Molecular monitoring of *Plasmodium falciparum* resistance to sulfadoxine-pyrimethamine in western Kenya, 14 years after its withdrawal

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Research article

Keywords: Sulfadoxine-pyrimethamine, drug resistance, *plasmodium falciparum*, pfdhfr, pfdhps.

Posted Date: April 16th, 2019

DOI: <https://doi.org/10.21203/rs.2.9218/v1>

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Abstract

Background: The application of chloroquine (CQ) as an antimalarial drug for over half a century and subsequent development of CQ-resistant *Plasmodium* strains has led to its withdrawal and replacement with sulphadoxine-pyrimethamine (SP). In 2004, SP was replaced with artemisinin-based combination therapy (ACT) as a first-line against uncomplicated malaria in Kenya. The sudden surge in ACT resistant against *P. falciparum* in Cambodia and neighboring countries had become a stumbling block in the management and control of this preventable and curable disease. The resistance associated with *P. falciparum* is linked to multiple mutations in the parasite's dihydrofolate reductase and dihydropteroate synthase genes. This study assesses the prevalence of *pf dhfr* and *pf dhps* gene mutation which encodes enzymes targeting SP. **Method:** Briefly, blood taken from a finger prick was collected on a filter paper from *P. falciparum* positive children attending health facility in Chulaimbo between May and November 2015. Using chelex-100 extraction DNA, genotyping was done for mutations on codon 51, 59 and 108 of *pf dhfr* and 437 and 540 of *pf dhps* genes using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technology. The enzymes used to digest the respective codons were Tsp5091, *Amx* I, *Alu* I, *Ava* II and *Fok* I respectively. **Results:** All the 76 *P. falciparum* isolates were successfully genotyped for the detection of *Pf dhfr* and *Pf dhps* mutations associated with SP resistance. The *P. falciparum* isolates were found to carry the mutant type N51I with a prevalence of 94% while C59R and S108N had 92% each. The prevalence of mutation at *Pf dhps* codons A437G and K540E stood at 94% and 91% respectively. **Conclusion:** The present study observed that there is no statistical significant on codon 51I and 437G ($\chi^2 = 3.3099$ df=1 $p > 0.05$) change in the proportion of resistant genotypes. However, there was a statistical significant on codon 59R and 108N ($\chi^2 = 4.338$ df=1 $p < 0.05$) and 540E ($\chi^2 = 5.391$ df=1 $p < 0.05$) indicating a slow but steady decreased resistance despite its withdrawal. In addition, the evidence of quintuple mutations that are likely to become fixed in the study population is threatening the future of SP especially in intermittent preventive treatment prophylaxis (IPTp) programs.

Background

Malaria continues to play a major threat to mankind globally and is a leading parasitic disease with 3.2 billion at risk with 247 million being infected and approximately a million death mostly in children under five [1, 2]. In the year 2013, it was estimated that 198 million cases of malaria occurred with 540,000 preventable deaths [3, 4]. The most affected continent is Africa where almost 90% of death occurs every year with approximately 78% of the most defenseless group being children below five years and expectant mothers [5]. In sub-Saharan Africa (SSA), *Plasmodium falciparum* infection is the main source of morbidity and mortality leading to a massive economic liability translating to 88% of all the diseases. However, resistance to antimalarial drugs continues to be one of the major hindrances for the fight against the disease globally. In Kenya, chloroquine-resistant *P. falciparum* was reported from in 1977 and by 1998 resistant levels had reached 70% prompting the Kenyan Ministry of Health to change the first line of treatment from chloroquine to sulfadoxine-pyrimethamine and later in 2004, Kenya officially changed the first line drug to artemether/lumefantrine (Coartem TM).

After the emergence of chloroquine resistance to *P. falciparum*, sulfadoxine-pyrimethamine (SP) was accepted as a drug of choice for malaria infection in many SSA countries including Kenya [7]. In addition, SP was given to pregnant mothers and children as a prophylaxis strategy to prevent malaria in malaria-endemic areas [7, 27]. Also, SP was recommended for use as a combination therapy with artemisinin derivatives [8].

However, soon after its introduction, resistance to SP gradually emerged and spread widely from Asia to Africa where it was replaced with artemisinin-based combination therapy (ACT).

Molecular studies have observed that mutations of parasites population is responsible for the antimalarial drug resistance globally. For example, several studies on antifolates resistant have implicated point mutation on *Plasmodium falciparum* dihydrofolate reductase (*Pf dhfr*) and *Plasmodium falciparum* dihydropteroate synthetase (*Pf dhps*) genes encoding for proteins involved in the folate biosynthesis pathway (Jacques-Mari et al., 2016). The antifolate pyrimethamine (PY) resistance is conferred through a key mutation with a change from serine to asparagine at amino acid position S108N in the *Pf dhfr* gene, while additional mutations at position N51I and C59R increase the levels of resistance [7]. In addition, the 164L mutation common in Southeast Asia has been shown to confer PY resistance. Similarly, a change in the amino acid at codon 437 on *Pf dhps* enzyme has shown to be the key determinant in mutation associated with sulfadoxine resistance while changes at position 540, 581, 613 and 436 appear to modulate the increasing level of resistance [6, 7].

In Kenya, during the year 2006, more than 95% of *P. falciparum* isolates carried *Pf dhfr* mutant alleles, a proportion that was already around 80% in the 1990s [7]. Therefore, following the array of mutations, approximating the single nucleotide polymorphism (SNP) level and assessing linkage among the SNPs in the population is useful in understanding the evolution of a particular gene. Hence, to achieve the goal of malaria elimination, molecular data on anti-malarial drug resistance with wide coverage in Kenya, particularly in highly endemic regions, is needed for proper implementation of antimalarial drug treatment policy. To achieve this objective, the prevalence of point mutations involving the antimalarial resistant genes *pf dhfr* and *pf dhps* for SP was analyzed from children with uncomplicated malaria infection in western Kenya.

Methods

Study site

This study was carried out in Chulaimbo Sub District Hospital in Kisumu County, Kenya.. Basing on the 2015 Kenya Malaria Indicator Survey, Kisumu is a malaria-endemic zone with stable *P.falciparum* transmission accounting for 38% of all outpatient visits and 40% of all admissions mainly children below five years and expectant mothers.

Study population

The study population consisted of children aged between 6 to 60 months. Children visiting the outpatient clinic at the hospital were enrolled in the study based on the following criteria; consent, monoinfection of *P. falciparum* with parasitemia between 1000-200,000 parasites per/ μ L of blood, axillary temperature $\geq 37.5^{\circ}\text{C}$ or with a history of fever with no history of anti-malarial drug intake during the previous week.

Study design

This was a cohort study [14, 15]

Sample size

The sample size was determined as described by Langa and Lemeshow (1991) Therefore the minimum sample size was 90. However, only 76 children whose guardian consented to complete follow up to 28th day after treatment with artemether-lumefantrine sampled.

Sample collection

All the children whose mother consented to participate in the study were screen for malaria by collecting blood from a finger prick. Thick and thin smears prepared and stained using Giemsa stain and examined under the microscope. In addition, blood film for malaria parasites was prepared to determine parasite density. Lastly, pre-treatment (day zero) blood samples were collected as dried blood spots on 3MM Whatman filter papers, packaged as an individual into zip lock bags with a desiccant and transported to Kenya Medical Research Institute where confirmation of species by Polymerase Chain Reaction (PCR) and genotyping analysis was done.

DNA extraction and amplification of *Pfdhfr* and *Pfdhps* genes

An aliquot amount of DNA extraction was processed from dried blood spot as described [16]. The amplification of the *Pfdhfr* and *Pfdhps* genes were analyzed using MJ Proflex PCR System. As previously described [17] with few modifications as shown in Table 1.

Restrictive fragment length polymorphism digest of the *Pfdhfr* gene

The restrictive fragment length polymorphism (RFLP) was done as previously described (Ranford-Cartwright *et al.*, 2002) using restrictive endonuclease *Tsp5091* (New England Biolabs, Beverly MA) for codons asn51ile of *Pfdhfr*. Each sample was mixed with 2 µL of ABgene™ 6x loading dye and loaded onto a 1.5% agarose gel in TAE buffer (the dye consist of: 15% (w/v) Ficoll R400, 0.06% (w/v) Xylene cyanol FF 30 mM EDTA). The gel was subsequently run for 35 minutes at a voltage of 80 volts on gel electrophoresis tank from Biorad and was viewed on the gel/photo-documentation system for analysis of the results.

***Pfdhps* gene amplification and digest**

Processed as above using specific primers and digestive enzymes; Ava II and Fok I (New England Biolabs, Beverly) for *Pfdhps* 437 and 540 respectively following procedure described above. Lastly, approximately 1.5% agarose gel was prepared and the products run in the presence of 100bp molecular ladder for 45 minutes. The products were then viewed on a gel documentation system. The primers, primers sequences, pair's fragments sizes and restriction enzymes are shown in Table 1.

Pf18sRNA gene amplification

The amplification of the 18s RNA gene was done to confirm whether the samples were from *Plasmodium falciparum* species.

Results

Demographic characteristics

The mean age and standard deviation were 32 months and SD $\pm$ 11.232 while the weight mean in Kg and the standard deviation was 14.07 and $\pm$ 2.970 respectively. The minimum and maximum age and weight were 12 and 58 months, 8 and 20 respectively. Of the 76 sample, males were 43 (56.6%) while 33 (43.4%) were females.

Study population characteristics

The geometric mean of microscopic parasite count on day 0 (before treatment) was 120,595 parasites/ μ L while the standard deviation was 163,395.1 (95% CI: 82319.4-15887.89). On day 1, the parasite clearance rate had a mean and standard deviation of 3508.93 and 11,783 respectively (95% CI: 797.78-6220.67), on day 2, the parasite clearance had a mean and standard deviation of 24 and 170.85 (95% CI -15-63.31) while on day 3, there were no parasites up to day 28 among all the children.

Analysis of *Pfdhfr*

The DNA amplification from the 76 samples was analyzed for SP resistant based on the presence of codon 51, 59 and 108 in *pfdhfr*. This amplification was successful giving bands of 560 bp, 260pb, and 700bp respectively. Similarly, amplification of the codon 437 and 540 mutations in *pfdhps* genes gave bands of 438bp and 161bp respectively. Out of the 76 samples, 5(6%) were classified as wild-type (SP sensitive) and 71(94%) as mutant meaning they carried the allele that conferred resistance to SP as shown in Figure 1. The codon 108 was subjected to RLFP using the enzyme Alu 1. Here, the control used was 3D7 as the wild-type control and DD2 as the mutant control. After the digestion, the wild-type was restricted to two fragments of 118bp and 180bp while the mutant control did not cut, appearing just like the uncut product. It's on this basis that the samples were categorized as either wild-type or mutant. Of the 76 samples, 6(8%) and 70 (92%) were classified as wild-type and mutant respectively (fig1). There is no statistical significant on codon 51(χ^2 =3.3099 df=1 p >0.05) change in the proportion of resistant genotypes. However, there was a statistical significant in the resistant genotypes on codon codon 108 (χ^2 =4.338 df=1 p<0.05).

Analysis of *Pfdhps*

The different mutant genotypes related to *dhfps* were identified using RFLP for codons 437 and 540. For codon 437, the enzyme used was Ava II and wild type control used was 3D7 while the mutant control was HB3. After digestion, the wild-type control gave a fragment of 438bp while the wild-type gave a fragment of 404bp. Out of the 76 samples, 5(6%) were classified as wild-type and 71(94%) as mutants as described in Figure 1. There was also no statistical significant on codon 437 (χ^2 =3.3099 df=1 p >0.05) change in the proportion of resistant genotypes. Similarly, for the 540 codons, the enzyme used for digestion was FokI with IEC513/86 and 3D7 being used as the wild-type and the mutant control respectively. The wild-type

control gave a fragment of 105bp and 138bp after restrictive digestion. Of the samples digested, 7(9%) were classified as wild-type and 69 (91%) as mutants as explained in Figure 1. However, there was a statistical significant on codon 540 ($\chi^2 = 5.391$ df=1 $p < 0.05$).

Discussion

Towards the turn of the Millennium, the prevalence of CQ resistant in Kenya was 100% leading to its replacement with SP against uncomplicated malaria in 1998 [1, 10]. Several years after the introduction of SP as the first-line antimalarial drug in Kenya, mutant's genotypes continue to increase in frequency. In this study, we investigated the prevalence of mutations at codon N51I, C59R and S108N of the *pfdhfr* gene and codon A437G and K540E of the *Pfdhps* gene, as major determinants for SP resistance. As shown in Figure 1, the respective codon values for *pfdhfr* and *pfdhps* are 94%, 92%, 92%, and 94% and 91%, a level indicating the persistent of these mutations in parasites circulating in endemic areas of western Kenya.

This finding is consistent with those of previous in vitro studies in western Kenya [10, 18], in Tanzania [20] and West Africa [27, 28] in which high prevalence of these mutations were reported. In contrast to our results, studies in India, Sri Lanka and Papua New Guinea [22] observed lower levels of the mutations, indicating a decrease in CQ resistance in these countries. The high frequencies of *pfdhfr* and *Pfdhps* we observed reflects the gradual increase in falciparum resistance to SP since the withdrawal of SP in Kenya. These data combined with past results from western Kenya, suggest that at the moment reintroduction of chloroquine therapy as an alternative to SP is not a viable option for this highly endemic region.

In Uganda for example, a study among expectant mothers on SP-IPTp demonstrated that the prevalence of single-nucleotide mutation in *Pfdhfr* at codon 51I, 59R, and 108N and in *Pfdhps* at codon 437G and 540E was 98% reaching 100% fixation after one dose of SP. This high prevalence was also reported in Cameroon where *Pfdhfr* triple mutant allele reached 100% fixation [27]. This observation is similar to our finding of over 90% prevalence rate of the quintuple mutations and with time gravitating towards 100% prevalence. However, observation from Gabon [28] observed 92% of the triple mutants while Senegal showed lower prevalence with 82.3% isolate of the *Pfdhfr* triple mutant with 40.4% of the *Pfdhps* double mutants [29].

The high prevalence of SP resistance *P. falciparum* parasites in our population mirrors results from other studies using samples from this location [10] and could partly due to SP use in IPTp as the pyrimethamine component of the drug selects for appropriate drug-tolerant variants. However, parasite proportions already bearing the resistance genotype before its introduction absolved SP use alone as the primary driver for the high mutant frequencies. Selection pressure could possibly have been enhanced by a similar-acting antifolate combination drug notably cotrimoxazole [10, 20, 21].

The use of SP-IPTp on a number of studies does not prevent malaria infection during pregnancy, especially in geographical areas with high levels of SP-resistance markers [23, 24]. However, during pregnancy's aftermaths, there is protection against complications such as low birth weight, maternal and neonatal mortality [25]. This has been observed especially when more than two doses of IPTp are administered [25]. This significant observation led to WHO's recommendation for SP-IPTp at any level of quintuple mutation

[25, 26]. Thus, aside from the WHO recommendation of more than two doses of SP-IPTp, the high prevalence of resistance markers observed in the current study and elsewhere in East Africa calls for careful and continuous evaluation of SP-IPTp efficacy and on the usefulness of SP in artemisinin combinations [30]. In the current study shows a slight decrease of *Pf dhps* SP resistance of this mutant at 100% in 2004 when SP replaced with artemether-lumefantrine to 91-94% among the five codons studied in the current study. In northern Tanzania, it is possible that cross-border spread of resistance contributes to these observations between Uganda and Kenya.

Conclusions

Our finding of a high prevalence of *Pf dhfr* and *Pf dhps* mutations that confer resistance to SP would provide a rationale of continued withdrawal of SP from this highly endemic region in Kenya. Of more value would be continuous molecular assays for the presence of *Pf dhfr* and *Pf dhps* mutations and regular surveillance to help monitor the level of resistance. The results could also help in recommending the first-line treatment of malaria in line the WHO recommendations.

Abbreviations

CQ	Chloroquine
SP	Sulfadoxine pyrimethamine
ACT	Artemisinin combination therapy
PCR	Polymerase chain reaction
RFLP	Restriction fragment length polymorphism
<i>Pf dhfr</i>	<i>Plasmodium falciparum dihydrofolate reductase</i>
<i>Pf dhps</i>	<i>Plasmodium falciparum dihydropteroate synthetase</i>
ITPp	Intermittent treatment preventive of malaria in pregnancy
ITPi	Intermittent treatment preventive of malaria in infant
SNP	Single nucleotide polymorphism
EDTA	Ethylene diamine tetra acetic acid
bp	base pair
WHO	World Health Organization
KEMRI	Kenya Medical Research Institute

Declarations

Authors' contributions

GK designed the study, collected samples from the field, analyzed data and drafted the manuscript. EN, GO, FK KT, DM and GK critically reviewed and revised the manuscript for important intellectual content. All authors read and approved the final manuscript.

Acknowledgement

My appreciation is to all the patients and their mothers for participating in the study, KEMRI management for allowing me to analyze the samples in the Laboratories especially malaria laboratory staffs.

Competing interest

The authors declare that they have no competing interests.

Availability of data and materials

The data from the current study is available from the corresponding author on reasonable request.

Funding statement

Funding was partly self-sponsored and partly sponsored and by National Council of Science Technology and Innovation (NACOSTI) Ref no. 5th call 2013/2014

Ethical considerations

The study was approved by the Scientific and Ethical Unit of the Kenya Medical Research Institute.

Consent to Participate

Consent was obtained from mothers of the participating children and also agreed that the results can be published without the names of the children appearing anywhere.

Consent to publish

Consent to publish the results was given by the mothers on condition the children names should not appear.

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Tables

Table 1: Primers, primers sequences, pairs fragments sizes and restriction enzymes used in detection of gene polymorphisms in *Pfdhfr* and *Pfdhps*.

Gene	Primers	Primers sequences	Size (bp)	Restriction enzyme	Fragment length (wild type)	Fragment length (mutant)
Pfdhfr (outer)	F M4	5'-GAATGTAATTCCCTAGATATGGAATATT-3' 5'-TTAATTTCCCAAGTAAACTATTAGAGCTTC-3'	326	AluI	189, 166	No digestion
(nested)	M3 F1	TTAATTTCCCAAGTAAACTATTAGAGCTTC 5'-AAATTCTTGATAAAACAACGGAACCTTTTA-3'	522	Tsp5091	55, 65, 120, 153	55 65, 120, 218
Pfdhps (outer)	R/ R2	5'AATTGTGTGATTTGTCCACAA-3' 5'-AACCTAAACGTGCTGTTCAA-3'	438	Fok I	438	33, 85, 320
(nested)	K K/	5'TGCTAGTGTTATAGATATAGGATGAGCATC-3' 5'-CTATAACGAGGTATTGCATTTAATGCAAGAA-3'	438	Ava II	438	405

Figures

Figure 1

Prevalence of Pfdhfr and Pfdhps wild type and mutant genotypes generated from the study. The *P. falciparum* isolates in the study population were found to carry the Pfdhfr mutant type N51I with a

prevalence of 94% while C59R and S108N had 92% each. The prevalence of mutation at Pfdhps codons A437G and K540E stood at 94% and 91% respectively.