

Original articles

Molecular monitoring of resistant *dhfr* and *dhps* allelic haplotypes in Morogoro and Mvomero districts in south eastern Tanzania

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Abstract

Background: Resistance to the antimalarial drug sulfadoxine-pyrimethamine (SP) emerged in *Plasmodium falciparum* from Asia in the 1960s and subsequently spread to Africa. In Tanzania, SP use as a national policy began in 1983 as a second line to chloroquine (CQ) for the treatment of uncomplicated malaria, until August 2001 when it was approved to replace CQ as a national first line.

Objective: The present study assesses the frequency of resistant *dhfr* and *dhps* alleles in Morogoro-Mvomero district in south eastern Tanzania and contrast their rate of change during 17 years of SP second line use against five years of SP first line use.

Methodology: Cross sectional surveys of asymptomatic infections were carried out at the end of rainy season during July-September of 2000, when SP was the national second line (CQ was the first line) and 2006 when SP was the national first line antimalarial treatment. Genetic analysis of SP resistance genes was carried out on 1,044 asymptomatic infections and the effect of the two policies on SP evolution compared.

Results: The frequency of the most resistant allele, the double *dhps*-triple *dhfr* mutant genotype, increased by only 1% during 17 years of SP second line use, but there was a dramatic increase by 45% during five years of SP first line use.

Conclusion: We conclude that National policy change from second line to first line SP, brought about an immediate shift in treatment practice and this in turn had a highly significant impact on drug pressure. The use of SP in specific programs only such as intermittent preventive treatment of infants (IPTi) and intermittent preventive treatment of pregnant women (IPTp) will most likely reduce substantially SP selection pressure and the SP resistance alleles alike.

Key words: *Plasmodium falciparum* infection, Sulfadoxine/Pyrimethamine resistance, Polymerase Chain Reaction, Sequence Specific Oligonucleotide Probing

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Introduction

The United Republic of Tanzania has a population of more than 36 million, all of whom are at risk of malaria. However, endemicity and risk of transmission varies and have been mapped by the MARA collaboration¹. This GIS-based analysis revealed that 75% of the population is subject to stable perennial or stable seasonal malaria transmission; 8% to unstable highly seasonal transmission; and 17% to no malaria transmission in the average year, but still at risk of epidemic malaria.

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The Morogoro-Mvomero population is among the 75% group which is subject to stable perennial transmission with some seasonal variations, where peak transmission occur during end of rainy season in July to September of each year. Control of malaria largely depends on early detection and treatment with efficacious drugs. However, Malaria parasites have become increasingly drug-resistant, and resistance has spread geographically^{2, 3, 4, 5, 6, 7, 8, 9}. A survey of SP efficacy prior to national policy change from SP to Artemether+Lumefantrine (ALU) showed a marked regional variability within Tanzania with clinical efficacy ranging between 33% in Ipinda, 43% in Mlimba to 49.3% in Mkuranga^{10,11}. Although SP was replaced by combination of Artemether+Lumefantrine (ALU) as a national first line treatment of uncomplicated malaria in 2006, it remains the only option for intermittent preventive treatment of malaria during pregnancy (IPTp) and an important drug in the intermittent preventive treatment of infant (IPTi). Currently, trials using SP for IPTi indicate variable protective efficacies (PEs) against clinical malaria ranging from 20.1 – 58.6%^{12, 13}. A number of factors including the intensity of transmission, the pattern of antimalarial resistance and the use of additional control measures such as insecticide treated nets (ITN) have been proposed to contribute to the observed variations in the PEs¹⁴.

Treatment of malaria in Tanzania is typically guided by official recommendations from the Ministry of Health and Social Welfare (MOHSW) regarding drugs of choice for various situations. “First-line” treatment refers to the drug officially recommended as the drug of first choice for the treatment of uncomplicated malaria. “Second-line” treatment refers to the drug officially recommended as an alternative primarily to be used for treatment of patients in whom the first-line treatment failed to clear the infection and other select patients (such as those who are hypersensitive to the first-line treatment). “Third-line” treatment typically refers to the drug recommended for severely ill patients (a rescue drug). In practice, few treatment failures are recognized and patients are often moved directly from first to third-line treatment, consequently, little second-line drug is used compared to the first-line drug. SP replaced CQ as the recommended first-line treatment on the Tanzanian mainland in August 2001, after 18 years of its use as a second-line treatment since 1983.

Evidence of CQ use in Malawi indicates, 12 years after its withdrawal for use in the country, CQ-

sensitive parasites re-emerged permitting its re-introduction for malaria treatment¹⁵. The Malawian CQ experience has stimulated interest among researchers and they are now exceedingly keen to learn whether a similar trend could be observed in SP following its replacement with ACT. Documentation of the prevalence of SP resistance markers (*dhfr* and *dhps* alleles) at the time of its withdrawal in Tanzania in 2006 is necessary to provide a baseline data for the monitoring of temporal trends of these alleles in the population thereafter.

We have examined the genetic change at *dhfr* and *dhps* loci under second and first line SP use in Morogoro-Mvomero districts. We collected malaria parasites from residents of randomly selected households in two cross sectional surveys. The first survey occurred during July-September 2000, when CQ was first line treatment and SP was second line. The second survey was conducted in July – September 2006, five years after adoption of a new national policy change from CQ to SP first line treatment of uncomplicated malaria which was also the time of SP replacement with ALU combination therapy as the national first line antimalarial therapy. Infections identified at community level were mainly asymptomatic. This method of sampling we believe is less biased compared to the health facility approach where by definition all infections are symptomatic and many are usually pre-treated cases.

Methods

Study area, subjects and samples

Community surveys were conducted during July, August and September of 2000 and 2006 in initially a one district of Morogoro (Population = 570,422) which was partitioned into Morogoro (Population = 290,316) and Mvomero (Population = 280,106) in 2002. The households that were sampled in 2000 survey were maintained in the 2006, this time belonging to two administratively separate districts of Morogoro and Mvomero but broadly similar in all other aspects and hence referred in this report as one district of Morogoro-Mvomero. The surveys were part of large combination therapy pilot implementation programme in Tanzania, the Interdisciplinary Monitoring Programme for Antimalarial Combination Therapy (IMPACT-TZ). A total of 6,301 adults and children belonging to randomly selected households participated in the study. A finger-prick blood sample for blood slide and filter paper bloodspot were collected from each

individual in the household. The filter paper bloodspots were air-dried and stored at room temperature in self-sealing plastic bags with desiccant and stored on dry area at room temperature. All blood slide samples were screened by microscopy for *P. falciparum*. Bloodspots from microscopically positive subjects were selected and preserved at room temperature for molecular genotyping.

Ethics

Scientific and ethical clearance was granted from the Medical Research Council of the National Institute for Medical Research in Tanzania, the Centers for Disease Control and Prevention, USA, and the London School of Hygiene and Tropical Medicine. Consent was obtained from all individuals or their guardians before collection of samples.

DNA extraction

The DNA was extracted from bloodspots dried on filter papers. A section of the dried blood spot filter paper was excised using a sterile blade or scissors, and soaked in 1 ml of 0.5% saponin in 1x phosphate buffered saline (PBS) overnight in a 96-deepwell plate. The segment was then washed twice in 1 ml of 1x PBS and was finally boiled for 8 min in 150µl solution containing 100µl PCR quality water and 50µl of 20% chelex suspension (pH 9.5).

PCR amplification

Nested PCR was used to amplify a 594 base pair (bp) fragment of *dhfr* and a 711 bp fragment of *dhps* each containing the sequences where mutations are found. Primer sequences and PCR reaction conditions were previously described in Pearce *et al*⁶. PCR was performed in 96 well plates with 25-µl PCR reaction volumes containing final concentrations of 0.25µM oligonucleotide primers, 2mM MgCl₂, 250µM each deoxyribonucleotide triphosphate (dNTPs), and 1x Taq polymerase. 1µl of DNA template was used in the outer (primary) PCR reaction mixture for *dhfr* and *dhps* amplifications. For the inner (secondary) *dhps* reactions 1 µl of the outer PCR product was used. The outer *dhfr* PCR products were diluted three fold before a 1 µl was introduced into the inner PCR reaction mixtures.

Molecular genotyping of point mutations by Sequence Specific Oligonucleotide Probing (SSOP)

The PCR amplified sequences from the coding regions of *dhfr* and *dhps* genes were fixed onto

membranes and probed with SSOP designed to detect each of the single base pair substitutions at all positions summarized in Table 1. The SSOP method has advantages for high throughput, while retaining sensitivity and specificity equivalent to those of other methods used for detection of *dhfr* and *dhps* SNPs. The amplified PCR products were screened for *dhfr* and *dhps* sequence variants at 10 loci where single nucleotide polymorphisms (SNPs) are known. The sequence changes (and the amino acid substitutions they code for) are summarised in Table 1.

Table 1: The nucleotide and amino acid substitutions at (a) *dhfr* and (b) *dhps* genes screened for by PCR-SSOP

a) <i>dhfr</i>					
Codon	50	51	59	108	164
Wild type	Cys (C) TGT	Asn (N) AAT AAC	Cys (C) TGT	Ser (S) AGC	Ile (I) ATA
Mutant*	Arg (R) CGT	Ile (I) ATT	Arg (R) CGT	Asn (N) AAC Thr (T) ACC	Leu (L) TTA
b) <i>dhps</i>					
Codon	436	437	540	581	613
Wild type	Ser (S) TCT	Ala (A) GCT	Lys (K) AAA	Ala (A) GCG	Ala (A) GCC
Mutant*	Phe (F) TTT Ala (A) GCT Cys (C) TGT	Gly (G) GGT	Glu (E) GAA	Gly (G) GGG	Ser (S) TCC Thr (T) ACC

* indicates altered sequences and the resultant amino acid residue at a given SNP locus

PCR products were spotted in a 12 by 8-grid and cross linked onto nylon membranes and probed for sequence polymorphisms by hybridisation to specific oligonucleotide probes described previously¹⁶. For analysis of samples collected in 2000, the visualization of hybridised digoxigenin labelled probes on membranes was performed by the alkaline phosphatase-catalysed breakdown of the CSPD substrate (Roche Boehringer Mannheim, Mannheim, Germany) and visualised by exposure on Hyperfilm-ECL (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, United Kingdom), according to Boehringer Mannheim recommendations and previously described by Conway *et al*⁷. For analysis of samples collected in 2006 the probed blots were

visualised using ECF substrate and detection using a phosphoimager (STORM). Inspection of autoradiographic films was carried out by light box illumination, while the phosphoimager output was recorded through viewing of digitally captured images of chemifluorescent signal. The change in the method by which probe hybridisation signal was visualised did not affect the results in any way since the probes and hybridisation conditions were unchanged.

The stringency and specificity of the hybridisation process was confirmed by inspection of a series of 4 controls with a known single genotype variant sequence. All blots with non-specifically bound probes were stripped and re-probed. A SNP was considered to be present in the PCR product when the intensity of signal was higher than that of the background. The blots were scored independently by two people.

In our analysis we aimed to establish the relative abundance of different point mutation haplotypes at *dhfr* and *dhps*. Since bloodstage *P. falciparum* is haploid, this is very straightforward when an infection consists of a single genotype because only one form of sequence at every SNP locus is seen. When infections are composed of multiple genotypes a mixture of different sequence variants occur making the inference of point mutation haplotypes within that infection more difficult.

The presence, absence, and relative abundance of hybridisation signal for every probe were recorded at each locus. A sample was considered to have a single haplotype when only one sequence variant was found at each locus. Blood samples were categorised as having a single, a majority or a mixture of sequence at every SNP locus. Majority and mixed genotype infections were differentiated according to the relative intensity of signal. To determine the relative abundance of different point mutation haplotypes in the parasite population, one haplotype only was counted from each infection and those mixed infections where haplotypes could not be resolved were omitted from the calculation of haplotype frequencies. Hence, frequency data is based upon a subset of isolates which were unmixed or had a predominating majority haplotype. A breakdown of the proportions of isolates which successfully PCR amplified and which were genotyped as single, majority or mixed haplotype infections is given in Table 2.

Statistical Analysis

Statistical comparison of allele frequencies at *dhfr* and *dhps* in the various sites was carried out using chi squared analysis in STATA version 9.2. The calculation of binomial exact 95% confidence intervals was carried out using STATA version 9.2. Linkage disequilibrium analysis was performed using Arlequin software¹⁸.

Role of the funding source

This study was funded through an interagency agreement between the United States Agency for International Development (USAID) and CDC and a cooperative agreement between CDC and the Ifakara Health Research and Development Centre (IHRDC). USAID did not participate in the design, collection, analysis, or interpretation of the data, in the writing of the report, or in the decision to submit for publication.

Results

Of the 6,301 people sampled in both 2000 and 2006 cross sectional surveys, 1,044 (17%) were asymptotically infected with *P. falciparum*. DNA was extracted from the 1,044 *P. falciparum* positive bloodspots and PCR amplification of *dhfr* and *dhps* performed, giving a combined rate of PCR amplification success of 73% for both genes (Table 2). The amplified products were screened for all the variant sequences described in Table 1. Out of the 774 isolates which amplified successfully for *dhfr*, 84% were single or majority genotype infections and the point mutation haplotypes could easily be determined. Of the 768 samples which amplified successfully for *dhps*, 77% were single or majority genotype with determinable haplotypes.

Allelic Haplotypes at *dhfr* and *dhps* genes

Three point mutations, N51I, C59R and S108N were found in the *dhfr* gene. Their haplotypic arrangements were CNCNI, CNCNI, CNRNI, CICNI and CIRNI. This pattern of point mutations is common throughout east Africa and has been previously reported in Tanzania¹⁶, Malawi¹⁹, Kenya^{20, 21} and Uganda^{22, 23}. One rare combination of mutation; N51I with C59R (CIRSVI) (previously reported in Uganda²²) was found in two individuals in this study.

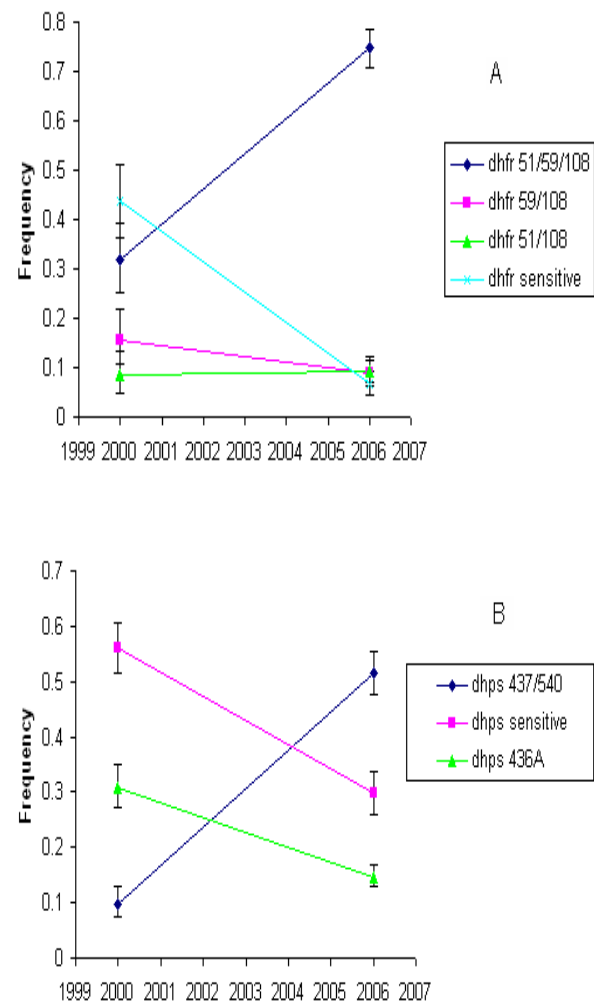
Table 2: Proportion of samples that were *P. falciparum* positive, PCR amplified *dhfr* or *dhps*, and single/majority *dhfr* or *dhps* in Morogoro-Mvomero

Item\Year	2000	2006
Survey population	1681	4620
<i>P. falciparum</i> positive	347 (21%)	697 (15%)
PCR amplified <i>dhfr</i>	246	528
PCR amplified <i>dhps</i>	244	524
Single or majority <i>dhfr</i>	180	471
Single or majority <i>dhps</i>	165	430
Single or majority <i>dhfr+dhps</i>	98	380

Five *dhps* mutations (S436A, S436F, S436C, A437G, and mely low frequency and have not been reported before, presumably because of their rarity. This is a reflection of the larger sample size collected in this study and the fact that some alternative typing methodologies do not have the capacity to routinely detect codon 436 SNP's, which normally occurs in a very rare frequency. Most of the typing methodologies routinely detect codons 437 and 540 SNP's.

The *dhfr* and *dhps* allelic frequency change over time in Morogoro was estimated by comparing isolates sampled at two time points, 2000 and 2006 (Figure 1). The *dhfr* triple mutant (CIRNI) and the *dhps* double mutant (SGEAA) haplotypes are the two alleles with greatest significance for SP efficacy. During the 2000 - 2006 period, the triple mutant *dhfr* allele frequency rose from 0.32 to 0.75 ($P \leq 0.0001$), dramatically displacing the sensitive *dhfr* allele frequency from 0.44 to 0.07 ($P \leq 0.0001$). The escalation of *dhfr* triple mutant in the population was also associated with significant displacement of *dhfr* double mutant (CNRN) haplotype frequency from 0.16 to 0.09 ($P \leq 0.02$). However, the *dhfr* double mutant (CICN) haplotype, did not change maintaining a constant frequency of 0.09. At the *dhps* gene, there was an escalation of the double mutant (SGEAA) haplotype by a five fold increase from 0.10 to 0.52 ($P \leq 0.0001$), displacing both the sensitive *dhps* and a single mutant (S436A) *dhps* haplotype from 0.56 to 0.30 ($P \leq 0.0001$) and from 0.31 to 0.15 ($P \leq 0.0001$), respectively (Figure 1b).

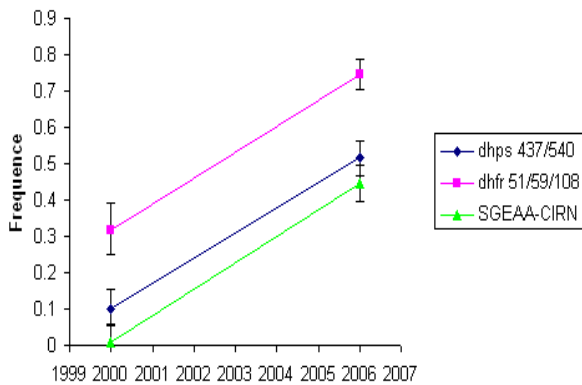
Figure 1: Temporal allele frequency trend in Morogoro. A) *dhfr* alleles and B) *dhps* alleles



Two Locus Genotypes

In a further subset of samples for which *dhfr* and *dhps* haplotypes could both be conclusively determined it was possible to measure the frequency of two locus genotype combinations of double *dhps*-triple *dhfr* (SGEAA-CIRN) mutant. Figure 2 shows change in frequency of this genotype in Morogoro population. Out of 98 *dhfr* - *dhps* two locus genotype combinations identified in 2000, only one was SGEAA-CIRN, while in 2006, 169 of the 380 *dhfr* - *dhps* two locus genotype combinations were SGEAA-CIRN. This estimate indicates a 45 fold increase of this genotype (1% - 45%) in six years time period. The SGEAA-CIRN genotype is predictive of *in vivo* SP failure²⁴.

Figure 2: Temporal trend of the alleles with greatest significance for SP efficacy, the double *dhfr* and triple *dhfr* mutant and their combination in Morogoro

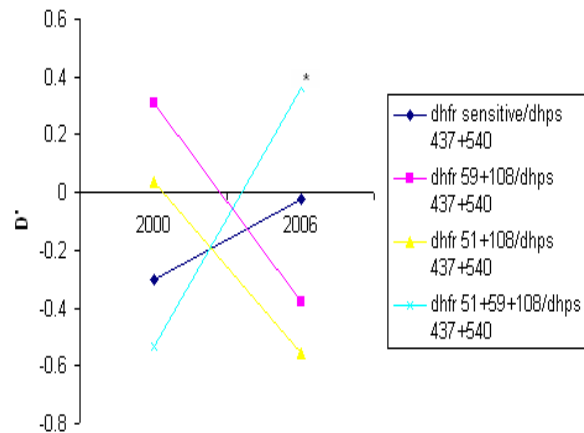


Linkage Disequilibrium

To examine the effects of simultaneous selection by pyrimethamine on *dhfr* and sulphadoxine on *dhps* we looked at two-locus genotypes sampled from Morogoro-Mvomero population in the two surveys. Taking the subset of samples for which point mutation haplotypes could be unequivocally resolved at both genes, we compared the observed with expected frequencies generated from contingency tables. Significant association between the *dhfr* triple mutant allele and the *dhps* double mutant allele occurred in 2006 but not in 2000 survey. The d' value an index of linkage disequilibrium are shown in Figure 3 which examines linkage between all *dhfr* alleles and the *dhps* A437G+K540E double mutant allele. There was a non-significant negative association between *dhfr* triple mutant allele and the *dhps* double mutant allele, and non-significant positive associations between *dhfr* double mutant alleles and the *dhps* double mutant allele in 2000. The strength of the association between *dhfr* triple mutant and *dhps* double mutant alleles increased in 2006 with ≤ 0.36 and chi square analysis showed this was highly significant ($p < 0.0001$). The non-significant positive association between *dhfr* double mutant alleles and *dhps* double mutant alleles during 2000 became negative in 2006 (Fig. 3).

Figure 3: Linkage disequilibrium between the *dhps* A437G+K540E allele and *dhfr* alleles

The d' values for 2000 ($n = 98$) and for 2006 ($n = 380$) are shown. Significant deviation between observed from expected occurred in 2006 indicated by $*(p < 0.0001)$



Discussion

We have demonstrated the SP drug pressure-driven escalation of sulphadoxine and pyrimethamine resistance in Morogoro-Mvomero district in south eastern Tanzania between 2000 - 2006. This escalation was coincident with change of national policy from CQ to SP first line treatment of uncomplicated malaria in August 2001, leading to shift in treatment practice as reported by house-hold survey of antimalarial drug use, which indicated sharp rise of SP use²⁵.

Morogoro and Mvomero are two of the six districts making up the Morogoro region. They both were one district of Morogoro when we conducted the 2000 survey, but later in 2002 divided into two and hence are broadly similar in many aspects. They are well matched in terms of predicted intensity and duration of malaria transmission and risk (MARA), relative access and overall utilization of health services such as IMCI (based on surveys) and fairly usage of insecticide treated nets (ITNs).

SP was approved a national second line treatment of uncomplicated malaria in 1983. Our data indicate it took 17 years of SP second line use for the most resistant triple *dhfr*-double *dhps* mutant genotype selection to attain a frequency of 1% in Morogoro-Mvomero population in 2000. Contrastingly, it took only five years for the genotype

to rise to 45% under SP first line selection (2001 – 2006) ($Pd^{*}0.0001$). The frequency of triple mutant *dhfr* and double mutant *dhps* resistance alleles rose by 32% and 10%, respectively in 17 years of SP second line use, but the selection strength was more potent during its first line use where the two resistant alleles rose rapidly by 43% and 42% in only five years ($Pd^{*}0.0001$). The increase of both the double mutant *dhps* and the triple mutant *dhfr* displaced alleles with lower fitness by virtue of their resistance properties at both genes. At the *dhfr* locus, the sensitive allele was displaced from 44% to 7% ($Pd^{*}0.0001$) in five years while the *dhfr* double mutant (C59R+S108N) was displaced from 16 % to 9% ($Pe^{*}0.05$). Surprisingly the *dhfr* double mutant (N51I+S108N) remained unchanged throughout maintaining a constant frequency of 10%. At the *dhps* locus, the rise of double mutant allele (A437G+K540E) displaced the sensitive allele from 56% to 30% ($Pd^{*}0.0001$) and the single mutant allele (S436A) from 30% to 15% ($Pd^{*}0.0001$). The contrasting response of both *dhfr* and *dhps* alleles to the increase of SP selection pressure following its widespread use as first line treatment of malaria, likely reflect their different contribution to the SP resistance. Under the population genetics perspective, for the successful spread of a resistance allele, it must be transmitted at a faster rate than the sensitive form. Since the transmission of both double mutant *dhfr* and single mutant *dhps* S436A was by far lower than the *dhfr* triple and *dhps* double mutant alleles, it is likely that they play a peripheral or transient role in the pyrimethamine and sulfadoxine resistance.

LD between the *dhfr* triple and *dhps* double mutants is an indirect measure of effective selection pressure. The combination of the two alleles confers greater fitness to the parasite than each allele separately, thus enhancing the chance of better survival of the parasite in the face of SP drug pressure. The two locus combination of the *dhfr* triple mutant and the *dhps* double mutant has been shown to have statistical association with treatment failure^{21, 24, 26}. The LD measured in 2000 indicates it was not statistically significant and its interpretation suggests the selection applied when SP was a second line was insufficient to keep the two unlinked alleles in association. By contrast, a strong association between the two alleles emerged in the 2006, confirming potent selection strength applied by SP first line. We predict that, following withdraw of SP as national first line antimalarial drug, the drug selection pressure will most likely decrease substantially as SP use remain

limited to specific programmes only. This decrease in drug selection pressure will disrupt the association between the triple mutant *dhfr* and double mutant *dhps* alleles leading to loss of LD.

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Conclusion

This report has clearly shown that the population rate of change of resistant *dhfr* and *dhps* alleles is contingent to the SP usage in the population. Greater rate of change was observed when SP was a national first line than when was the second line antimalarial treatment policy. The 2006 *dhfr* and *dhps* allele frequencies reported here will be useful in the monitoring of rate of reversal of resistant forms of these alleles following withdrawal of SP use as a first line treatment in Tanzania in the late 2006.

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Erratum:

Prevalence of overweight and obesity in young adults in Uganda

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The author's correct initials are **Kabagambe EK** and NOT Kabagambe KE