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Different origin and dispersal of sulfadoxine-resistant *Plasmodium falciparum* haplotypes between Eastern Africa and Democratic Republic of Congo

Vito Baraka [a,b,*,1], Christopher Delgado-Ratto [b,1], Sidsel Nag [c,d], Deus S. Ishengoma [a], Rashid A. Madebe [a], Hypolite Muhindo Mavoko [b,e], Carolyn Nabasumba [b,f], Pascal Lutumba [e], Michael Alifrangis [c,d], Jean-Pierre Van Geertruyden [b]

[a] National Institute for Medical Research, Tanga Research Centre, P.O. Box 5004, Tanga, United Republic of Tanzania
[b] Global Health Institute, University of Antwerp, Antwerp, Belgium
[c] Centre for Medical Parasitology at the Department of Immunology and Microbiology, University of Copenhagen, Copenhagen, Denmark
[d] Department of Infectious Diseases, National University Hospital (Rigshospitalet), Copenhagen, Denmark
[e] Département de Médecine Tropicale, Faculté de Médecine, Université de Kinshasa, B.P. 747 Kin XI, Kinshasa, The Democratic Republic of the Congo
[f] Epicentre Mbarara Research Base, P.O. Box 930, Mbarara, Uganda

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ABSTRACT

Sulfadoxine/pyrimethamine (SP) is still used for malaria control in sub-Saharan Africa; however, widespread resistance is a major concern. This study aimed to determine the dispersal and origin of sulfadoxine resistance lineages in the Democratic Republic of the Congo compared with East African *Plasmodium falciparum* dihydropteroate synthetase (*Pfdhps*) haplotypes. The analysis involved 264 isolates collected from patients with uncomplicated malaria from Tanzania, Uganda and DR Congo. Isolates were genotyped for *Pfdhps* mutations at codons 436, 437, 540, 581 and 613. Three microsatellite loci (0.8, 4.3 and 7.7 kb) flanking the *Pfdhps* gene were assayed. Evolutionary analysis revealed a shared origin of *Pfdhps* haplotypes in East Africa, with a distinct population clustering in DR Congo. Furthermore, in Tanzania there was an independent distinct origin of *Pfdhps* S**GE**GA resistant haplotype. In Uganda and Tanzania, gene flow patterns contribute to the dispersal and shared origin of parasites carrying double- and triple-mutant *Pfdhps* haplotypes associated with poor outcomes of intermittent preventive treatment during pregnancy using SP (IPTp-SP). However, the origins of the *Pfdhps* haplotypes in DR Congo and Eastern Africa sites are different. The genetic structure demonstrated a divergent and distinct population cluster predominated by single-mutant *Pfdhps* haplotypes at the DR Congo site. This reflects the limited dispersal of double- and triple-mutant *Pfdhps* haplotypes in DR Congo. This study highlights the current genetic structure and dispersal of high-grade *Pfdhps* resistant haplotypes, which is important to guide implementation of SP in malaria chemoprevention strategies in the region.

1. Introduction

The widespread emergence of *Plasmodium falciparum* resistance to antimalarial drugs is a major public health concern threatening malaria control efforts. Sulfadoxine/pyrimethamine (SP) and amodiaquine were adopted to replace chloroquine as the first-line treatment against uncomplicated malaria following widespread resistance to chloroquine [1]. However, a few years after the adoption of SP, escalating treatment failure rates indicated that SP resistance was spreading. This prompted policy changes to adopt artemisinin-based combination therapies (ACTs) as the first-line treatment in malaria-endemic countries in Africa.

SP is still recommended by the World Health Organization (WHO) in sub-Saharan Africa for intermittent preventive treatment during pregnancy (IPTp-SP) to protect women and to improve foetal outcomes against the consequences of malaria infection. It is also used in seasonal malaria chemoprevention (SMC) strategies in combination with amodiaquine in areas prone to high seasonal malaria transmission across the Sahel subregion as well as in intermittent preventive treatment in infants (IPTi-SP) in areas of moderate to high transmission [2,3]. In some countries (including the Democratic Republic of the Congo), SP is used in combination with artemisinin for the treatment of uncomplicated malaria [4]. However, due to widespread SP resistance, concerns have been raised about whether this strategy is still effective [5,6].

* Corresponding author. National Institute for Medical Research, Tanga Research Centre, P.O. Box 5004, Tanga, United Republic of Tanzania. Fax: +255 27 264 2010.
E-mail address: vitobaraka@gmail.com (V. Baraka).
[1] These two authors contributed equally to this work.

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Pyrimethamine resistance is conferred by point mutations in the *P. falciparum* dihydrofolate reductase (*Pfdhfr*) gene leading to substitutions at codons N51I, C59R and S108N [7]. Sulfadoxine resistance is mediated by substitutions in the *P. falciparum* dihydropteroate synthetase (Pfdhps) at codons S/A436F, A437G, K540E, A581G and A613S/T [8,9]. Evidence suggests that SP resistance tends to increase as a result of stepwise accumulation of single nucleotide polymorphisms (SNPs) in the *Pfdhps–Pfdhfr* genes [10]. Interestingly, parasite isolates from East Africa were shown to harbour *Pfdhps* haplotypes with double mutations (at codons A437**G** and K540**E**) leading to the S**GE**AA haplotype, whereas in Western African countries the mutations are mainly limited to a single A437**G** mutation [11,12]. Recently, in East Africa, expansion of *Pfdhps* mutation at codons A581**G** and K540**E** has worsened SP resistance leading to the emergence of the triple-mutant haplotype (S**GEG**A) [13]. *Pfdhfr–Pfdhps* haplotypes confer high-grade resistance associated with poor outcomes of IPTp-SP [6,14]. Recently, a *Pfdhps* sextuple haplotype, defined as a combination of the triple *Pfdhfr* and triple *Pfdhps* mutations, was associated with reduced birth weight [6]. In addition, a recent meta-analysis estimated that if the prevalence of *Pfdhps* 581**G** is >10.1%, then IPTp-SP is no longer protective against low birth weight and alternative strategies should be considered [15]. In the East African region where the levels of S**GE**AA and S**GEG**A haplotypes are expanding, the effectiveness of IPTp-SP in controlling parasite growth and improving pregnancy outcomes is deteriorating [6,14,15]. In contrast, in West Africa, the majority of haplotypes are either *Pfdhps* wild-type or a single A437**G** (S**G**KAA) or S436**A** (**A**AKAA) mutation, with limited occurrence of the K540**E** and A581**G** mutants, and thus IPTp-SP retains its effectiveness [11]. These observations underscore the need for monitoring the distribution of parasite populations in order to mitigate the dispersal of resistant haplotypes in the region.

Studies from Southeast Asia revealed multiple and limited origins of *Pfdhps* mutant alleles [16,17], and analysis of the *Pfdhps* mutant alleles in malaria parasites from East and West Africa demonstrated that resistance emerged independently in multiple sites in Africa [17]. After segregating the *Pfdhps* triple mutant S**GEG** haplotype lineages by country, the *Pfdhps* haplotypes seemed of local origin [18]. In DR Congo, Taylor et al [19] demonstrated genetically distinct resistant *Pfdhps* haplotypes between Eastern and Western provinces. However, owing to the escalating levels of *Pfdhps* A581**G** and K540**E** mutations associated with poor IPTp outcomes in Eastern Africa, there is a need to define the genetic structure and dispersal of *Pfdhps* haplotypes in the Central African corridor and their relatedness to the Eastern African lineages. Therefore, this study determined the origin and dispersal of sulfadoxine-resistant lineages in Central Africa compared with Eastern Africa *Pfdhps* haplotypes.

2. Methods

2.1. Sample collection

Samples for this study were collected between 2012 and 2014 in Uganda (Kihurura) and DR Congo (Lisungi-Kinshasa) as part of the QuinACT clinical trial [20]. Samples from Tanzania were collected in 2014 in Muheza District, Tanga Region, as part of a study to assess the efficacy and safety of artemether/lumefantrine versus dihydroartemisinin/piperaquine for the treatment of uncomplicated malaria (Fig. 1). Children aged 6 months to 10 years with uncomplicated falciparum malaria were enrolled. Fingerprick blood samples were collected on Whatman® 3 mm filter papers (Whatman plc., Maidstone, UK) and were stored at room temperature until further use.

2.2. DNA extraction

Parasite DNA isolation was performed using a QIAamp® DNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Extracted DNA was stored at –20 °C until further use for PCR amplification.

2.3. PCR and sequence-specific oligonucleotide probes enzyme-linked immunosorbent assay (SSOP-ELISA)

P. falciparum DNA extracts were amplified by nested PCR and the products were analysed for detection of *Pfdhps* SNPs at codons 436, 437, 540, 581 and 613 using SSOP-ELISA as described previously [21].

2.4. Microsatellite (MS) genotyping

MS genotyping was performed using neutral *Pfdhps* microsatellites located on chromosome 8 at 0.8, 4.3 and 7.7 kb downstream from codon 437. Semi-nested PCR was used to amplify the microsatellites as previously described by Roper et al [22]. The sizes of the MS PCR products were determined using the GeneScan™ 500 LIZ size standard on an ABI 3730 DNA Analyzer (Applied Biosystems,

Fig. 1. Location of the study sites and major cities within the Democratic Republic of the Congo, Uganda and Tanzania (adapted from Google Maps).