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High Prevalence of Molecular Markers of *Plasmodium falciparum* Resistance to Sulphadoxine–Pyrimethamine in Parts of Ghana: A Threat to ITPTp-SP?

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ABSTRACT

Malaria in pregnancy is a huge public health problem as it is the cause of maternal anaemia, still birth, premature delivery, low birth weight among others. To tackle this problem, WHO recommended the administration, during pregnancy, of intermittent preventive treatment with sulphadoxine–pyrimethamine (IPTp-SP). The introduction of this policy is likely to create SP drug pressure which may lead to the emergence of parasite strains resistant to the drug. This study investigated the prevalence of the molecular markers of SP resistance as pointers to potential failure of IPTp-SP among pregnant women attending antenatal clinic, women at the point of baby delivery and out patients department (OPD) attendees. The study was conducted in health facilities located in parts of Ghana. Prevalence of mutations in *dhfr* and *dhps* genes of *Plasmodium falciparum* was determined using the method described by Duraisingh *et al*. The outcome of the study indicated the presence of high prevalence of strains of *P.falciparum* with the resistant alleles of the *dhfr* or *dhps* genes in the three categories of participants. There was a high prevalence of triple mutations (IRN) in the *dhfr* gene of *P.falciparum* isolates: 71.4% in peripheral blood of antenatal attendees; 74.1% in placenta cord blood of delivering mothers and 71.1% in OPD attendees. Quintuple mutations were only found in 2 (0.5%) isolates from OPD attendees. This observation might have occurred due to the increased use of SP for IPTp among others. There is the need for an interventional measure in order to protect pregnant women and their unborn children.

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LAY SUMMARY

When pregnant women get infected with the malaria parasites they are exposed to all manner of dangers including pre-term delivery, still birth, maternal anaemia and low birth weight. Taking sulphadoxine–pyrimethamine (SP) at predetermined periods during pregnancy, referred to as 'intermittent preventive treatment with SP' (IPTp-SP)' helps to curtail these problems. However, the frequent taking of these drugs is likely to create SP drug pressure which may lead to the emergence of parasite strains that are not readily killed by the drugs. In order to ascertain this phenomenon and advice stakeholders, this study determined the prevalence of certain 'materials' certified as markers of parasite resistance to SP. Alarmingly, more than 5% of all the category of women recruited to participate in this study were found to harbour the parasites that causes malaria. The outcome, also suggest the existence of high levels of strains of the malaria parasite, carrying the materials that make them to become resistant to SP. Policy makers must pay attention to these observations and institute measures to avoid escalation of the situation.

KEYWORDS: sulphadoxine–pyrimethamine, mutation, genotype, prevalence, pregnancy, *Plasmodium falciparum*, intermittent preventive treatment

BACKGROUND

Apart from children under 5 years, another group that is vulnerable to malaria in terms of risk and outcomes of infection is pregnant women. This is because, compared with non-pregnant, pregnant women attract twice the number of *Anopheles gambiae*, the predominant vector for the *Plasmodium* parasite in Africa [1]. Pregnancy is also associated with 4-fold increased risk of malaria infection and a 2-fold risk of mortality from malaria. The physiology of pregnancy and the pathology of malaria infection have a synergistic effect on each other's course. The consequences of all these means, malaria infection in the pregnant women is direr.

Globally, some 125 million pregnancies are said to be at risk of malaria infection annually [2, 3] of which majority live in sub-Saharan Africa. Malaria in these women does not pose a threat only to themselves, but to their unborn babies as well. Malaria in pregnancy is a known cause of some 400 000 cases of maternal anaemia which contributes to an estimated 10 000 maternal deaths per year [4]. It is also known to cause about 50% of all low birth weight, therefore contributing significantly to neonatal and infant morbidity and mortality. An estimated 75 000–200 000 infants die annually due to malaria in pregnancy [3, 4].

To control malaria in pregnancy, the WHO recommended the intermittent preventive treatment of malaria in pregnancy using SP (IPTp-SP) in addition to the use of insecticide-treated nets, and effective case management of malaria and anaemia during pregnancy. IPTp-SP consists of the administration of curative doses of SP (500/25 mg) at determined intervals during pregnancy. SP is given very early during the second trimester and 1-month interval, up to the time of delivery. The drug is administered under the direct observation of healthcare giver during antenatal clinics regardless of the malaria status of the pregnant women. IPTp-SP is said to decrease peripheral and placental parasitaemia, improves maternal haemoglobin and birth weight of neonates, especially in primi and secundigravidae [4].

SP act by inhibiting the enzymes *Dihydrofolate reductase (dhfr)* and *Dihydroptroate synthetase (dhps)* respectively, during folate biosynthesis in the *Plasmodium* parasite. Parasite resistance to SP is attributable mainly to mutations in the genes coding for these enzymes. Although mutations at codons N51I, C59R and S108N of the *dhfr* gene are associated with pyrimethamine resistance, mutations at codons A437G and K540E of the *dhps* gene lead to sulphadoxine resistance. High prevalence of these molecular markers of resistance of *Plasmodium sp.* to SP can therefore threaten the IPTp-SP programme.

The degree of resistance for which IPTp-SP can be said to be ineffective is unknown. The WHO however recommends that, IPTp-SP can be implemented in malaria-endemic regions of the world with a prevalence rate of mutant K540E <50% and A581G <10% [5], or in countries where SP failure rate among children is <50% with other

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interventions such as ITN usage [6]. In Ghana, SP has been observed to have a failure rate of 28% when used for treatment of malaria in children [7]. IPTp-SP may however be more effective because of pre-immunity to *Plasmodium* parasites among pregnant women living in endemic regions such as Ghana. Triple mutations in the *dhfr* gene (N51I, C59R and S108N) are said to be responsible for SP resistance in Ghana. They confer about 10-fold increase risk of treatment failure. *Dhps* mutations appear not to be predictive of SP resistance among children in Ghana [8].

Increased prevalence of SP resistance markers does not seem to be the only predictor of SP-IPTp failure. However, it is a significant contributory factor and a threat to the overall success of the IPTp programme, as shown in studies in the eastern parts of Africa. Indeed, there are increasing reports of SP resistance in children and pregnant women spreading from the eastern parts of Africa to West Africa as a result of increasing prevalence of molecular markers of SP resistance. This study therefore ascertained the prevalence of the markers of SP resistance of *Plasmodium falciparum* in order to provide a baseline information for the documentation of resistance strains in the study area which can be extrapolated to all malaria endemic areas. This information will serve as an early warning signal to alert public health managers of the potential threat posed to SP-IPTp by increasing prevalence of resistant markers in areas of intense malaria transmission. The parasite isolates were collected from peripheral blood of parasitaemic pregnant women at the antenatal clinic (ANC) and from cord blood of delivering mothers. In order to know the extent of circulation of these mutations in the general population, the prevalence of the mutations were also determine among OPD attendees.

MATERIALS AND METHODS

Study population

The study was carried out in Sekondi–Takoradi, the administrative capital of the Western Region of Ghana (Fig. 1). It is located in the South-Eastern part of the Western Region and lies on latitude 4.91°N and longitude 1.77°W. The climate of the Metropolis is equatorial, with an average annual temperature of about 22°C. Rainfall is bi-modal, with the major season occurring between March and July and the minor season occurring between August and November. The mean annual rainfall is about 1380 mm [9].

Malaria transmission rate in this area is nearly stable all year round with rates increasing in the rainy seasons, mainly in the months of May–July. The incidence of malaria in the general population is 28.7%, whereas that in pregnant women is 1.84%.

Two health facilities were chosen. These were the Takoradi and the Essikado Hospitals. Takoradi Hospital is the metropolitan hospital with an obstetric bed capacity of 18. The average monthly antenatal attendance is about 1200 and average monthly new antenatal attendees are 120. The Essikado hospital is a well-attended sub-district hospital. It serves mostly women in the relatively lower socio-economic suburbs of the metropolis. The average monthly ANC attendance is about 1050, with 100 of them being new attendees.

The study was a hospital-based cross sectional study. It was conducted during the rainy season in April–June. The study involves three types of participants. The first group were pregnant women of any age and parity who were at 16–26 weeks of gestation, yet to receive their first SP dose. The second group of participant were women in the delivery room: Placenta cord blood from these women during delivery were collected and used in this study. The third group comprised of attendees at the general OPD who were screened for participation on the day of recruitment. There was no age limit for this group of participants.

Sample collection and diagnosis of malaria

Procedures for obtaining the samples were adequately explained to all participants in each group and informed consent sought. After an aseptic preparation, about 3 ml of blood (peripheral venous blood in the ANC and general OPD attendees and cord blood in the case of the delivering mothers) was obtained from each participant using a sterile disposable hypodermal syringe fitted with a 23-gauge needle and dispensed from the syringe barrel into sterile Ethylenediaminetetraaceticacid tubes for further processing. The samples obtained were used for

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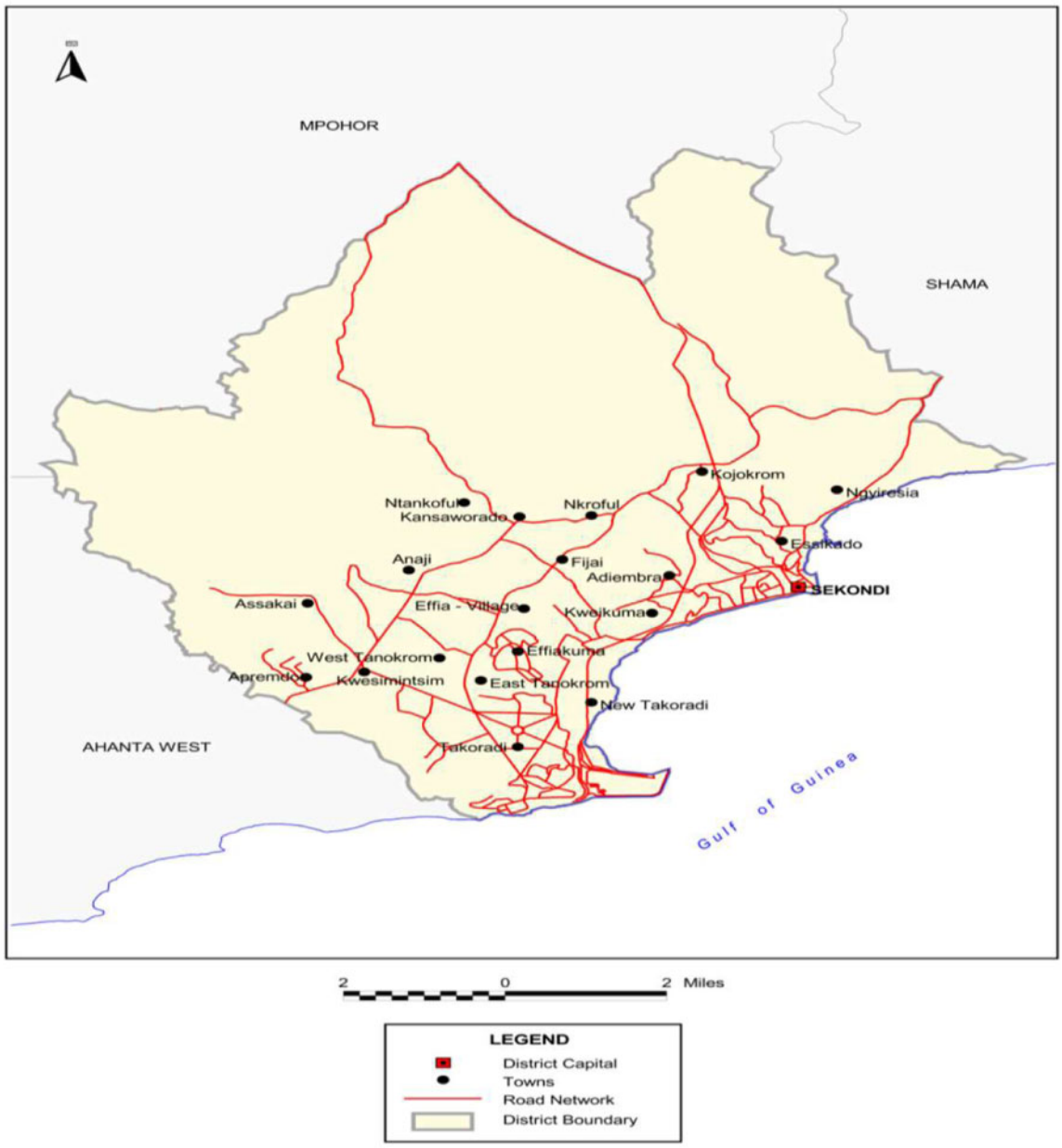

Fig. 1. Map of Sekondi–Takoradi metropolis. (Source: Ghana Statistical Service report, 2014).

diagnosis of malaria by Malaria Rapid Diagnostic Test as well as microscopy after staining with Geimsa. About 10 µl of the sample blood was blotted on Whatman filter papers into circular spots. These spots were allowed to air dry at room temperature for at least 4 h until they became uniformly dark brownish in colour. Each of these was put into a single, clean, sealed and gas-impermeable plastic envelopes to avoid contamination. They were, appropriately labelled and stored at room temperature for DNA extraction and subsequent molecular analysis, not more than 14 days thereafter.

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Diagnosis by rapid diagnostic test (RDT)

The SD Bioline Malaria Ag Pf® test kit was used. This detects Histidine Rich Protein-2.

Both negative and positive controls were set for each box of test kits to ensure reliability of kits. About 5–10 µl of whole blood sample was delivered into the sample well. About 60 µl of assay buffer provided was then delivered into the buffer well. Test was allowed to run for 15–20 min at room temperature. A test was said to be positive when two rose-pink colour bands were seen at both the control (C) and test (T) labels. If the colour band was seen at only the control label, then the test was said to be a negative test. Test was said to be invalid if there were no bands at both the control and test labels.

Diagnosis by microscopy

Thick and thin blood smears were prepared for all samples and stained with Geimsa. The thin blood films were used for speciation. The thick films were used to estimate the parasite density. Malaria parasites were counted against 300 leucocytes. Parasite densities were expressed per microlitre using an assumed count of 8000 white blood cells (WBCs) per microlitre of blood. Parasite Density = 8000 WBC/µl × Parasites counted against 300 WBC. To declare a film as negative (without parasites), minimum of 100 high power fields were examined. Slides were read independently by two experienced microscopists. If there were disparities in any result, a third and a more experienced microscopist read the slide. The result of the third microscopist was deemed final.

Diagnosis by polymerase chain reaction (PCR)

Detection of malaria infection and identification of *Plasmodium* species were done using species specific primer nested PCR amplification of the genomic DNA. DNA was extracted by the classic Chelex method. Extracted DNA was stored at −32°C until used. Species specific PCR was conducted on all samples. It involved the use of both genus and species-specific primers in a 25 µl reaction mixture in all cases. This consisted of 12.5 µl of the DreamTaq green® premix; 0.3 µl each of the forward and reverse primers; 10.2 µl nuclease free water and 2 µl of extracted DNA. The Nested 1 PCR involved the use of the genus-specific primers; rPLU1: 5′ TCA AAG ATT AAG CCA TGC AAG TGA3′ and rPLU5: 5′ CCT GTT GTT GCC TTA AAC TCC3′ under the following reaction conditions: Initial denaturation at 94°C for 4 min; 35 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 1 min and extension at 72°C for 1 min. Final elongation was at 72°C for 4 min. Nested 2 amplifications involved the use of *P. falciparum* specie specific primers: rFAL1: 5′ TTA AAC TGG TTT GGG AAA ACC AAA TAT ATT3′ and rFAL2: 5′ ACA CAA TGA ACT CAA TCA TGA CTA CCC GTC3′. Reaction conditions for Nested 2 were identical to that of Nested 1 except that the annealing temperature in Step 3 was 56°C. PCR mixture without a DNA template was used as negative control and PCR-confirmed *P.falciparum* 3D7 laboratory clones donated by Professor Neils Quashie, Noguchi Memorial Institute for Medical Research, University of Ghana was used as reference. The PCR products were electrophoresed on 2% agarose gel stained with ethidium bromide. The gel was visualized in a digital ultra violet (UV) light gel documentation system.

Detection of molecular markers of resistance

Detection of mutations in the *dhfr* and *dhps* genes was done using the protocol described by Duraisingh *et al.* [8] with slight modification. The method involves nested PCR amplification of the genes, followed by enzyme restriction digestion. Primers used for the PCR included those so designed to allow detection and distinction of the polymorphisms in the *dhfr* and *dhps*. For this study, our main interest was for detection of mutations in S108N, N51I and C59R (the so called triple mutations in the *dhfr* gene responsible for pyrimethamine resistance) and K540E and A437G mutations in *dhps* gene responsible for sulphadoxine resistance. All primers were purchased from The Midland, Certified Reagent Company, TX, USA. The PCR reaction mixtures for both nested 1 and 2 were made to a volume of 25 µl; comprising 12.5 µl of the Gotaq green premix, 10.2 µl of nuclease free water, 0.3 µl of both the forward and reverse primers and 2 µl of the DNA template. Primers and reaction conditions were as shown. For *dhfr* genes, primers for *Nest 1*: **M1**5′ TTTATGATGGAACAAGTCTGC-3′ and **M5**5′ AGTATATACATCGCTAACAGA-3′. Reaction conditions were: initial denaturation at 94°C for 3 min;

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then 94°C for 1 min, annealing at 45°C for 1 min and extension at 72°C for 1 min, repeated for 40 cycles, with a final extension step at 72°C for 10 min. Primers for Nest 2 were: **M3**5′ TTTATGATGGAACAAGTCTGC GACGTT3′, **F**/5′ AAATTCTTGATAAACAACGG AACCTttTA3′,**F**5′GAAATGTAATTCCCTAGATAT GgAATATT3′, **M4**5′ TTAATTTCCCAAGTAA AACTATTAGAgCTTC3′. Reaction conditions for Nest 2 were same as Nest 1 above, only that in this case, the first five annealing steps were carried out for 2 min.

For the *dhps* gene, primers for Nest 1 reaction were: **R2**5′AACCTAAACGTGCTGTTCAA3′and **R**/5′AA TTGTGTGATTTGTCCACAA3′. Reaction conditions were; initial denaturation at 94°C for 3 min; then 94°C for 1 min, annealing at 45°C for 1 min and extension at 72°C for 1 min, repeated for 40 cycles, with a final extension step at 72°C for 10 min. Primers for *dhps* Nest 2 reactions: **K**5′ TGCTAGTGTTATAGATAT AGGatGAGcATC3′, **K**/5′ CTATAACGAGGTATTg CATTTAATg CAAGAA3′. Reaction conditions for Nest 2 were same as in Nest 1, only that in this case, the first five annealing steps were carried out for 2 min.

Enzymatic restriction digestions were carried out overnight. The enzymes Tsp509I and BsrI were used to digest at codon 51 and 108 on the *dhfr* gene, respectively. XmnI was used to digest the gene at codon 59 of the *dhfr* gene. Codons 437 and 540 on the *dhps* genes were digested with enzymes MwoI and FokI, respectively. In all cases, reaction volume was 20 µl comprising10 µl of the PCR product, 8.5 µl of nuclease free water, 1 µl of buffer and 0.5 µl of the restriction enzyme. The external control used was the 3D7 laboratory strain of *P.falciparum* also supplied by Professor Neils Quashie of Noguchi Memorial. However, the primer designs used also provided for internal controls in the PCR products after Nest 2 amplifications.

Gel electrophoresis was done on 2.5% agarose gel stained with ethidium bromide. A total of 100 bp molecular markers were used to estimate the sizes of the band. The gel was visualized in a digital UV light gel documentation system.

Data analysis

Data generated in this study were entered into STATA version 13.0. Prevalence of mutations in *dhfr* I51, R59, N108, and *dhps* G437 and E540 was determined. Prevalence of the quintuple mutant, which involves mutants of the above named five codons from the two genes (synergistically confers *in vivo* SP failure) was also determined.

RESULTS

The number of pregnant women screened and recruited at the ANC and during delivery to participate in the study is shown in Table 1. Detailed information on the selection of OPD attendees is also shown in Table 1. The outcome of the three methods used for detection of the presence of *P.falciparum* is shown in the table. The number of samples that were successfully typed, using PCR, for the presence of the *dhfr* and *dhps* is also indicated in Table 1. Per PCR analysis, 13% and 8.5% pregnant women screened at ANC and in the delivery room respectively were positive for the presence of *P.falciparum*. This compares with 22% recorded for OPD attendees.

Table 2 is a summary of the outcome of the molecular analysis of the samples. Generally, the results indicate a very high prevalence of single mutations at codons 108, 59 and 51 on the *dhfr* gene and moderate prevalence at codons 540 and 437 on the *dhps* gene.

DISCUSSION

The high level of *P.falciparum* infection observed among pregnant women in this study (Table 1), especially in the placenta cord blood should be of concern to stake holders as it once again emphasize the danger to which women are exposed during pregnancy in malaria endemic areas of the world. This situation calls for strengthening of existing control measures and the formulation of other innovative ways to mitigate or prevent malaria infection during pregnancy. Persistence of asymptomatic parasitaemia could also result in the reservation of a pool of parasites which would sustain malaria transmission and thereby thwart the efforts of the malaria control strategy geared towards disease elimination.

Overall, very high prevalence of single mutations at codons 108, 59 and 51 on the *dhfr* gene and moderate prevalence at codons 540 and 437 on the *dhps* gene

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Table 1. Summary of samples screened

	Total number of samples screened	RDT positives	Microscopy positives	PCR positives	Number that amplified *dhfr* and *dhps* genes
ANC Attendees	413	57	42	54	54
Placenta blood	424	21	18	36	27
OPD Attendees	2891	513	521	549	529
Total	3728	591	581	639	610

Table 2. Summary of outcome of the molecular analysis

Mutations	Source of blood sample/percent (%) prevalence of genotype		
	ANC attendees ($n = 54$)	Placenta blood ($n = 27$)	OPD attendees ($n = 529$)
Single mutation			
N108	85.7	92.3	92.9
I51	83.3	85.2	82.3
R59	73.8	88.9	87.6
E540	7.1	7.4	6.2
G437	19.1	22.2	20.3
Double mutations			
IN	78.6	70.3	73.8
RN	81	77.8	75.3
IR	73.8	70.3	67.6
GE	0	3.7	0.95
Triple mutation			
IRN	71.4	74.1	71.1
Quadruple mutations			
IRNG	11.9	11.1	13.3
IRNE	0	3.7	3.7
Quintuple mutation			
IRNGE	0	0	0.5

were observed in this study (Table 2). When compared with the 71.1% prevalence observed in the OPD attendees, the prevalence of the triple mutations in *dhfr* 108, 59 and 51 could be said to be similar to that reported by Mockenhaupt *et al.* in 2006; but much higher than the 22% reported by Duah *et al.* [10] in 2008 from a study carried out in Tarkwa (closest major town to Takoradi) and the 11.4% observed by Tahita *et al.* [11] in Burkina Faso in 2015. Reasons for these observed differences in prevalence are not quite clear, but could be attributed to factors such as geographical location, differences in drug pressure, sample size of the different studies, among others. The prevalence of mutations in *dhfr* observed in this study is lower when compared with observations from eastern Africa where the general increase in the prevalence of *dhfr* and *dhps* mutant genes and SP resistance is rife. For instance, in Angola, report indicate that mutations in N108, I51 and R59 were evident in 100%, 93% and 57% of isolates, respectively [9] and in Tanzania, 100%, 100%, 100% and 100% of isolates for R59, N108, S436/G437 and E540, respectively, in 2007 from a low transmission area [12]. Data from Uganda also

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showed about 99%, 99% and 57–94% of isolates, respectively for the N108, I51 and R59 mutations [13].

The prevalence of mutations in codons of the *dhps* were observed as 20.6% at codon G437 and 6.3% at codon E540 and the prevalence of double mutations at codons GE was 1.0%. These frequencies were lower compared with the observations made in the *dhfr* genes. These relatively lower frequencies seem to be the trend in west and central Africa [ ]. In eastern and southern Africa however, *dhps* mutations have similar or sometimes higher prevalence compared with *dhfr* mutations [9, 12]. A little over 13.0%, quadruple mutations were found in the sample set at codons I51, R59, N108 and G437 (IRNG) and 3.7% were found at codons I51, R59 N108 and E540 (IRNE).

Mutation in *dhfr* and *dhps* is stepwise, with every additional point mutation adding to the intensity of resistance to SP. The 13% prevalence of quadruple mutations is high and therefore increases the risk of resistance of parasites to SP. It is however lower than the 44% and 25% reported by Duah *et al.* for forest and savannah zones, respectively for parasites isolated between 2007 and 2010, respectively in Ghana. However as noted earlier SP efficacy may not only be dependent on the prevalence of mutations in the *dhfr* and the *dhps* genes but several other factors. Thus said, this high prevalence of mutations are contributors of treatment failures with SP and therefore should alert public health policy makers as to the need for preparedness for options to SP as IPTp.

Quintuple mutation (IRNGE) is known to be associated with high-grade parasite resistance to SP [14]. In this study, two (2) of the parasites isolated showed the presence of quintuple. The Mockenhaupt study also reported the presence of quintuple mutation in one isolate. It does not appear therefore that the prevalence of the quintuple mutation is fixed or increasing significantly across the country as it is the case in most places in eastern and southern Africa [15, 16].

CONCLUSION

The outcome of the study indicated the presence of high prevalence of strains of *P.falciparum* with the resistant alleles of the *dhfr* or *dhps* genes in the three categories of participants.

This observation could be as a consequence of SP drug pressure created by an increased use of SP for IPTp as well as the unauthorized use of the drug for the treatment of malaria or fever.

The high prevalence of the resistance marker among pregnant women, which may not be any different in disease endemic areas of the world stocking SP, should be of concern to the relevant authorities.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was conducted according to the Helsinki Declaration on Research regarding human subjects. The protocol for the study was reviewed and approved by the Ghana Health Service's Ethical Review Committee (GHS-ERC). Approval was also sought and given by respective Medical Superintendents of the health facilities used in the study. Participants' consent was also sought before taking blood samples.

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