PCR amplification followed by direct sequencing.

The distribution of the *dhps*-431V mutation was widespread throughout Nigeria with the highest prevalence in Enugu (46%). In Ibadan where we had sequential sampling, its prevalence increased from 0% to 6.5% between 2003 and 2008. Although there were various combinations of *dhps* mutations with 431V, the combination 431V + 436A + 437G+581G+613S was the most common.

All these observations support the view that *dhps*-431V is on the increase. In addition, *P. falciparum* DHPS crystal structure modelling shows that the change from Isoleucine to Valine (*dhps*-431V) could alter the effects of both S436A/F and A437G, which closely follow the 2nd β-strand. Consequently, it is now a research priority to assess the implications of *dhps*-VAGKGS mutant haplotype on continuing use of SP in seasonal malaria chemoprevention (SMC) and intermittent preventive treatment in pregnancy (IPTp). Our data also provides surveillance data for SP resistance markers in Nigeria between 2003 and 2015.

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1. Introduction

Malaria is a major public health challenge in sub-Saharan Africa. In 2015, there was an estimated 214 million cases and 438,000 deaths due to malaria globally with Nigeria accounting for 25% of these (WHO, 2015). Malaria poses health risks to both neonate and mother during pregnancy. It leads to low birth-weight, placental malaria, severe maternal anaemia (especially in primigravidae), and perinatal mortality (Shulman et al., 1999; Menendez et al., 2010; Oyibo and Agomo, 2011). Pregnant women are usually at higher risk of malaria infection than their non-pregnant counterparts due to temporary depression of immunity during foetal development (Menendez, 1995). The World Health Organization (WHO) has recommended intermittent preventive treatment during pregnancy (IPTp) with sulfadoxine-pyrimethamine (SP) as part of strategies to control malaria in most endemic countries (WHO, 2009). IPTp-SP involves the administration of a supervised curative treatment dose of SP at each scheduled antenatal care visit starting as early as possible in second trimester and at an interval not less than 4 weeks apart and up to the time of delivery (WHO, 2014). WHO recommended IPTp-SP as a strategy for prevention of malaria in pregnancy in 2001 but IPTp-SP was only adopted in 2005 as national policy in Nigeria (WHO, 2000; FMOH, 2005). The implementation of this strategy is being faced with challenges such as timing of SP administration (Onoka et al., 2012), knowledge and practices of the population (Onwujekwe et al., 2012; Diala et al., 2013) and rising levels of parasite resistance to SP in the general population (Happi et al., 2005; Mockenhaupt et al., 2008). Seasonal malaria chemoprevention (SMC) is another malaria control intervention, which uses SP. It is the administration of a complete treatment course of amodiaquine plus SP to children aged between 3 and 59 months at monthly intervals, beginning at the start of the transmission season to a maximum of four doses during the malaria transmission season (WHO, 2012). SMC is only recommended in areas with highly seasonal malaria transmission in the Sahel subregion of sub-Saharan Africa, where *P. falciparum* is sensitive to both antimalarial medicines. SMC has been fully deployed in Katsina and Jigawa states of northern Nigeria.

Surveillance of SP resistance levels must be achieved by monitoring of molecular markers (WHO, 2004). SP resistance is linked with substitutions of amino acids in the enzymes dihydropteroate synthetase (*DHPS*) and dihydrofolate reductase (*DHFR*) in the folate biosynthetic pathway (Cowman et al., 1988; Triglia and Cowman, 1994; Brooks et al., 1994). Pyrimethamine targets the enzyme *DHFR* disrupting catalysis of the NADPH-dependent reduction of 7, 8-dihydrofolate to 5,6,7,8-tetrahydrofolate (Blakeley, 1984) while sulfadoxine blocks the folate biosynthetic pathway at the *DHPS* level by disrupting the coupling of 7,8,-dihydro-6-hydroxymethylpterin pyrophosphate with para-amino benzoic acid (pABA) to yield 7, 8- dihydropteroate (Walter, 1991).

Resistance to SP has evolved worldwide, and is caused by point mutations that accumulate at multiple sites in both the *dhfr* and *dhps* genes (Wang et al., 1997). In both genes, each successive mutation has been shown to incrementally increase the parasite's tolerance to the drug *in vitro* (Triglia et al., 1997, 1998). An asparagine substitution at codon 108 of *dhfr* followed by substitution at codons 51 and 59 seem to be necessary for pyrimethamine resistance while an additional mutation at codon 164 (I164L) has been associated with high grade pyrimethamine resistance (Plowe et al., 1997). Mutations at codons 437 and 540 of *dhps* play the most significant role in sulfadoxine resistance among African parasites. In East and South Africa, mutations at the 437 and 540 codons are found together while in West and Central Africa the 437 is found on its own (Pearce et al., 2009). Laboratory studies show that the A437G and K540E substitutions in combination raise sulfadoxine tolerance of sensitive *DHPS* by 200 fold, compared to just 10 fold for the A437G substitution alone (Triglia et al., 1997). Hence East African parasites are predicted to withstand higher doses of SP than West African parasites. The efficacy of IPTp-SP is being further compromised in east Africa by the additional emergence of *dhps* mutation at codon 581 in northern Tanzania (Gesase et al., 2009) which has been shown to reduce the efficacy of IPTp-SP (Minja et al., 2013) termed super resistance (Naidoo and Roper, 2013). WHO recommended that prior to implementation of IPTp-SP in any region with moderate to high malaria transmission, the prevalence of K540E and A581G should be determined. IPTp-SP should be used in regions with a prevalence rate K540E less than 50% and A581G less than 10% (WHO, 2013a).

Hitherto the *dhps* K540E and A581G mutations have been rare in West and Central Africa and this is consistent with evidence of IPTp-SP efficacy during the same period (Falade et al., 2007; Aziken et al., 2011).

Reports of novel *dhps* mutations at codon 431(I431V) from UK imported malaria infections originating from Nigeria (Sutherland et al., 2009) and pregnant women from Cameroon (Chauvin et al., 2015) suggest this mutation is emerging. In Nigeria, there has been a dearth of molecular surveillance data (Naidoo and Roper, 2011; Drug resistance maps, http://www.drugresistancemaps.org/ipti/) which makes this difficult to substantiate. Crucially this needs to be addressed to underpin the continuing use of SP for IPTp and seasonal malaria chemoprevention (SMC).

In order to fill the gap in SP resistance surveillance data in Nigeria, we analysed retrospectively 1000 filter paper blood spots collected from malaria-infected pregnant women (with or without IPTp-SP intervention) and children with uncomplicated falciparum malaria from four geopolitical zones in Nigeria. We also evaluated the patterns of genetic changes in the parasite between 2003 and 2015. This study is the first of its kind providing patterns of SP resistance in different regions of Nigeria.

2. Materials and methods

We identified molecular markers of SP resistance by nested PCR and direct sequencing in 1000 malaria positive blood spots collected from pregnant women and children attending hospitals across Southwest, Southeast, South-south and Northeast Nigeria (Fig. 1). Southern Nigeria comprises of the tropical rain forest with perennial malaria transmission occurring in rural and urban areas while the northern part is mostly characterized as arid savannah with less annual rainfall and more seasonal transmission (Ekanem et al., 1990). In the past, chloroquine and sulfadoxine-pyrimethamine were used but later abandoned in 2005 due to increased threat of drug resistance. The antimalarial drug regimens for all parts of Nigeria is currently artemether-lumefantrine and amodiaquine-artesunate. Filter paper blood spots and placental impressions were collected from pregnant women attending St Mary's Catholic Hospital Eleta Ibadan between May 2003 and October 2004 (Falade et al., 2007), Damboa General hospital between 2010 and 2012 (Damboa LGA, Borno State), Polyclinic (an extension of Park Lane hospital) and Balm of Gilead Specialist hospital between 2010 and 2012 (both in Enugu, Enugu State). Filter paper blood spots were also collected from children with uncomplicated malaria presenting at General Outpatient Department of the University College Hospital (UCH), Ibadan, and the Primary Health Care Center (PHC), Idi-Ayunre, Oluyole Local Government Area (both in Oyo State) between August 2007 and May 2008 (Falade et al., 2014); University of Benin Teaching Hospital (UBTH) and Sickle-cell centre (both in Edo State) between September 2014