Epidemiology International
Volume 4, Issue 1 - 2019, Pg. No. 20-25
Peer Reviewed & Open Access Journal

Research Article

Title

Authors Name

Afiliation: (Post, Department, Insitiute Name, City, State, Country

I N F O

Corresponding Author:

E-mail Id:

Orcid Id:

How to cite this article:

Date of Submission: 0000-00-00
Date of Acceptance: 0000-00-00

A B S T R A C T

Background: Malaria persists to be one of the major significant diseases in the world. A range of antimalarial drugs are readily accessible but management of the disease remains a problem. Despite the broadened spread of resistance to Sulfadoxine Pyrimethamine (SP), it still remains the suggested drug to treat and prevent malaria in expecting women and children below five years. This study sought to assess the current trend of SP resistance markers a decade after it was withdrawn as the first-line anti-malarial in Msambweni, Kwale County, Kenya.

Materials and Methods: Smear-positive samples (N=134) collected from June 2013 cross-sectional study amid infants visiting Msambweni District Hospital were evaluated for mutations in *dhfr* and *dhps genes*. Extraction of DNA was done using Chelex method followed by PCR amplification of *dhfr* and *dhps* genes. Specific enzymes were used to cleave the successfully amplified DNA to establish the samples as either mutated or wild type.

Results: Pfdhps/pfdhfr A437G/K540E/N51I/C59R/S108N quintuple mutant linked with SP-resistance did not change significantly (p=0.967).

Conclusion: This survey proves fixation of key mutations in the *Pfdhfr* and *Pfdhps* genes conferring resistance to SP. Further research involving more samples and endemic sites need to be conducted to endow the stakeholders with information on the emergence and increase of SP resistance.

Keywords: Malaria, *Plasmodium falciparum*, Sulfadoxine Pyrimethamine Resistance

Introduction

A high percentage of the populace living in malaria endemic areas world-over experiences numerous malaria-related problems that affect their well-being. The disease that accounts for more than 67% of global malaria fatalities poses greater danger to pregnant women and children under five years. Nearly 92% of the cases of malaria reported globally occur in the continent of Africa.[1] In Kenya, the disease remains a major illness that leads to death, with an estimated 70% of the population at risk of getting the disease.[2] Various intervention measures have been employed to combat this life-threatening disease. These have had substantial gains as the proportion of the population in danger of contracting malaria has decreased by 41% globally between 2000 and 2015 and by 21% between 2010 and 2015 globally.[1] Therefore, prevalence of infection with malaria parasite in Sub-Saharan Africa has declined from 17% in 2010 to 13% in 2015.[3] Despite the tremendous progress made in combating the disease, malaria is still a leading cause of children mortality in malaria-endemic zones worldwide.[1,4] Proper diagnosis of the disease coupled with the timeliness in treatment with effective drugs is core to the fight against the disease.[2,5]

Molecular markers have been used by researchers to determine parasite resistance to antimalarial drugs. Molecular markers are mostly used to categorize an exact sequence of DNA in a pool of unidentified DNA in that they are linked to a specific region within the genome.[6] For instance, mutations in the parasite genes that code for *Dihydrofolate reductase (Dhfr)* and *Dihydropteroate synthase (Dhps)* have since been connected with SP resistance since they cause total treatment failure.[7] Other markers that are responsible for resistance to malaria drugs include *P. falciparum* Chloroquine resistance transporter gene (*Pfcrt*) for Chloroquine (CQ) and *P. falciparum* multi-drug resistance 1 (*Pfmdr-1*) for CQ as well as other anti-malarial drugs.[8] Extensive resistance of *P. falciparum* to Chloroquine in Kenya led to its discontinuation and replacement with the antifolates combination, Sulfadoxine-Pyrimethamine (SP), as the preferred regimen in 1999.[9]

The molecular markers allied with SP resistance are *P. falciparum* dihydrofolate reductase (*pfdhfr)* and *P. falciparum* dihydropteroate synthase (*pfdhps*).[10] The regions flanking mutations to SP are *dhfr* codons N51I, C59R and S108N and *dhps* at points A437G and K540E. Sulfadoxine Pyrimethamine works by inhibiting parasite *dhfr* and *dhps* enzymes involved in folate biosynthesis. Sulfadoxine inhibits *dihydropteroate Synthase (dhps)* in *P. falciparum* while Pyrimethamine is a competitive inhibitor for *dhfr*.[11]

The combination of Pyrimethamine and Sulfadoxine (Fansidar®) exhibit a potentiating synergetic effect and are efficient against Chloroquine resistant strains of *P. falciparum*.[12] However, resistance to SP has led to its replacement with Artemisinin-based combination therapy as the first line regimen for *P. falciparum* malaria. Despite its ban to treat malaria, SP still stays the advised drug to treat malaria in pregnant women and infants less than five years in malaria prevalent areas.

The use of SP as Intermittent Presumptive Treatment (IPT) raises more concern as its effectiveness could be compromised due to the broadened emergence of resistance. The current study was therefore designed to determine the frequency of SP resistance markers in clinical isolates from Msambweni District Hospital of Kwale County. Data from the study will guide malaria treatment policy making. Additionally, genomic advances may be of assistance in identifying molecular markers for resistance to Artemisinin and their partner drugs.

Materials and Methods

Study Site

A molecular epidemiological study of *P. falciparum* parasite resistance markers to SP was undertaken in Msambweni, Kwale County between June 2013 and June 2014. The region is sited about 56 km south of Mombasa and lies south of Tanzania.

Msambweni lies less than 300 m above sea level. The region is known for its hot and humid climate with an average rainfall of 900mm and 1500mm annually. According to the Kenya Malaria Indicator Survey (KMIS),[4] Kwale County is a malaria prevalent region. Malaria incidences in this area mount to 40% of all outpatients' cases and 40% of all inpatient admissions.

Study Population and Sample Collection

The study used samples archived from confirmed malaria patient between the age of 6 months -10 years and residing within the Msambweni District hospital, Kwale County in 2013. Axillary temperature ≥37.5°C or with a history of fever and no history of anti-malarial drug intake in the previous week, were used to confirm presence of *P. falciparum* infection, and absence of any differential diagnosis as defined by the WHO.

Patients were excluded from the study if there was administration of any additional anti-malarial drugs, emergence of any non-malarial febrile illness that would interfere with the classification of the treatment outcome, patient relocation from the study site, and withdrawal from the study.

The study population was 300 patients whose blood specimen was obtained by finger pricking and making a 3mm smear on Whatman® filter papers. The dried specimens were packed in zip lock bags and taken to Kenya Medical Research Institute (KEMRI), Nairobi for storage and further analysis.

ISSN: 2455-7048
DOI: https://doi.org/

Ethical Considerations

The study was approved by the Ethical Review Committee (ERC) of Kenya Medical Research Institute (KEMRI) Nairobi.

DNA Extraction and Amplification of *Pfdhfr* and *Pfdhps*

Extraction of DNA was done according to Warhust DC et al.[13] with slight adjustments. The amplification of the Single Nucleotide Polymorphism (SNP) in *dhps and dhfr* gene was executed on an MJ Thermo cycler® PCR machine. The outer PCR for *dhfr* codons in each reaction tube was made up of 10 X PCR buffer (Roche®), 20mM dNTP mix and 10 µM each of oligonucleotide primer pair AMP1 and AMP2, for each targeted codon. Each of the reaction tubes was made to a volume of 30 microliter (µL) with double distilled water which was free from DNA. The PCR programming was set at an initial denaturation temperature of 94°C for 3 min, and final denaturation at 94°C for 1 min, the annealing temperature which is critical was optimized at 50°C for 2 min. Extension of oligonucleotide primers was done in two steps; initial extension at 72°C for 2 min and final extension at 72°C for 10 minutes. The number of cycles required for complete amplification were 40 cycles and the process brought at a standstill at 4°C. The polymorphic regions of *dhfr* were amplified by nested PCR using 3 µL of the outer amplification products to target the respective codons using oligonucleotide primer pair SP1 and SP2 using the enlisted PCR parameters; 94°C for 2 min, 94°C for 1 min, 45°C for 1 min, 72°C for 2 min, 35 cycles, final extension at 72°C for 10 min and halted at 4°C. After obtaining the amplified products, gel electrophoresis was done on a 2% Ethidium stained agarose. Clear bands of the accurate size were stored at -20°C for restriction digestion.

Analysis of *Pfdhps* and *Pfdhfr* Genes by Restriction Digests

Analysis of the genes was done following the methods by Duraisingh MT et al.[14] To confirm presence of mutation specific enzymes were used which cuts only the mutated region. For instance, FOK1 enzyme, was used to completely cuts the Glu540 mutant sequence and Ava11 was used to wholly cleave the mutant Gly437 sequence. In the analysis of *dhfr* mutation, samples amplified using AMP1/ AMP2 for outer PCR and SP1/ SP2 primers for nested PCR were assessed for mutation by digestion of each codon using specific enzyme TSP5091 was used to cleave the mutated are in *dhfr* 51 Mutation at codons 59 and Asn108/ Thr108/ Ser108 were carried out using enzymes Xmn1 and Bsr1/ Scrf1/ Alu1 respectively.

Each restriction digest premix contained 0.5 µL of the enzymes and the protocols were run as per the supplier's specifications (New England Biolabs). All reactions were topped up to a volume of 20 mL comprising of 5 mL of unpurified PCR product, buffered and incubated as per protocol (NEB). After digestion the cleaved products were run on 2.0% Ethidium bromide stained agarose gel and visualized under U.V light on transilluminator and results photographed using Polaroid® camera and stored as soft copy.

Statistical Analysis

Differences between allele frequency was calculated using chi-square on 2*2 table using Yates correlation and Fishers exact test.

Result

Table 1.Occurrence of *Pfdhfr, Pfdhps* and combined *Pfdhfr*/*Pfdhps* genes calculated as a percentage of total number of samples successfully analysed per codon in *P.falciparum* isolates from Msambweni District Hospital, Kwale County

Gene	Haplotypes	No. of samples	%
Pfdhfr	I51	110	80.6
	R59	97	72.4
	N108	125	93.3
	I51/ R59/ N108	85	63.4
Pfdhps	G437	110	82.1
	E540	105	78.4
	G437/E540	98	79.9
Pfdhfr/ Pfdhps	I51/ R59/ N108+G437/ E540	72	53.7

A total of 134 out of 150 samples were effectively amplified and analyzed for all targeted single nucleotide polymorphism (SNPs) in both *Pfdhfr* and *Pfdhps*. The two major mutations in *dhps* genotype were at codon 437 and 540 classified as single or double mutant. Mutation present in the three major codons of *Pfdhfr* genes and two major codons of *dhps* genes were classified as quintuple mutant which was the most prevalent combination among all the analyzed samples in *Pfdhfr/ Pfdhps* genes.

In each *P. falciparum* parasite at all examined codons, no mixed genotype was detected. In *Pfdhfr*, mutation at codon *Pfdhfr* 108 was the most prevalent, 125 (93.3%), the prevalence at codon *Pfdhfr* 51 and *Pfdhfr* 59 were 110 (80.1%) and 97 (72.4%) respectively. The prevalence of *Pfdhps* 437 and *Pfdhps* 540 were 110 (82.1%) and 105 (78.4%) respectively.

The most prevalent combination of *Pfdhfr* gene (N51I/ C59R/ S108N) among all the isolates analyzed was the triple mutant at 63.4% while single mutant and double mutant were the least prevalent at 6.7% and 24.6% respectively The single or double mutant at codon 437 and 540 of *Pfdhps* gene accounted for 14.2% and 79.9% respectively

ISSN: 2455-7048
DOI: https://doi.org/

of all the isolates examined The frequency of genotypes in combined *Pfdhfr* and *Pfdhps* genes was the most prevalent at 53.7% (Figure 1).

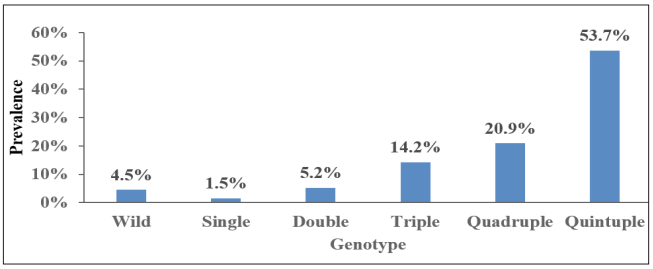

Figure 1.Frequency of combined genotypes in *pfdhfr*/ *pfdhps* quintuple mutant in the analyzed *P. falciparum* isolates from Msambweni District Hospital, Kwale County

Discussion

Resistance to SP has resulted to its diminishing healing efficiency in many regions of the countries where malaria is endemic.[15] Since its inception as the first-line treatment drug for unsophisticated malaria in Kenya in 1999, its efficacy has diminished due to resistant and most malaria endemic regions in the African countries have since substituted it with ACT.[15,16] In 2004, Kenya changed the malaria treatment regimen from SP and Artemether Lumefantrine (AL) became the preferred chemotherapy and thus the first line regimen for malaria up to date.[17]

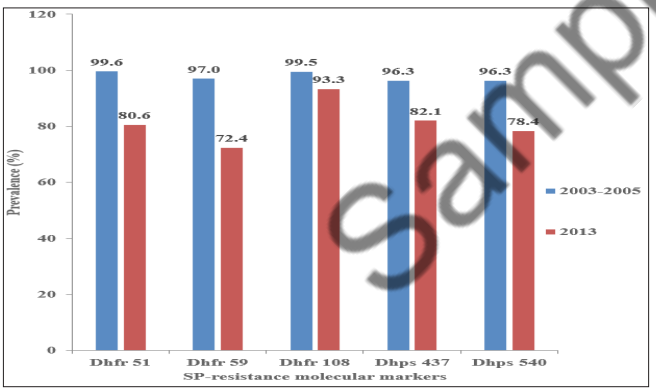

Figure 2.Trends of SP drug-resistance molecular markers in *P. falciparum* isolates from Msambweni District Hospital, Kwale County

The results of this survey show that the quintuple mutant *Pfdhfr*/*Pfdhps* alleles are maintained at high frequency a decade after withdrawal of SP and its frequency stands at 53.7% in a population sampled in 2013. In a 2003/05 study carried out at Western Kenya, the prevalence of *Pfdhfr* mutations at C59R, N51I and S108N was 72.4%, 80.6% and 93.3% respectively.[15] When compared to the results of this study, there were some remarkable reductions in proportions of isolate. *Pfdhfr* mutations at N51I with the prevalence in 2003/ 05 being 99.6% while in 2013 the prevalence was 80.6% (p<0.001) and *pfdhfr* mutations at C59R was. The proportion of isolates containing *Pfdhfr* mutations at S108N decreased from 99.5% in 2003/ 05 to 93.3% in 2013 (p=0.001) (Figure 2). Mutation at *Pfdhps* at A437G and *Pfdhps* at K540E also reduced significantly. From the results of this study its evident that resistance to SP is linked to mutation at codons N51I, C59R, S108N of the *P. falciparum pfdhfr* gene, and codons A437G and K540E of the *P. falciparum pfdhps* gene. The mutations in these five codons commonly referred to as quintuple mutant results in an amino acid change in all the codons and this has been proven to be the chief determinant of SP resistance.[16]

Although 2003-2005 marked the period which SP was discontinued and AL introduced as first-line regimen for malaria, the occurrence of *Pfdhfr/ Pfdhps* N51I, C59R, S108N/A437G, K540E quintuple mutant genotype which is sturdily linked with clinical SP treatment failure continued to raise at a distressing rate during this period. Iriemenam NC et al. and Spalding MD et al. studies observed the occurrence of the quintuple mutant genotype increase from 7% and 21% in sample isolates collected in 1996-2000 and 1999-2000 to 88% and 53% in samples collected in 2008-2009 and 2003-2005 respectively.[15, 17] In the current study the frequency of quintuple mutant is similar to that from Spalding MD et al. The small difference could be the fact that the two studies were carried at different period and maybe a reflective of the malaria transmission rates found in those locations. The transmission rate at the coast has been lower compared to other endemic region and this can be attributed to the use of scaled interventions such as use of treated mosquito nets among other control measures.[18]

The frequency and status of mutations in *pfdhfr* and *pfdhps* genes in Msambweni represents various dynamics which could be as a result of number factors yet to be determined. However, compared to other endemic regions in Kenya and across East Africa the occurrence of malaria is on a steady decline in the Kenyan coast (Unpublished). A study carried out between August 2010 to February 2012 in Mukono district (a town near Lake Victoria in Central Uganda), the frequency of quintuple mutation (*dhfr* 51, 59, 108 and *dhps* 437, 540) was 88.9%.[19] Similar results of high frequencies of resistant haplotypes have been recorded in Tanzania and some regions in Kenya.[20]

In a 2014 study carried out in Malindi, the same geographical area as this survey, the occurrence of the quintuple mutant *pfdhfr/ pfdhps* allele was 53.4%. There was a very negligible decrease in the rate of recurrence of the quintuple mutant between the two studies conducted in the coastal region, where malaria is endemic. The small disparity could be attributed to the spatial variation in the periods of data collection and disparities of population from which samples

ISSN: 2455-7048
DOI: https://doi.org/

were collected. Our current survey used samples obtained from general population in 2013 while the study carried out by Juma *et al* was between 2008 and 2012.[17]

This clearly indicates and confirms that the quintuple mutant genotype is sturdily allied with SP treatment failure. The high frequency of the quintuple mutations can be attributed to lack of immediate total withdrawal of SP drug from the pharmacy outlets with SP use still high regardless of AL being the recommended first-line malaria treatment in Kenya. The drug could have possibly remained in circulation and sold over the counter (in government facilities/ chemists). Over the counter SP drugs and continued use of SP in pregnant mothers and infants as IPT maintain a selection pressure for the mutations and fixation in the population.[21] Lack of rigorous awareness to educate the public on the reduced efficacy level of SP as an antimalarial could have contributed to the continued use of SP hence the presence of drug pressure resulting to fixation of key mutations in the population. Fixation of the resistance alleles have been attributed to parasites that may have acquired additional compensatory mutations. Moreover, use of other antifolates drugs e.g. Cotrimoxazole (used for treatment of respiratory tract infections among HIV patients) could be another reason for the high frequency of SP resistance.[22] Cotrimoxazole is said to provide additional selective pressure possibly due to cross resistance between trimethoprim and Pyrimethamine in *P. falciparum* malaria parasites. Spread and sustenance of the mutations in parasites circulating within the coastal region of Kenya is independent of the SP drugs that induced these mutations.[18,22] The risk associated to the mutation is minimal instead mutant parasites haven't been selected against during period of reduced drug pressure following SP withdrawal.

Conclusion

The current study has established that the occurrence of molecular markers for SP remains significantly high in the coastal region. The five codons sampled in *pfdhfr* and *pfdhps* were all maintained at high frequencies. In the combined allele, the quintuple mutant was the most prevalent, hence associated with resistance to SP. The WHO needs to set a maximum threshold of drug resistance before an antimalarial drug can be banned on the basis of compromised efficacy. Policy makers need to be advised on the way forward since SP is widely used as IPTp/i. A clear policy recommendation on this cut off point is required. Although the occurrences at the coast are lower compared to other region it would be necessary to carry out more surveys to find out if the results of this study could be replicated in other regions with similar transmission rates. More research based on molecular examination and in vitro susceptibility tests should be conducted to inform the stakeholders about the emergence and increase of SP resistance since SP remains the suggested drug for preventing and treating malaria in expectant women and infants. Further studies with larger number of samples are recommended to give a more comprehensive result on the genetic variation of *P. falciparum* isolates in the study area.

Acknowledgement

We thank the director of KEMRI for permission to publish these findings and to the KEMRI malaria laboratory staff who assisted with this study. We wish to thank the Department of Zoological sciences staff of, Kenyatta University for the valuable input. This study was funded by National Commission for Science, Technology & Innovation (NACOSTI/RCD/STI/7TH CALL/MSC/193).

Conflict of Interest: None

References

1. World Health Organization (WHO). Monitoring health for the SDGs Sustainable Development Goals, World Health Statistics; 2017.https://www.who.int>gho>publications.
2. Ministry of Health Malaria Surveillance guidelines; National guidelines for the diagnosis, treatment and prevention of malaria in Kenya, 2014.repository.eac.int>bitstream>handle.
3. Kenya Malaria Indicator Survey (KMIS). Division of Malaria Control [Ministry of Public Health and Sanitation], Kenya National Bureau of Statistics, and ICF Macro. 2011.https://www.knbs.or.ke.
4. Okiro EA, Alegana VA, Noor AM et al. Changing malaria intervention coverage, transmission and hospitalization in Kenya. *Malaria Journal* 2010; 9: 285.
5. Moody A. Rapid diagnostic tests for malaria parasites. *Clin Microbial Rev* 2002; 15(4): 66-78.
6. Kublin JG, Dzinjalamala FK, Kamwendo D et al. Molecular markers for failure of sulfadoxine-pyrimethamine and chlorproguanil-dapsone treatment of *Plasmodium falciparum* Malaria. *Journal of Infectious Diseases* 2002; 185: 380-388.
7. Khan B, Omar S, Kanyara JN et al. Antifolate drug resistance and point mutations in *Plasmodium falciparum* in Kenya. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 1997; 91(4): 456-460.
8. Djimde A, Ogobara PD, Doumbo K et al. Molecular marker for chloroquine-resistant falciparum malaria. *N Engl J Med* 2001; 344(4): 257-263.
9. Amin A, Zurovac D, Kangwana B et al. The challenges of changing national malaria drug policy to artemisinin-based combinations in Kenya. *Malaria Journal* 2007; 6: 72.
10. Sridaran S, McClintock SK, Syphard LM et al. Anti-folate drug resistance in Africa: meta-analysis of reported dihydrofolate reductase (dhfr) and dihydropteroate

synthase (dhps) mutant genotype frequencies in African *Plasmodium falciparum* parasite populations. *Malar J* 2010; 9: 247.

11. Thaithong S, Chan SW, Songsomboon S et al. Pyrimethamine resistant mutations in *Plasmodium falciparum. Mol Biochem Parasitol* 1992; 52(2): 149-157.
12. Gregson A, Plowe CV. Mechanisms of resistance of malaria parasites to antifolates. *Pharmacological Reviews* 2005; 57(1): 117-145.
13. Warhurst DC, Kariem FM, Miles MA. Simplified preparation of malarial blood samples for polymerase chain reaction. *Lancet* 1991; 337: 303-304.
14. Duraisingh MT, Curtis J, Warhurst D. Plasmodium falciparum: detection of polymorphism in the dihydrofolate reductase and dihydropteroate synthase genes by PCR and restriction digestion. *Experimental Parasitology* 1998; 8(1): 1-8.
15. Spalding MD, Eyase FL, Akala HM et al. Increased prevalence of the Pfdhfr/phdhps quintuple mutant and rapid emergence of Pfdhps resistance mutations at codons 581 and 613 in Kisumu, Kenya. *Malar J* 2010; 9: 338.
16. Iriemenam NC, Shah M, Gatei W et al. Temporal trends of sulfadoxine-pyrimethamine (SP) drug-resistance molecular markers in *Plasmodium falciparum* parasites from pregnant women in western Kenya. *Malar J* 2012; 11: 134.
17. Juma DW, Omondi AA, Ingasia L et al. Trends in drug resistance codons in Plasmodium falciparum dihydrofolate reductase and dihydropteroate synthase genes in Kenyan parasites from 2008 to 2012. *Malaria Journal* 2014; 13: 250.
18. Okombo J, Kamau AW, Marsh K et al. Temporal trends in prevalence of Plasmodium falciparum drug resistance alleles over two decades of changing antimalarial policy in coastal Kenya. *Int J Parasitol Drugs Drug Resist* 2014; 4(3): 152-163.
19. Mbonye AK, Birungi J, Yanow SK et al. of Plasmodium falciparum resistance markers to sulfadoxine pyrimethamine among pregnant women receiving intermittent preventive treatment for malaria in Uganda. *Antimicrob Agents Chemother* 2015; 59: 5475-5482.
20. Alifrangis M, Lusingu JP, Mmbando B et al. Five-year surveillance of molecular markers of Plasmodium falciparum antimalarial drug resistance in Korogwe District, Tanzania: accumulation of the 581G mutation in the P. falciparum dihydropteroate synthase gene. *Am J Trop Med Hyg* 2009; 80: 523-527.
21. Amin AA, Snow RW. Brands costs and registration status of antimalarial drugs in the Kenyan retail sector. *Malar J* 2005; 4: 36.
22. Manyando C, Njunju EM, D'Alessandro U et al. Safety and efficacy of co-trimoxazole for treatment and prevention of Plasmodium falciparum malaria: a systematic review. *PLoS One* 2013; 8(2): 56916.

ISSN: 2455-7048
DOI: https://doi.org/