Research Square

Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

Saturation of *Pfdhfr* and *Pfdhps* haplotypes in general population increasingly threatens the benefits of using sulfadoxine-pyrimethamine for intermittent preventive treatment in pregnant women in Tanzania

George Bwire (georgebwire@gmail.com)
Muhimbili University of Health and Allied Sciences School of Pharmacy
Wigilya P. Mikomangwa
Muhimbili University of Health and Allied Sciences School of Pharmacy
Manase Kilonzi
Muhimbili University of Health and Allied Sciences School of Pharmacy

Research article

Keywords: Pfdhfr, Pfdhps, Sulfadoxine-pyrimethamine, Resistance, Tanzania

Posted Date: January 6th, 2020

DOI: https://doi.org/10.21203/rs.2.20112/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License

Version of Record: A version of this preprint was published at BMC Infectious Diseases on July 22nd, 2020. See the published version at https://doi.org/10.1186/s12879-020-05253-7.

Abstract

Background : High levels of Plasmodium falciparum resistance prompted withdrawal of sulfadoxine-pyrimethamine (SP) as the first-line treatment for uncomplicated malaria in Tanzania. However, SP was limited for intermittent preventive treatment during pregnancy (IPTp) especially where there is moderate to high malaria transmission. This study reports the patterns of P. falciparum dihydrofolate reductase (Pfdhfr) and dihydropteroate synthetase (Pfdhps) mutations.

Methods: Parasite genomic DNA was extracted from dried blood spots prepared by finger prick. Batched samples (384) were sequenced in a single MiSeq lane combining all PCR products. Samples were de-plexed using the multiplexing adapters and individual CRAM files were aligned to a modified amplicon reference genome. Genotyping of Pfdhfr and Pfdhps mutations were done using bcftools as well as custom scripts to filter and translate genotypes into drug resistance haplotypes.

Results: The Pfdhfr was analyzed from 445 samples, the wild type (WT) Pfdhfr haplotype NCSI was detected in only six (1.3%) samples. Triple Pfdhfr IRN I haplotype was dominant, contributing to 84% (n=374) of haplotypes. The total of 446 samples were studied for Pfdhps . WT for Pfdhps was found in 6.7% (n=30) of all samples detected. Double Pfdhps haplotype (S GE AA) accounted for 83% of mutations of the Pfdhps gene. The overall prevalence of K540E was 90.4% (n=396) while A581G was 1.1% (n=5). Additionally, 91.4% (n=447) genotypes where detected from 489 sequenced samples. Of 447 genotypes detected only 0.9% (n=4) of samples were WT (SAKAA-NCSI). Quintuple mutation, S GE AA-IRN I accounted 71.4% of concomitant Pfdhfr/Pfdhps mutations where 0.2% (n=1) had septuple mutation, AG K GS - IRN I.

Conclusions : Despite the high prevalence of mutations in Pfdhfr and Pfdhps gene but the current mutations at Pfdhfr K540E and Pfdhps A581G are within the recommended WHO range, stopping IPTp-SP is recommended in areas where the Pfdhfr K540E prevalence is >95 % and Pfdhps A581G is >10 % as SP is likely to be ineffective). Nevertheless, saturation in Pfdhfr and Pfdhps haplotypes is alarming, therefore screening for alternative antimalarial drug for IPTp-SP is recommended.

Background

Antimalarial drugs use and vector control are the commendable tools for global malaria prevention and control respectively (1). However, resistance of Plasmodium to antimalarial drugs and resistance of Anopheles mosquitoes to insecticides impair the progressive fight against malaria (2). Resistance of P. falciparum to previous generations of medicines i.e. chloroquine (CQ) (3) and sulfadoxine-pyrimethamine (SP) (4) influenced the replacement of CQ with SP (5), then artemisinin-based combination therapy (ACT) as the first-line treatment for uncomplicated malaria in Tanzania (6).

The changes in malaria treatment policy were supported by evidence from molecular epidemiological resistance surveillance against SP (7), which are P. falciparum dihydrofolate reductase (Pfdhfr) and dihydropteroate synthetase (Pfdhps) enzymes mutations (8,9). Mutations in the Pfdhps gene at codons

Serine 436 to Alanine (S436A), Alanine 437 to Glycine (A437G), Lysine 540 Glutamic acid (K540E), Alanine 581 to Glycine (A581G) and Alanine 613 to Serine (A613S) predicts sulfadoxine resistance whereby mutations in the Pfdhfr gene at codons Cysteine 50 to Arginine (C50R), Asparagine 51 to Isoleucine (N51I), Cysteine 59 to Arginine (C59R), Serine 108 to Asparagine/Threonine (S108 N/T), and Isoleucine 164 to Leucine (I164L) confer pyrimethamine resistance (10).

Mutations in both the Pfdhfr/Pfdhps genes which greatly influence SP clinical treatment failures (11), resulted SP being unfit for first -line treatment of uncomplicated malaria in Tanzania (12). However, in 2006 the drug was limited for intermittent preventive treatment during pregnancy (IPTp-SP) in areas with moderate to high malaria transmission (13), pregnant women regardless of the presence or absence of malaria should administer at least three curative doses of SP with an interval of at least 1 month between the two doses, starting in the second trimester of pregnancy (14) to prevent pregnancy associated malaria and improve pregnancy outcomes (13).

Unfortunately, the rapid widespread of Pfdhfr/Pfdhps genes mutations and saturation of haplotypes including quintuple (CIRNI-SGEAA) and sextuple (CIRNI-SGEGA) reported in Tanzania (10) are likely to compromise effectiveness of IPTp-SP strategy. In this regard, World Health Organization (WHO) recommends stopping IPTp-SP in areas where the Pfdhfr K540E prevalence is > 95% and Pfdhps A581G is > 10% as SP is likely to be ineffective (15).

For the purpose of guiding IPTp-SP policy and inform on the current SP resistance status, routinely surveillance of SP effectiveness using molecular markers should be implemented as recommended by WHO (15). Therefore, a study was conducted in general population, more than a decade since SP was limited for IPTp use in pregnant women in Tanzania.

Methods

Study design, area, period and population

A hospital based surveillance of molecular markers for SP resistance was conducted between April and August 2019 at Kibiti Health Center (KHC). The study was conducted to determine the current antimalarial status of SP, more than a decade since SP were limited for IPTp for pregnant women in Tanzania. The coastal region experiences malaria transmission throughout the year with regional prevalence ranging between 3.1 and 14.8% (16). Patients attending clinic at KHC who presented with symptoms suggestive of malaria infection were recruited in the study. The symptoms such as fever, general body weakness and headache were confirmed by the attending physician (17). Malaria screening in patients were done using CareStart™ malaria HRP2/pLDH (Pf/pan) Combo test (Access Bio, Ethiopia). Then positive samples by rapid tests were subjected to blood smear (BS) microscopy for confirmation. A total of 489 dried blood samples (DBS) from patients tested positive with BS microscopy were prepared.

DBS preparation and DNA extraction

DBS were prepared according to MalariaGEN SpotMalaria, DBS collection protocol (18). A sterilized patient's finger was pricked, and four blood spots from each patient were prepared; two on each paper card. The blood spots were allowed to dry and placed in the desiccant sachet for storage. DNA from the DBS was extracted using QIAamp DNA Investigator Kit for isolation of total DNA from filter papers (Qiagen, Valencia, California, United States) and as described elsewhere (19).

Genotyping of Pfdhfr and Pfdhps mutations

Molecular genotyping of Pfdhfr and Pfdhps gene mutations associated with SP-resistance was performed by Wellcome Sanger Institute, UK.

Briefly, targets for genotyping were identified and multiplex PCR primers were designed using a modified version of the mPrimer software (20). Primers were designed to amplify products between 190–250 bp and combined into 3 pools. A two-step protocol was used to first amplify the target regions of the parasite genome, followed by a second PCR to incorporate sequencing and multiplexing adapters. Batched samples (384) were sequenced in a single MiSeq lane combining all PCR products. Samples were de-plexed using the multiplexing adapters and individual CRAM files were aligned to a modified amplicon reference genome. Genotyping was done using bcftools as well as custom scripts to filter and translate genotypes into drug resistance haplotypes.

Results

Haplotypes for *Pfdhfr* and *Pfdhps* mutations

The *Pfdhfr* was analyzed from 445 samples, the wild type (WT) *Pfdhfr* haplotype NCSI was detected in only 1.3% (n=6) samples. Triple *Pfdhfr* **IRN**I haplotype was dominant, contributing to 84% (n=374) of haplotypes. The total of 446 samples were studied for *Pfdhps*. The WT for *Pfdhps* was found in 6.7% (n=30) of all samples detected. The most common mutation was the change of amino acid alanine to glycine (A437G) and lysine to glutamic acid (K540E). Double *Pfdhps* haplotype (S**GE**AA) accounted for 83% of mutations of the *Pfdhps* gene. The overall prevalence of K540E was 90.4% (396) while A581G was 1.1% (5) (Table 1).

Table 1: *Pfdhfr* and *Pfdhps* haplotypes

Concomitant *Pfdhfr* and *Pfdhps* haplotypes

Overall 447 (91.4%) genotypes were detected from 489 sequenced samples. The concomitant mutations (*Pfdhfr-Pfdhps*) were present to 443 samples (99.1%). Of 447 genotypes detected only 4 samples (0.9%) were WT (SAKAA-NCSI). Concomitant mutations with haplotypes/quintuple (S**GE**AA-**IRN**I) dominated by 71.4% where one (0.2%) sample detected septuple mutation (**AG**K**GS**-**IRN**I) (Table 2).

Table 2: The frequency distribution of *Pfdhfr-Pfdhps* haplotypes

Discussion

This study was conducted to determine the current SP resistance status, more than a decade since SP was limited for IPTp use in pregnant women in Tanzania.

The study found high level (99.1%) concomitant mutations (Pfdhfr-Pfdhps) where quintuple mutation (SGEAA-IRNI) dominated by 71.4%. Additionally, one sample detected septuple mutation (AGKGS-IRNI) and only 0.9% contained the WT (SAKAA-NCSI) protein. These results were close to the findings from the previous study which reported high-level of P. falciparum SP resistance with the concomitant occurrence of septuple haplotype (10). There was a high number of quintuple (SGEAA-IRNI) and few sextuple mutations. These haplotypes have been highly associated with sub-optimal IPTp-SP effectiveness in study conducted between 2008–2010 in Korogwe district, Tanzania (21). Moreover, high prevalence of the Pfdhfr/Pfdhps sextuple haplotype was associated with reduced birth- weight (22–24).

In this study, triple Pfdhfr IRNI haplotype mutation was dominant, contributing to 84% of all Pfdhfr haplotypes whereas double Pfdhps haplotype (SGEAA) accounted for 83% of mutations of the Pfdhps haplotypes. Compared to study by Baraka et al.,2015 (10), Pfdhfr IRNI haplotype were predominant in all sites with significantly higher frequencies at Muheza (93.3%) compared to Muleba (75.0%) and Nachingwea districts (70.6%). In this regard, there was 13.4% increase in Pfdhfr IRNI haplotype prevalence when Nanchingwea (70.6%) and Kibiti district (84%) both found along the coastal region were compared. Additionally, the previous study found that double Pfdhps haplotype SGEAA was significantly high at Muheza (27.2%) and Muleba (20.8%) while none (0%) was detected at Nachingwea while the current study at Kibiti district detected 83% Pfdhps SGEAA mutations. Meaning that, from 2015 to 2019 there is an increase from 0–83% Pfdhps SGEAA mutation in the coastal region.

WHO malaria report of 2019 has clearly mentioned pregnant women as the most affected group and use of effective IPTp should be highly implemented to combat risks associated with pregnancy malaria (25). On top of that, a recent study by Mikomangwa et al., (26) concluded that pregnant woman who was malaria positive had 11 times more risk of giving birth to low birth weight (LBW) child when compared to those who tested malaria negative.

Nevertheless, Pfdhfr K540E and Pfdhps A581G, major predictors for IPTp-SP failure, were found to have the prevalence of 90.4% for Pfdhfr K540E and 1.1% for Pfdhps A581G. This confirm the continual IPTp use. WHO recommends stoppage of SP for IPTp if Pfdhfr K540E prevalence > 95% and Pfdhps A581G > 10% as SP is likely to be ineffective (15). However, rapid increase and saturation in Pfdhfr and Pfdhps mutations with spread of sextuples and septuple is of great concern. The effort to search for alternative drug (s) to replace SP for IPTp should be increased.

The current study was conducted in the general population, hence limited data on clinical, birth outcomes and malaria infection status in pregnant women in relation to SP resistance genotypes.

Conclusions

Despite the high prevalence of mutations in Pfdhfr and Pfdhps gene but the current mutations at Pfdhfr K540E and Pfdhps A581G are within the recommended range (WHO recommends stopping IPTp-SP in areas where the Pfdhfr K540E prevalence is > 95% and Pfdhps A581G is > 10% as SP is likely to be ineffective). Nevertheless, saturation in Pfdhfr and Pfdhps gene is alarming, therefore screening for alternative antimalarial drug for IPTp-SP is recommended.

Abbreviations

SP: Sulfadoxine-pyrimethamine (SP) ; WT: Wild Type; IPTp: intermittent preventive treatment during pregnancy; *Pfdhfr*: *Plasmodium falciparum* dihydrofolate reductase; *Pfdhps*: *Plasmodium falciparum* dihydropteroate synthetase; WHO: World Health Organization

Declarations

Ethics approval and consent to participate

Ethical approval to conduct this study was obtained from Muhimbili University of Health and Allied Sciences Ethical Review Board (Ref. DA.282/298/01A.C/) and National Institute for Medical Research (Ref. NIMR/HQ/R.8A/Vol.IX/3107). Permission to conduct the study at KHC was obtained from both Kibiti District Medical Officer and KHC in charge Medical officer. Written informed consents after explaining the purpose of the study was requested before enrollment.

Competing interests

The authors declare that they have no competing interests.

Availability of data

The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

Consent for publication

Not applicable

Funding

This study was funded by Swedish International Development Cooperation Agency (Sida), Sweden through Muhimbili University of Health and Allied Sciences. The funder did not participate in the design of the study, data collection, analysis, interpretation, and manuscript preparation.

Authors' contributions

GMB participated in conception; study design, data collection, analysis and manuscript writing, MK, WPM participated in data analysis and manuscript writing participated in data analysis and interpretation. All authors read and approved the final manuscript.

Acknowledgements

This publication uses data generated using Tanzanian samples in collaboration with MalariaGEN SpotMalaria Project (https://www.malariagen.net/projects/spotmalaria); the project is coordinated by the MalariaGEN Resource Centre with funding from Wellcome (206194, 090770). The authors would like to thank the staff of Wellcome Sanger Institute Sample Management, Genotyping, Sequencing and Informatics teams for their contribution. Furthermore, we thank Sonia Goncalves for providing the statement on the molecular typing for antimalarial resistance markers. The author (GMB) acknowledge the training support on antimalarial resistance markers when he attended Global Health Fellowship, Novartis Institutes for Biomedical Research, Emeryville, California, USA.

References

1. Patouillard E, Griffin J, Bhatt S, Ghani A, Cibulskis R. Global investment targets for malaria control and elimination between 2016 and 2030. BMJ Glob Heal [Internet]. 2017 May 1;2(2):e000176. Available from: http://gh.bmj.com/content/2/2/e000176.abstract
2. WHO. Global report on antimalarial drug efficacy and drug resistance: 2000-2010. Who. 2010;115.
3. Mohammed A, Ndaro A, Kalinga A, Manjurano A, Mosha JF, Mosha DF, et al. Trends in chloroquine resistance marker , Pfcrt-K76T mutation ten years after chloroquine withdrawal in Tanzania. Malar J [Internet]. 2013;12(1):1. Available from: Malaria Journal
4. NDEJEMBI M, BECK H-P, MALISA A, ABDULLA S, LEMNGE M, NKYA W, et al. Therapeutic Efficacy of Sulfadoxine-Pyrimethamine and Prevalence of Resistance Markers in Tanzania Prior To Revision of Malaria Treatment Policy: Plasmodium Falciparum Dihydrofolate Reductase and Dihydropteroate Synthase Mutations in Monitoring in Vivo Re. Am J Trop Med Hyg. 2018;71(6):696–702.
5. Mubyazi GM, Gonzalez-Block MA. Research influence on antimalarial drug policy change in Tanzania: Case study of replacing chloroquine with sulfadoxine-pyrimethamine as the first-line drug. Malar J. 2005;4:1–13.

6. P. Y, J.L. C, S. A, J. A, P.S. L, J. M. Trends in availability and prices of subsidized ACT over the first year of the AMFm: Evidence from remote regions of Tanzania. Malar J [Internet]. 2012;11:299. Available from: http://ovidsp.ovid.com/ovidweb.cgi?T=JS&PAGE=reference&D=emed14&NEWS=N&AN=52185324
7. Mugittu K, Abdulla S, Falk N, Masanja H, Felger I, Mshinda H, et al. Efficacy of sulfadoxine-pyrimethamine in Tanzania after two years as first-line drug for uncomplicated malaria: assessment protocol and implication for treatment policy strategies. Malar J [Internet]. 2005;4. Available from: https://doi.org/10.1186/1475-2875-4-55
8. Brooks DR, Wang P, Read M, Watkins WM, Sims PFG, Hyde JE. Sequence Variation of the Hydroxymethyldihydropterin Pyrophosphokinase: Dihydropteroate Synthase Gene in Lines of the Human Malaria Parasite, Plasmodium falciparum, with Differing Resistance to Sulfadoxine. Eur J Biochem. 1994;224(2):397–405.
9. Triglia T, Menting JGT, Wilson C, Cowman AF. Mutations in dihydropteroate synthase are responsible for sulfone and sulfonamide resistance in Plasmodium falciparum. Proc Natl Acad Sci U S A. 1997;94(25):13944–9.
10. Baraka V, Ishengoma DS, Fransis F, Minja DTR, Madebe RA, Ngatunga D, et al. High-level Plasmodium falciparum sulfadoxine-pyrimethamine resistance with the concomitant occurrence of septuple haplotype in Tanzania. Malar J. 2015;14(1):1–9.
11. Mugittu K, Ndejembi M, Malisa A, Lemnge M, Premji Z, Mwita A, et al. Therapeutic efficacy of sulfadoxine-pyrimethamine and prevalence of resistance markers in Tanzania prior to revision of malaria treatment policy: Plasmodium falciparum dihydrofolate reductase and dihydropteroate synthase mutations in monitoring in vivo re. Am J Trop Med Hyg. 2004;71(6):696–702.
12. Kavishe RA, Kaaya RD, Nag S, Krogsgaard C, Notland JG, Kavishe AA, et al. Molecular monitoring of Plasmodium falciparum super-resistance to sulfadoxine-pyrimethamine in Tanzania. Malar J. 2016;15(1):1–8.
13. IT Committee: This Friday we have two items on our agenda. 1. Al Wysocki. 2017;226.
14. Rogerson SJ, Mwapasa V, Meshnick SR. Malaria in pregnancy: Linking immunity and pathogenesis to prevention. Am J Trop Med Hyg. 2007;77(SUPPL. 6):14–22.
15. Gana R, Leke F, Slutsker L. WHO Evidence Review Group on Intermittent Screening and Treatment (ISTp) and ACT Treatment of Malaria in Pregnancy. 2015;(September):1–17. Available from: http://www.who.int/malaria/mpac/mpac-sept2015-erg-mip-presentation.pdf
16. TMIS. Tanzania Malaria Indicator Survey (TMIS) 2017 [Internet]. 2017. Available from: https://dhsprogram.com/pubs/pdf/MIS31/MIS31.pdf
17. United THE, Of R. THE UNITED REPUBLIC OF TANZANIA STANDARD TREATMENT GUIDELINES AND ESSENTIAL MEDICINES LIST. 2013;
18. MalariaGEN Resource Centre members based at the Wellcome Trust Sanger Institute U. Protocol for collecting dried blood spots from malaria patients, for the purposes of sequencing Plasmodium parasites. https://www.malariagen.net/network/capacity-building/methods#DBS.

19. Oyola SO, Ariani C V., Hamilton WL, Kekre M, Amenga-Etego LN, Ghansah A, et al. Whole genome sequencing of Plasmodium falciparum from dried blood spots using selective whole genome amplification. Malar J. 2016;15(1):1–12.
20. Shen Z, Qu W, Wang W, Lu Y, Wu Y, Li Z, et al. MPprimer: A program for reliable multiplex PCR primer design. BMC Bioinformatics. 2010;11.
21. Minja DTR, Schmiegelow C, Mmbando B, Boström S, Oesterholt M, Magistrado P, et al. Plasmodium falciparum mutant haplotype infection during pregnancy associated with reduced birthweight, Tanzania. Emerg Infect Dis. 2013;19(9):1446–54.
22. McCollum AM, Mueller K, Villegas L, Udhayakumar V, Escalante AA. Common origin and fixation of Plasmodium falciparum dhfr and dhps mutations associated with sulfadoxine-pyrimethamine resistance in a low-transmission area in South America. Antimicrob Agents Chemother. 2007;51(6):2085–91.
23. Naidoo I, Roper C. Mapping “partially resistant”, “fully resistant”, and “super resistant” malaria. Trends Parasitol [Internet]. 2013;29(10):505–15. Available from: http://dx.doi.org/10.1016/j.pt.2013.08.002
24. Harrington WE, Morrison R, Fried M, Duffy PE. Intermittent Preventive Treatment in Pregnant Women Is Associated with Increased Risk of Severe Malaria in Their Offspring. PLoS One. 2013;8(2):2–7.
25. World Health Organization (WHO). World Malaria Report [Internet]. WHO. 2019. Available from: https://www.who.int/publications-detail/world-malaria-report-2019
26. Mikomangwa WP, Oms M, Aklillu E, Kamuhabwa AAR. Adverse birth outcomes among mothers who received intermittent preventive treatment with Sulphadoxine-Pyrimethamine in the low malaria transmission region. BMC Pregnancy Childbirth. 2019;19(1):1–11.

Tables

Table 1.

	Gene Haplotypes	Frequency (%)
Pfdhfr (n=445)	NCSI (WT)	6 (1.3)
	I*	1 (0.2)
	IC**N**I	30 (6.7)
	IN**I***	5 (1.1)
	IR*	5 (1.1)
	IRNI	374 (84.0)
	N**I***	1 (0.2)
	N**R***	1 (0.2)
	N**RN**I	22 (4.9)
Pfdhps (n=446)	SAKAA (WT)	30 (6.7)
	AAAA*	1 (0.2)
	AAKAA	6 (1.3)
	AGEAA	6 (1.3)
	AGK**GS**	1 (0.2)
	FGKAA	1 (0.2)
	GEAA*	2 (0.4)
	HAKAA	2 (0.4)
	SAAA*	1 (0.2)
	SA**E**AA	3 (0.7)
	S**E**AA*	3 (0.7)
	S**G***	2 (0.4)
	S**G**AA*	5 (1.1)
	S**GE***	6 (1.3)
	S**GE**A*	1 (0.2)
	S**GE**AA	370 (83.0)
	S**GEG**A	4 (0.9)
	S**GK**AA	2(0.4)

*Indicates missing genotype(s) because the genotype was not detected during sequencing. Each letter represents an amino acid, A, alanine; C, cysteine; E, Glutamic acid; F, Phenylalanine; G, Glycine; H, Histidine; I, Isoleucine; K, Lysine; N, Asparagine; R, arginine; S serine.

Bold underline represent amino acid changes.

Table 2.

Concomitant *Pfdhfr-Pfdhps* haplotypes	Frequency n(%)
WT	4 (0.9)
Single (1) mutation	0
Double (2) mutations	11 (2.5)
Triple (3) mutations	30 (6.7)
Quadruple (4) mutations	73 (16.3)
Quintuple (5) mutations	319 (71.4)
Sextuple (6) mutations	9 (2.0)
Septuple (7) mutations	1 (0.2)