Research Article

Molecular Monitoring of *Plasmodium falciparum* Resistance to Sulfadoxine-pyrimethamine in Western Kenya, 14 Years after its Withdrawal

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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 with SP being recommended for intermittent preventive in pregnancy (IPTp). Several mutations of *Plasmodium falciparum* have been implicated in SP resistance but it is unclear if the prevalence of these mutations is decreasing or increasing after it was restricted to expectant mothers. This study was design to assess the current status *pfdhfr* and *pfdhps* gene mutation which encodes enzymes targeting SP. **Method:** Blood from a finger prick was collected onto 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 pfdhr and 437 and 540 of *pfdhps* genes using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technology. **Results:** All the 76 *P. falciparum* isolates were successfully genotyped for the detection of *Pfdhfr* and *Pfdhps* 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 *Pfdhps* 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 (χ^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 (χ^2 =4.338 df=1 p<0.05) and 540E (χ^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 in the study population is threatening the future of SP especially in intermittent preventive treatment prophylaxis (IPTp) programs and also future combination with another antimalarial drug. These findings highlight the need for continual monitoring of parasites genotypes as indicators of therapeutic efficacy of antimalarial.

Keywords: Sulfadoxine-pyrimethamine; Drug resistance; *Plasmodium falciparum*; *pfdhfr*; *pfdhps*

Introduction

Worldwide approximately 219 million cases occurred in 2017 compared with 239 million in 2010 with African region bearing the greatest burden of malaria morbidity with approximately 200 million cases (92%) in 2017, followed by WHO South-East Asia Region (5%) and the WHO Eastern Mediterranean Region (2%). [1] This slight reduction suggesting that progress of the fight against malaria had generally stalled. Worldwide, 3.4% of all the estimated cases of malaria infection were caused by *P. vivax* with 56% of the vivax cases being in the WHO South-East Region. *Plasmodium vivax* is the predominant parasite in the WHO Region of the Americas (74%), and is responsible for 37% of the cases in the WHO South-East Asia Region and 31% in the Eastern Mediterranean Region. [1] Of great concern, about 80% of all malaria cases globally were in 15 African countries and India with 50% in Nigeria, DRC 11%, Mozambique and Uganda 4% (WHO, 2018). However, 266,000 (61%) deaths were children under 5 years globally with Africa region accounting for 93% in 2017. [1]

Through concerted global fight against malaria infection, there have been a decline of deaths from 607 000 to 435 000 cases while estimates of malaria mortality rate (deaths per 100 000 population at risk) show that most countries had recorded reductions by 2017, except the WHO Region of the Americas, mainly due to a rapid increase in malaria in Venezuela. [1]

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However, Africa accounted for 93% of all deaths with 88% of the 172,000 cases reported in 2017. [1] Although many countries continue to reduce the malaria burden, the rate of change has slowed in the highest-burden countries with some indicating a rise in malaria cases. Majority of these countries are in sub-Saharan Africa (Burkina Faso, Cameroon, DRC, Ghana, Mali, Mozambique, Niger, Nigeria, Uganda, and Tanzania) and account for more than 70% of the global malaria cases and deaths. [1] However, a number of countries are progressing on malaria elimination. In 2017, United Arab Emirates, Armenia Turkmenistan, and Morocco attained zero indigenous cases for 3 years. In 2018, Paraguay was awarded WHO certification of elimination of malaria while Algeria formally requested WHO certification of malaria-free status in 2017 and Argentina continues to work towards certification. [1]

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 with children under five and expectant mothers bearing the greatest burden. [2,3] Malaria during pregnancy has been associated with fetal growth retardation, pre-term birth, low birth weight, increased perinatal mortality and maternal anaemia. [3] In Kenya, chloroquine-resistant *P. falciparum* was reported 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). [2]

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. [4] In addition, SP was given to pregnant mothers and children as a prophylaxis strategy to prevent malaria in malaria-endemic areas and was recommended for use as a combination therapy with artemisinin derivatives. [3,5] 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). Reports of resistance to the currently recommended artemisinin-based combination therapy are consequently of major concern and highlight the importance of the evolution of drug resistance. [5] Therefore, effective surveillance of malaria cases and deaths is essential for identifying the areas or population groups that are most affected by malaria and for targeting resources for maximum impact.

Molecular studies have observed that mutations of parasites population are responsible for the antimalarial drug resistance globally. For example, several studies on antifolates resistant have implicated point mutation on *Plasmodium falciparum* dihydrofolate reductase (*Pfdhfr*) and *Plasmodium falciparum* dihydropteroate synthetase (*Pfdhps*) genes encoding for proteins involved in the folate biosynthesis pathway. [4] 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 *Pfdhfr* gene, while additional mutations at position N51I and C59R increase the levels of resistance. [4] 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 *Pfdhps* 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. [3,4]

In Kenya, during the year 2006, more than 95% of *P. falciparum* isolates carried *Pfdhfr* mutant alleles, a proportion that was already around 80% in the 1990s. [2] 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 *pfdhfr* and *pfdhps* for SP was analyzed from children with uncomplicated malaria infection in western Kenya.

Methods

Study site

This study was carried out in the lake region of Kisumu constituency in Chulaimbo Sub District Hospital. 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. *Plasmodium falciparum* is transmitted by two vectors namely Anopheles gambiae and Anopheles funestus. Though these vectors exist throughout the year, they peak between long and short rains in the months of April and June as well as October and November respectively.

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, mono infection of *P. falciparum* with parasitemia between 1000-200,000 parasites per/ µL of blood, axillary temperature ≥ 37.5°C or with a history of fever with no history of anti-malarial drug intake during the previous week. The subjects were excluded from the study they had taken drugs within 24 hours, patient relocation from study site and withdrawal from the study by the guardian.

Study design

This was a cohort study. [6]

Sample size

$n = Z^2 p(1-P)/ e^2$. [7]

Where;

Z = standard normal deviation of the required confidence.

n = the desired sample size

p = Proportion in the target population estimated to have characteristic being measured. According to KMIS, 2015 the prevalence of malaria stands at 40% or 0.4.

e = the level of statistical significance set.

Therefore substituting Z for 1.96, 0.38 for P and 0.1 for e, then the minimum sample size will be

$1.962 \times 0.38 (1 - 0.68) / 0.12 = 90$

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 3 mM 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 of *Pfdhfr* and *Pfdhps* genes

An aliquot amount of DNA extraction was processed from dried blood spot as described. [8] Briefly, each dried filter paper was cut into small pieces and soaked in Saponin-phosphate buffered saline (PBS) overnight at 4°C. This was followed by washing with 1x PBS and incubated for 30 minutes. The brown solution from the tube was discarded and 50 microliters of the stock 20% solution and 150 microliters of DNAse free water were added followed by vigorous vortexing. The tubes were then heated at 100°C and centrifuged at 10,000 g for two minutes. Lastly, the supernatant was transferred to a new tube, spun again and a final transfer was done. The DNA product was then stored at -20°C.

Amplification of *pfdhfr*

The amplification of the *Pfdhfr* genes were analyzed using GeneAmp® PCR system 9700 machine. The PCR amplification was done with few modifications. Outer PCR: each reaction tube consisted 10x PCR buffer (Roche®) to a final concentration of 1x, 25 mM $MgCl_2$ to a final concentration of 1.5 mM, 20 mM dNTP mix to a final concentration of 400 µM, 10 µM primers each of *Pfdhfr* F (5'-GAA TGT AAT TCC CTA GAT ATG GAA TAT T-3') (Inqaba) and M4 reverse (5'-TTA ATT TCC CAA GTA AAA CTA TTA GAG CTT C-3') (inqaba) to a final concentration of 100 nM, 5U/ µL Taq polymerase (Bioline) to a final concentration of 1.0 U of enzyme/reaction tube and 1 µL of the DNA template. The mixture was topped up to a volume of 30 µL with DNAse water. PCR programme was set at 95°C for 5 minutes, 92°C for 30 seconds, 45°C for 45 seconds, 72°C for 45 seconds, 45 cycles, final extension at 72°C for 3 minutes then held at 4°C. In the outer PCR, the primers M4-F was used to amplify the region consisting of 326 base pairs containing cys59arg and ser108thr.

Nested PCR: each reaction tube consisted of 0.2 µL of outer PCR product thawed on ice, 10x PCR buffer (Roche®) to a final concentration of 1x, 25 mM $MgCl_2$ to a final concentration of 1.5 mM, 20 mM dNTP mix to a final concentration of 400 µM, 10 µM primers each of *Pfdhfr* F1 (AAA TTC TTG ATA AAA CAA CGG AAC CTT TTA)(inqaba) and *Pfdhfr* M3 (TTA ATT TCC CAA GTA AAA CTA TTA GAG CTT C)(inqaba) forward and reverse respectively each to a final concentration of 100nM, 5U/ µL Taq polymerase (Bioline) to a final concentration of 2.5 U/reaction tube. The mixture was topped up to a volume of 30 µL with nuclease-free PCR water. The PCR programme was set at 95°C for 5 minutes, 92°C for 30 seconds, 45°C for 30 seconds, 72°C for 45 seconds, 45 cycles, final extension at 72°C for 3 minutes then held at 4°C. The first set of nested reaction used the primers F-M4 (forward and reverse respectively) amplifying the region of 326 base pairs containing Cys 59 arg and ser 108 thr while the second set of nested reaction also used primers M3-F1 was used to amplify the region consisting of 522 base pairs containing asn51ile and ser108ile. The primers, primers sequences, pair's fragments sizes, and restriction enzymes are shown in Table 1.

Restrictive fragment length polymorphism digests of the *Pfdhfr* gene

The restrictive fragment length polymorphism (RFLP) was done as previously described [9] using restrictive endonuclease Tsp5091 (New England Biolabs, Beverly MA) for codons asn51ile of *Pfdhfr*. Briefly, in 15 µL, 1.5 µL of 10x buffer 3, 0.15 µL of bovine serum antigen, 8 µL of amplified DNA, 0.5 µL of Tsp5091 restrictive endonuclease was added to 9.85 µL of nuclease-free PCR water. This was followed by incubation for 14 hours at 50oC with no agitation. Each sample was mixed with 2 µL of ABgeneTM 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

For the outer PCR reaction, the amplification of the *Pfdhps* gene was done using R/ and R2 (Inqaba) as forward and reverse primers respectively. Each tube consisted 10x PCR buffer (Roche ®) to an absolute concentration of 1x, 25 mM $MgCl_2$ to an absolute concentration of 1.5 mM, 20 mM dNTP mix to a final concentration of 200 µM, 100nM primers each of *Pfdhps* R/R2 (5'AATTGTGTGATTTGTCCACAA-3') and R2 reverse (5'-AACCTAAACGTGCTGTTCAA-3') (inqaba) to a final concentration of 100nM, 5 U/ µL Taq polymerase (Bioline) to a final concentration of 2.5 U/reaction tube and 3 µL of the DNA template. The mixture was topped up to a volume of 30 µL with DNAse water. The samples were then loaded onto the thermocycler set at 95°C for initial denaturation for 3 minutes.

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 59	F M4	TTAATTTCCCAAGTAAAACTATTAGAGCTTC 5'-AAATTCTTGATAAAACAACGGAACCTTTTA-3'	326	AluI	189, 166	No digestion
Pfdhfr (Nested) 51	M3 F1	5'-GAATGTAATTCCCTAGATATGGAATATT-3' 5-'TTAATTTCCCAAGTAAAACTATTAGAGCTTC-3'	522	Tsp5091	55, 65, 120, 153	55 65, 120, 218
Pfdhps (outer) 437	R/ R2	5'AATTGTGTGATTTGTCCACAA-3' 5'-AACCTAAACGTGCTGTTCAA-3'	438	Fok I	438	33, 85, 320
(nested) 540	K K/	5'TGCTAGTGTTATAGATATAGGATGAGCATC-3' 5'-CTATAACGAGGTATTGCATTTAATGCAAGAA-3'	438	Ava II	438	405

This was followed by denaturation at 92°C for 30 seconds, annealing temperature of 50°C for 45 seconds, 72°C extension for 1 minute. These conditions were repeated for 30 cycles followed by a final extension for 3 minutes at 72°C, then halted at 4°C.

Nested PCR was done using primers K and K/ (Inqaba) as forward and reverse primers respectively. Each reaction tube consisted of 10x PCR buffer to an absolute concentration of 1x, 25 mM magnesium chloride to an absolute concentration of 1.5 mM, 20 mM dNTPs to an absolute concentration of 200 µM, 10 µM each of the nested primers K (5'-TGCTAGTGTTATAGATATAGGATGAGCATC-3') and K/ (5'-CTATAACGAGGTATTGCATTTAATGCAAGAA-3') to a final concentration of 100 nM and 5 U/ µL Taq polymerase (Roche) to a final concentration of 2.5 U/reaction tube. In addition, 0.2 µL of the amplified DNA samples thawed on the ice were transferred to each tube. The mixture was topped up to a volume of 60 µL with DNAse free PCR water. PCR was then run with the initial denaturation being set at 94°C for 3 minutes, followed by a denaturation temperature of 94°C for 30 seconds, the annealing temperature of 45°C for 1 minute and extension at 72°C for 1 minute. The steps were repeated for 40 cycles and then followed by a final extension at 72°C for 3 minutes before halting the reaction at 4°C. The nested PCR products (K-K/ and R/-R2) were digested using Ava II and Fok I (New England Biolabs, Beverly) for *Pfdhps* 437 and 540 respectively following the procedure described above. The primers, primers sequences, pair's fragments sizes, and restriction enzymes are shown in Table 1.

Restrictive Fragment Length Polymorphism of *Pfdhps*

The products of amplification of *Pfdhps* were subjected to enzyme digestion to detect which of these samples had mutations. The digestion was done according to the instructions of the enzymes supplier. For the target ala437gly, enzyme Ava II was used with the *Plasmodium falciparum* wild- type strains 3D7 and HB3 acting as a control. For this reaction, 1.5 µL of the NEB buffer, 0.4 µL of the restricted enzyme Ava II, 1.7 µL of nuclease-free water and 11 µL of the PCR product were used. The product was then incubated at 37°C for 24 hours followed by another incubation at 80°C for 20 minutes. A 1.5% agarose gel at 80 volts for 30 minutes was prepared and the products run in the presence of a 100 bp molecular ladder for 45 minutes. The products were then viewed on a gel documentation system for analysis of the results.

Similarly, digestion was done for the target lys540glu with Fok I acting as the restrictive enzyme. The reaction was carried out according to manufactures instructions with IEC51/86 and 3D7 being used as controls. The digestion was done for a reaction volume of 15 µL which included 1.5 µL of NEB buffer, 1.7 µL of nuclease-free water, 0.8 µL of the restriction enzyme and 11 µL of PCR product. The samples were incubated at 37°C overnight and the enzyme inactivated at 65°C for 20 minutes. A 1.5% agarose gel at 80 volts for 30 minutes was prepared and the products run in the presence of 100 bp molecular ladder for 45 minutes. The products were then viewed on a gel documentation system.

Amplification of Pf18sRNA gene

The amplification of the 18s RNA gene was done to confirm whether the samples were from *Plasmodium falciparum* species. For this amplification, the primers pairs 18R-18F were used with the base pairs (5'-CTGAGTCGAATGAACTAGCT-3') and (5'-CCATTTTACTCGCAATAACG-3') respectively. For the reaction, 1x of PCR buffer included MgCl2), 400 nM, 200 nM of primers, 1U of Taq Polymerase and 1 µL of DNA template was used. PCR was then run with the initial denaturation set at 94°C for 3 minutes, followed by denaturation at 94°C for 1 minute, annealing at 55°C for 2 minutes and extension at 72°C for 2 minutes. The final extension was set at 72°C for 10 minutes with a total of 30 cycles and finally halting the reaction at 4°C.

Results

Demographic characteristics

The mean age and standard deviation were 32 months and SD ± 11.232 while the weight mean in Kg and the standard deviation was 14.07 and ± 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/ µL 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 438 bp and 161 bp 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. 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 statistically significance in the resistant genotypes on 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 statistically significant on codon 540 (χ^2 =5.391 df=1 p<0.05).

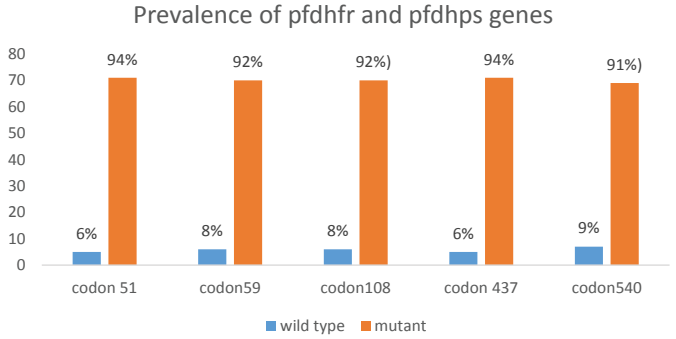

Figure 1: Prevalence of *Pfdhfr* and *Pfdhps* wild type and mutant genotypes.

Discussion

The instant and extensive increase of anti-malarial drug resistance are directly holding back and hindering the process of malaria control, prevention and elimination. [10,11] The tremendous advancement in molecular markers has allowed early detection of drug resistance and susceptibility providing fundamental for drug policy and has played an imminent role in the epidemiological survey. [10] Towards the turn of the Millennium, the prevalence of CQ resistant in Kenya was 100% leading to its replacement with SP and Amodiaquine (AQ) as the first- and second-line antimalarial treatment, respectively, against uncomplicated malaria in 1999. [2] However, clinical resistance to SP prompted the adoption of artemisinin-based combination therapy (ACT) with Coartem ™ as the first-line regiment and SP relegated to intermittent use during pregnancy. [12] In this study, we sought to establish the current status of SP resistance in western Kenya, 14 years after it was officially banned as an antimalarial drug.

The outcome of this study demonstrates that *Pfdhfr* and *Pfdhps* polymorphism associated with sulfadoxine-pyrimethamine resistance persist at high frequency. The present study as shown in Figure 1 shows the prevalence of these mutants for *pfdhfr* stands at 94% for codon N51I, 92% each for codon C59R and S108N while *pfdhps* codon A437G and K540E accounted for 94% and 91% respectively. This observation of high frequency of triple mutant *Pfdhfr* is similar to previous studies in Kilifi and western Kenya, [12] in Tanzania, [13] West Africa, [14,15] Equatorial Guinea and Democratic Republic of Congo [10] which observed that the mutant *Pfdhfr* alleles are maintained at high frequencies more than a decade after withdrawal of sulfadoxine-pyrimethamine. In contrast to our results, studies in Iran, India, Sri Lanka, and Papua New Guinea observed lower levels of the triple mutations, suggesting a continuous use of SP as the treatment of choice for uncomplicated malaria. [15] The high frequencies of *pfdhfr* and *Pfdhps* we observed reflect the gradual increase in *P. falciparum* resistance to SP since its withdrawal in Kenya. The high prevalence of the three well-characterized mutations in *Pfdhfr* (N51I, C59R, and S108N) indicates that the *P. falciparum* isolates from the study display high resistance that needs to be addressed urgently by the policymakers. These data combined with past results from the coast and other parts of Kenya; suggest that SP is not a viable option in the near future for this highly endemic region.

In Mozambique for example, SP resistance quintuple mutation was reported to be above 78% in while triple mutant had reached 100% fixation. [13] The current study indicates quintuple mutation of above 90% demonstrating high levels in frequency pointing to a high SP resistance in the East African region as opposed to West Africa region where SP resistance based on the quintuple mutation is very low in most countries suggesting that SP-IPT is still effective in those regions. The mutations of *Pfdhfr* and

Pfdhps genes exhibit a relatively high prevalence in the current study. By contrast, the *Pfdhps* K540E has a low prevalence in West and Central Africa. For example the mutation prevalence in the countries neighboring Cameroon was 6.25% in Gabon in 2007, 0.8% in Congo in 2004, 5.2% in the Central African Republic in 2004, 11% in Sao Tome and Principe islands in 2004 and 24% in Nigeria in 2004 [14] and Equatorial Guinea 5.06%. [10]

In Uganda for example, a study among expectant mothers on SP-IPTp demonstrated that the prevalence of the 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. [16] This high prevalence was also reported in Yaoundé Cameroon where *Pfdhfr* triple mutant allele reached 100% fixation. [14] This observation is similar to our findings indicating over 90% prevalence rate of the quintuple mutations and with time gravitating towards 100% prevalence. However, observation from Gabon [15] showed 92% of the triple mutants while Senegal has a slightly lower prevalence with 82.3% isolate of the *Pfdhfr* triple mutant with 40.4% of the *Pfdhps* double mutants. [17,18]

The high prevalence of SP resistance *P. falciparum* parasites in our population mirrors results from other studies using samples from this location and could partly due to SP use in IPTp as the pyrimethamine component of the drug selects for appropriate drug-tolerant variants. [12] 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. [12,19,20] This drug possess only mild antimalarial potency but is a common prescription against opportunistic respiratory tract infections among HIV patients. However, there is need to continually monitor expectant mothers and pediatrics cases which are potential source of amplification and dissemination of parasites bearing this allele due to their predisposition to IPT.

In East Africa high levels of this haplotype are likely to compromise the importance of SP-IPTp. [13] Several studies have shown that although implementation of SP-IPTp does not prevent malaria infection during pregnancy, especially in the presence of high of SP-resistance markers, there is a significant protection against severe outcome of pregnancy in malaria such as low birth weight, maternal and neonatal mortality, especially when two or more doses of IPTp are administered. [13] This significant observation led to WHO's recommendation for SP-IPTp at any level of quintuple mutation. [1] 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 future artemisinin combinations. [21] The current study shows a slight decrease of *Pfdhps* SP resistance of this mutant at 100% in 2004 when SP replaced with artemether-lumefantrine to 91-94% among the five codons studied. In northern Tanzania, it is possible that cross-border spread of resistance contributes to these observations between Uganda and Kenya.

Conversely, due to continued use of SP for IPTp, SP is readily available in both public and private sector making its restriction for only IPTp impossible. In the current situation it is unlikely that self-medication with SP can be prevented especially due to its low cost compared to ACT, which may also explain the observed high prevalence of SP resistance markers despite its replacement with ACT. Furthermore, it is expected as the quintuple mutation continues to rise towards fixation, the *Pfdhps* 581G mutation considered to confer SP super-resistance when in combination with 540E will continue to rise. Therefore, it's important for the policy makers to consider restricting SP to IPTp only, through restricting its general prescriptions. However, the present study provides an update on the prevalence of mutation conferring SP resistance in Kenya. Despite the small sample size, a single-center study and limited funding, these findings calls for continued effort for studies in the wider part of the region to prevent the spread of highly resistance parasites.

Conclusion

In this study, prevalence of SP resistance based on quintuple mutations in Kenya is high, approaching fixation levels. This trend has been observed in other parts of East Africa and the continued use of SP may lead to poor SP-IPTp outcome despite continued recommendation by the WHO. Therefore, there is need for a search for alternative drugs for IPTp in East Africa. Also, the observation has shown that molecular markers is fundamentals for surveillance in malaria control program in order to prolonged the lifespan of antimalarial drug therapy in Africa.

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.

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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.

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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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