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Analysis of Sulphadoxine-Pyrimethamine Drug Resistance in *Plasmodium falciparum* from Khurda District of Odisha, India.

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ABSTRACT

Around 1.0million confirmed cases of malaria are annually reported in India of which about 50% cases reported are due to *Plasmodium falciparum*. Odisha alone contributes 25% of malaria cases and 46% of *P. falciparum* (*Pf*) cases reported in India. Since 2010, Sulphadoxine-Pyrimethamine (SP) is being used as first line drug in combination with Artesunate in India. To investigate the resistance of *P. falciparum* to SP if any, in *Pf* positive blood collected from Banpur Community Health Centre (CHC) of Khurda district of Odisha. Genes for dihydrofolate reductase (*dhfr*) and dihydropteroate synthase (*dhps*) were used as resistance marker genes. Genomic DNA was extracted from collected blood samples followed by polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) to detect the SP resistant-associated point mutations on *dhps* and *dhfr* genes. In the present study, 5 codon sites (16,51,59,108 and 164) of *dhfr* gene and 4 codon sites (436,437,540 and 581) of *dhps* gene were considered. It was observed that, mutations were not found in the codons of *dhfr* gene. Similarly, in *dhps* gene, there was no mutation in the 436 and 540 codons, rather Gly-437 and Gly-581 mutations were found in 33.3% and 100% of samples respectively. Mutations at both of these codons indicate substitution of the amino acid glycine in place of alanine and confirm the existence of *P. falciparum* resistance to sulphadoxine. Study on a larger population is required to confirm our findings on the status of Sulphadoxine drug resistance at different time interval in this area. Further, constant surveillance throughout the country for the detection of development of antimalarial drug resistance is highly essential.

Keywords: *Plasmodium falciparum*, drug resistance, Sulphadoxine-Pyrimethamine, Odisha, India.

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INTRODUCTION

Malaria remains one of the major global health problems. Among the Southeast Asian countries, India alone contributes more than 80% cases of malaria [1]. In India, around 1.0 million laboratory confirmed cases of malaria are reported annually, of which about 50% are due to *Plasmodium falciparum* [2].

Odisha, an eastern state of India contributes 25% of malaria cases, 46% of *Plasmodium falciparum* cases in the country [3]. Among four species of *Plasmodium*, *P. falciparum* causes the cerebral malaria, which often leads to death. Management of this disease is dependent largely on antimalarial drugs. Lack of effective vaccines and development of resistance to most commonly used antimalarial drugs in parasite, poses a major problem for malaria control programs [4]. In India, emergence of chloroquine resistance had necessitated the use of alternative antimalarials against falciparum malaria. Hence, artesunate plus sulfadoxine-pyrimethamine was introduced as the second-line drug in 2005 for chloroquine treatment failures and as the first-line antimalarial treatment in areas with documented drug resistance. In 2007, artesunate plus sulfadoxine-pyrimethamine was selected as the first-line treatment in high-risk districts and areas with identified resistance, with the goal of covering most of the nation's *P. falciparum* burden and in 2010 this treatment became the first-line treatment throughout India [5].The drugs interfere with the synthesis of DNA from guanosine triphosphate (GTP) and thus act as folate pathway blockers in the malaria parasite. Pyrimethamine inhibits the dihydrofolatereductase (*dhfr*), whereas sulfadoxine inhibits the dihydropteroatesynthetase (*dhps*) [6,7]. The molecular basis of SP resistance has been well documented [7-11]. Point mutations in *Plasmodium falciparum* dihydrofolatereductase (*dhfr*) are known to give rise to pyrimethamine resistance. The first mutation occurs at the S108N codon, followed by C59R, N51I, and I164L mutations [12]. Previous studies have identified mutations at five *dhps* codons (S436A/F; A437G; K540E; A581G; and A613S/T) to be associated with sulfadoxine resistance in *P. falciparum* [7,8,11,13,14].

This preliminary study was carried out in 2010 to evaluate the frequency of point mutations if any, in *dhfr* and *dhps* among *P. falciparum* isolates from Banpur CHC of Khurda district of Odisha to get a clear picture of the level of drug pressure in the field from the time when SP was introduced as first line drug for malaria treatment in the country and also give information to policy makers for opting to other new effective, cheap and safe antimalarial drug combinations.

MATERIALS AND METHODS

Study area and collection of blood samples

Plasmodium falciparum positive blood samples were collected from Banpur CHC of Khurda district in 2010. The ethical clearance was obtained from Institute's Human Ethical Committee. A total of 25*Pf* positive blood samples were obtained on autoclaved Whatman filter paper no.1 strips and the strips with dried blood spots were stored at room temperature until when needed for further analysis.

Extraction of genomic DNA from *P. falciparum* infected blood spots

Genomic DNA was extracted from dried *Pf* positive blood spots collected on Whatman filter paper by QIAmp mini kit (Qiagen) as per the manufacture's protocol. The extracted DNA from each blood sample was frozen in aliquots at -20°C until amplification by PCR.

Amplification of parasite DNA by PCR

For amplification of the parasite genomic DNA, two primer pairs M1/M5 and R2+R/ were used as forward and reverse primers in primary (Nest I) PCR reaction for *dhfr* and *dhps* genes respectively. Similarly, in secondary (Nest II) PCR reaction M3+F and F+M4 primers were used to amplify the 5 regions on *dhfr* where the point mutation is amplified to occur [15]. On the other hand, K+K/ and L+L/ primers were used to amplify 4 regions on the *dhps* gene where resistance associated mutations are said to occur [16]. For this amplification reaction, detail of primer sequences is shown in Table-1. Total reaction volume in both nest I and nest II PCR was 50 µl. The final concentration of each reagent was 1× PCR reaction buffer (10× PCR buffer-$MgCl_2$, Qiagen, Geneiad), 1.5 mM $MgCl_2$, 200 µMdNTP (Qiagen), 200 nM primers (Operon) and 0.025 U/µlTaq Polymerase (Qiagen). To each PCR tube, 10 µl of DNA was added in nest I PCR and 5 µl was re-amplified in the nest II PCR

reaction. The genomic DNA from laboratory parasite isolates RKL 9 and MZR 2 from Parasite Bank, NIMR, Delhi were used as positive controls. PCR conditions were as earlier mentioned by Mbugi et al., [17]. In brief, for nest I PCR, reaction conditions were 94°C-3 min, 94°C-1 min, 50°C-2 min, 72°C-2 min, ×40, 72°C-10 min, 4°C-hold and for nest II PCR, conditions were 94°C-2 min, 94°C-1 min, 45°C-1 min, 72°C-2 min, ×35, 72°C-10 min, 4°C-hold respectively. Before declaring negative, samples with no detectable PCR products were re-examined at least two times from the DNA preparation. In each isolate all mutations were taken into consideration. The mutation specific primers are listed in Table-1.

Table 1: Primers for amplification of *dhfr* and *dhps* gene of *P. falciparum*

Gene	Mutation	Primers	Primer Sequences
dhfr Primary		M1 M5	5'TTTATGATGGAACAAGTCTGC3' 5' AGTATATACATCGCTAACAGA3'
dhfr Nested	N51I	M3 F/	5'TTTATGATGGAACAAGTCTGCGACGTT3' 5'AAATTCTTGATAAACAACGGAACCTttTA3'
	C59R	F M4	5'GAAATGTAATTCCCTAGATATGGAATATT3' 5'TTAATTTCCCAAGTAAAACTATTAGAGCTTC3'
	S108N	F M4	5'GAAATGTAATTCCCTAGATATGGAATATT3' 5'TTAATTTCCCAAGTAAAACTATTAGAGCTTC3'
	I164L	M3 F/	5'TTTATGATGGAACAAGTCTGCGACGTT3' 5'AAATTCTTGATAAACAACGGAACCTTTTA3'
	A16V	M3 F/	5'TTTATGATGGAACAAGTCTGCGACGTT3' 5'AAATTCTTGATAAACAACGGAACCTTTTA3'
dhps Primary		R2 R/	5'AACCTAAACGTGCTGTTCAA3' 5' AATTGTGTGATTTGTCCACAA3'
dhps Nested	S436A	K K/	5'TGCTAGTGTTATAGATATAGGATGAGcATC3' 5'CTATAACGAGGTATTGCATTTAATGCAAGAA3'
	A437G	K K/	5'TGCTAGTGTTATAGATATAGGATGAGcATC3' 5'CTATAACGAGGTATTGCATTTAATGCAAGAA3'
	K540D	K K/	5'TGCTAGTGTTATAGATATAGGATGAGcATC3' 5'CTATAACGAGGTATTGCATTTAATGCAAGAA3'
	A581G	L L/	5'ATAGGATACTATTTGATATTGGACCAGGATTCG3' 5'TATTACAACATTTTGATCATTCGCGCAACCGG3'

Source: Duraisingh et al., [18]

Restriction Fragment Length Polymorphism analysis (RFLP)

Site-specific restriction enzymes were used to digest the PCR amplicons, namely *AluI, BsrI, BstNI, Tsp509I, XmnI, DraI& N1aIII* for *dhfr* domain and *MnlII, MspAI, AvaII, MwoI, FokI, BstUI, BslI, BsaWI & AgeI* for *dhps* domain respectively [18,19]. Essentially, 5 µl of PCR products was incubated with restriction enzymes (New England Biolabs, Beverly, MA, USA) according to manufacturer's protocol in 15 µl final reaction volume. As previously described [16,18, 20], the *dhfr* and *dhps* variants were identified.

Gel electrophoresis

Nested PCR products were electrophoresed on 2% agarose gels before subsequent RFLP analysis. Similarly, electrophoresis of restriction digests was also carried out on 2% agarose gel. The gels of PCR amplified and digested products were stained and then visualized. Then, polymorphism of the PCR products was analyzed for studying drug resistance status of the collected blood samples.

RESULTS

PCR amplification of dhfr and dhps

Different primers were used to amplify regions in *dhfr* and *dhps* containing various point mutations associated with resistance to SP, thus different DNA fragments with different band sizes were obtained. M3 +

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F/ amplification(Figure 1a) produced DNA amplicons of about 522bp while DNA amplicons of approximately 326bp band size were obtained following DNA amplification by F + M4 primers (Figure 1b). PCR amplification using K+K/ (Figure 1c) and L+L/ primers (Figure 1d) produced fragments of 438 and 161 bp respectively on 2% agarose gel.

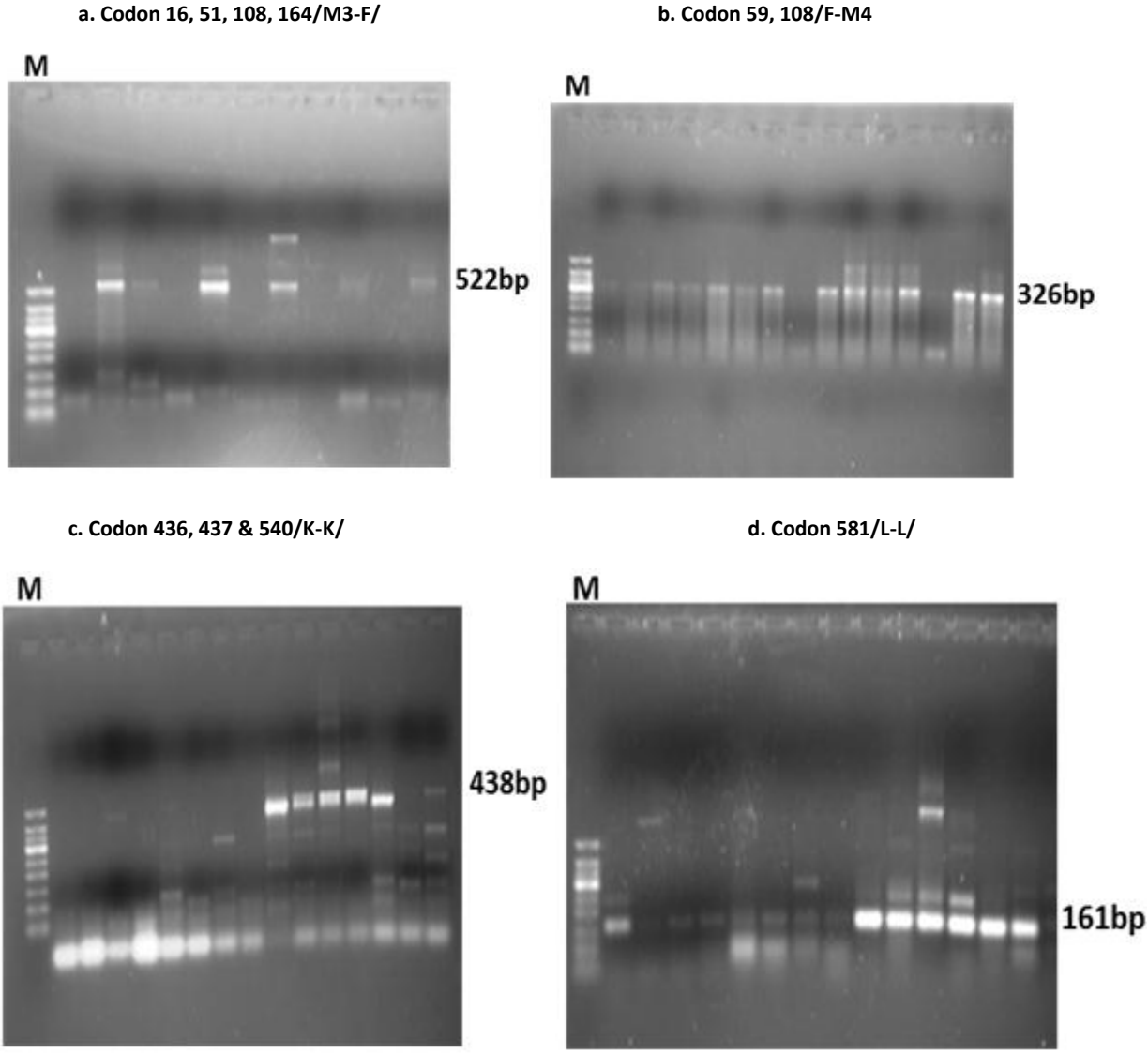


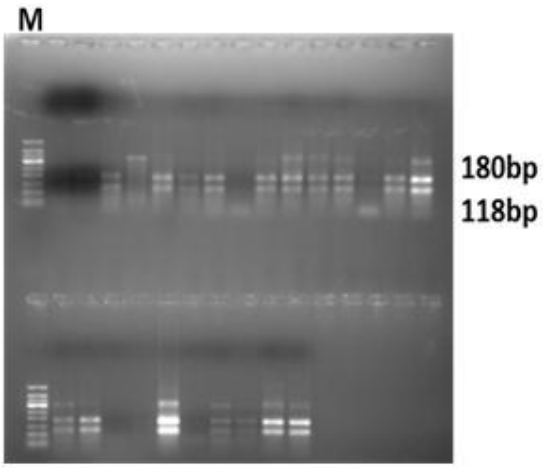
Figure 1: Agarose gels of the PCR products ((a) M3+F/; (b) F+M4; (c) K+K/; (d) L+L/) of the tests for the polymorphisms of *dhf* and *dhps*. Fragment sizes are in bp. M-Marker (50bp)

RFLP analysis

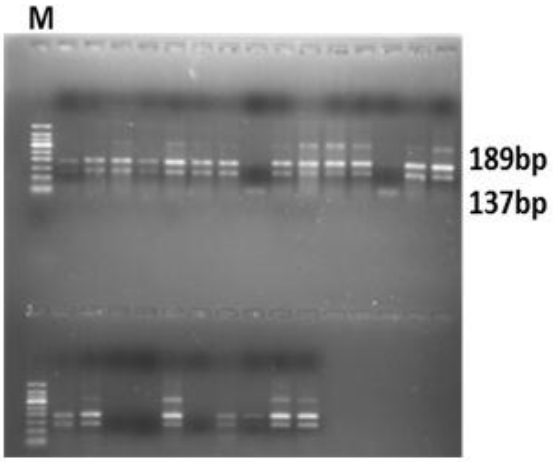
Figure-2 represents 2% agarose gels of the restriction digests used to detect the fragment patterns corresponding to the different variants at each codon on the *dhfr* and *dhps* domains. The restriction enzyme *Alu*I was used specifically to cut a PCR product with the primers F + M4 sized 326 bp(Figure 1b) having the codon 108 into fragments discriminating the two alternative forms, wildtype (180 and 118 bp) and mutant (299 plus 27 bp) at that codon. The wild type indicates presence of serine in the amino acid sequence of the enzyme system while the mutant form of the gene indicates the substitution by asparagine. In all the samples the wild type of codon (180 and 118bp fragment) was found (Figure 2a). Restriction digestion of the same PCR fragment with *Xmn*I was used to distinguish wild type (189 and 137 bp) and mutant (162, 137 + 27 bp fragments) variants on codon 59 of the gene. Mutation at this codon reflects substitution of amino acid cystine (wild type) by arginine (mutant). In this wild type of codon(189 and 137 bp fragment) was also found in all the samples(Figure 2b). The restriction enzyme *Bst*N1 was used specifically to cut the same PCR product to

discriminate the mutant type codon 108 representing amino acid threonine. In all the samples, the wild type of codon (326bp fragment) was found (Figure 2c). The M3 + F/ amplified PCR product sized 622 bp (Figure 1a) were digested with *Dra*I, *BsrI, NlaIII* and *TSP509*I to distinguish variants at codon 164, 108, 16 and 51 respectively. Digestion with *Dra*I was expected to produce fragments of sizes 245, 171 and 106 bp indicative of presence of amino acid isoleucine (wild type) and 145, 143, 106 and 28 bp indicative of presence of amino acid leucine (mutant) distinguishing polymorphisms at codon 164. In this wild type of codon(245, 171 and 106 bp fragments) were found in all the samples(Figure 2d). Digestion with *BsrI* produced fragments size 309 and 190 bp indicative of presence of amino acid asparagines (mutant type) at codon 108. In this also wild type of codon (522 bp fragment) were found in all the samples (Figure 2d). A restriction site for *Nla III* in this PCR product is destroyed by the mutation from codon 16-alanine to 16-valine. In all the samples, wild type codon was found (Figure 2d). On the other hand, digestion by *Tsp509*I produce fragments of sizes 153 and 120 bp indicative of presence of amino acid asparagine (wild type) and 217 and 120 bp indicating presence of amino acid isoleucine (mutant) at codon 51. In this also wild type of codon (153 and 120 bp fragments) were found in all the samples (Figure 2d).

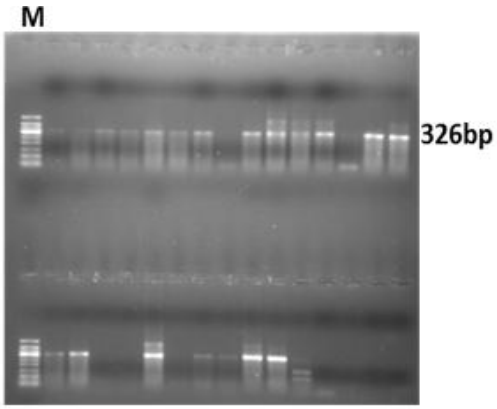
a. **Codon-108/*Alu*I**



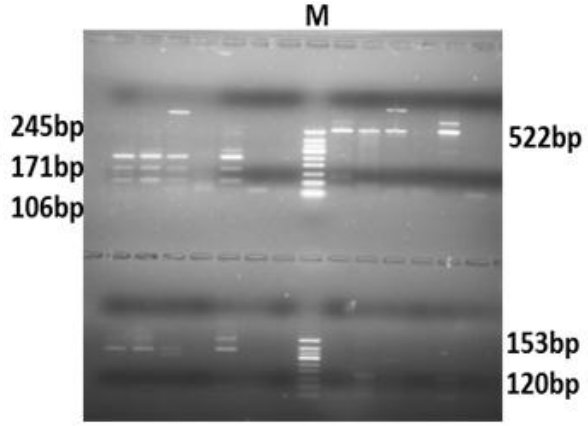
b. Codon-59/*Xmn*I



c. Codon-108/*BstN1*



d. Codon-164,108,16 &51 (*Dra*1, *Bsr*I, *Nla*III &*Tsp*509I)



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e. Codon-436, 437, 540 & 581
(*Mnl*I, *Ava*II, *Fok*I &*Bst*UI)

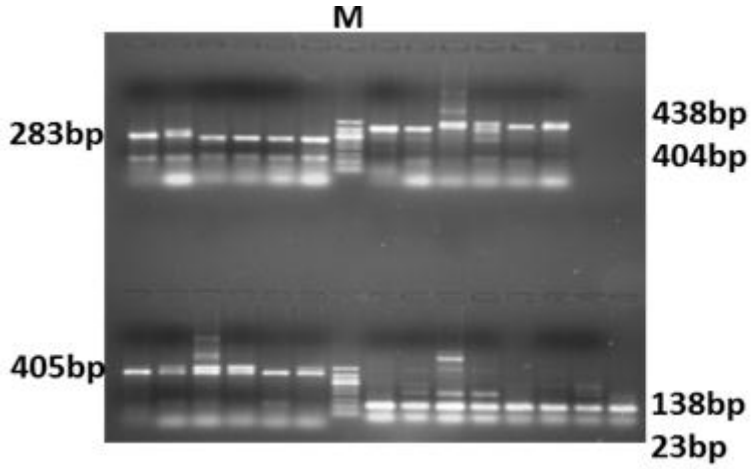

Figure 2: Polyacrylamide gels of the restriction digest of the PCR products: (a)*Alu*I (b) *xmn*I (c) *BstN1* (d) *Dra*I,*Bsr*I, *Nla*III and *Tsp509*I; (e)*Mnl*I, *Ava*II, *Fok*I *and BstU*I of the tests for the polymorphisms of the *dhfr* and *dhps* genes. Fragment sizes are in bp. M-Marker (50bp).

PCR products of approximately 438 bp (Figure 1c) were obtained from amplification with the K and K/ primers. A 438 bp PCR product was digested by *Mnl*Ito detect 436-serine (283 bp). In this wild type (438bp fragment) codon was found in all the samples (Figure 2e). The variants at codon 437 are discriminated by restriction digestion using the restriction enzyme *Ava*II. In this case uncut fragment (438 bp) indicated wild type while mutation at this site is shown by a cut fragment sized 404 bp. Mutation at this codon indicates substitution of glycine for alanine. In this mutant type(404 bp fragment) codon was found in two samples out of six samples and in another four samples wild type(438bp) codon was found(Figure 2e). The K + K/ amplified PCR products have been also used to detect polymorphisms occurring at codon 540. A restriction enzyme, *Fok*I produce fragments sized 405 bp (wild type) and 320 and 85 bp (mutant) discriminating variants at that codon. The 405 bp fragment indicates presence of amino acid Lysine while the 320 and 85 bp fragments reflect substitution of glutamate for lysine at this codon. In this wild type (405bp fragment) of codon was found in all the samples (Figure 2e). Following amplification with L+ L/ primers, PCR products of size 161 bp were produced (Figure 1d). These PCR fragments have been used to describe polymorphism on the *dhps* gene occurring at codon 581 using a restriction enzyme *Bst*UI. This restriction enzyme produced restriction digests of sizes 105, 33 and 23 bp (wild type) and 138 and 23 bp (mutant) from the PCR products of size 161 bp. The former reflects presence of amino acid, alanine at this codon and the latter indicates substitution of glycine for alanine. In this mutant type(138 and 23 bp fragments) codon was found in all the samples analysed(Figure 2e). Five codons (16, 51, 59, 108 and 164) on *dhfr* and 4 codons (436, 437, 540 and 581) on *dhps* were evaluated and the results are presented in figure-2. Mutations were not found in the codons of *dhfr* gene. In *dhps* gene, there was no mutation in the 436 and 540 codons. However, Gly-437 and Gly-581 mutations were found in 33.3% and 100% of samples respectively. Mutations of these codons indicate substitution of the amino acid Glycine in place of Alanine which indicates the existence of *P. falciparum* resistance to sulphadoxine in Khurda district of Odisha.

DISCUSSION

Introduction of a new drug into a parasite population causes genomic changes in the parasite DNA to enhance its survival under the drug pressure. These genomic changes are usually the point mutations in the target gene, which further cause the rapid spread of the mutant allele [21]. Emergence of chloroquine resistance in India has required the use of alternative antimalarials against falciparum malaria. In 2007, artesunate plus sulfadoxine–pyrimethamine was introduced as the first-line treatment in high-risk districts and areas with identified resistance, with the goal of covering most of the nation's *P. falciparum* burden. In 2010, this treatment became the first-line treatment throughout India [5].This preliminary study was based on the use of PCR-based molecular diagnostic tests to characterize mutations in *dhfr* and *dhps* genes of *P. falciparum* where its use is becoming common. Several studies have investigated the association between mutations in *dhfr* and *dhps* and the parasitological and/or clinical response to SP medication at individual level [13, 22-24].

Most of these studies produced tangible results regarding the use of *dhfr* and *dhps* genotypes as resistance marker genes for SP [25]. Pyrimethamine resistance has been widespread in the Indian subcontinent [26,27]. Lumb et al.[21] reported an asymmetric selective sweep around mutated *pfdhfr* alleles observed with isolates from Assam, Orissa, and Madhya Pradesh. Similarly, SP resistance has been reported from the north-eastern, central, southern and eastern regions [28]. In the present investigation, it was found that *P. falciparum* parasites from Khurda district of Odisha carried wild genotypes of *dhfr*, but carried mutations in 437 and 581 codons of *dhps* genes reflecting resistance to sulphadoxine. A change from alanine to glycine at codon 437 (A437G) is the initial step to resistance to sulfa drugs, followed by sequential mutations at codons 436 (S436A), 540 (K540E), 581 (A581G), and 613 (A613S/T), that cause a further increase in drug resistance which confirms our findings of sulphadoxine resistances as earlier reported by Triglia et al. [10]. Resistance to Sulphadoxine was at initial stage at Khurda district of Odisha. Therefore, further study is required to know the status of Sulphadoxine drug resistance at different time interval. Further, studies by Biswas et al. [29] showed *P. falciparum* parasites from India carry mutations in *dhfr* (at high rate) and *dhps* (at low rate) genes reflecting resistance to SP. However, more studies are needed to confirm the status of *Pf* resistance to SP from different districts of Odisha state.

CONCLUSIONS

Results of this preliminary study indicates that *P. falciparum* parasites from Khurda district of Odisha carried wild genotypes of *dhfr*, but mutations in 437 and 581 codons of *dhps* genes were observed which reflects resistance to Sulphadoxine. Therefore, constant surveillance is highly essential throughout the country for the early detection of development of drug resistance particularly to Sulphadoxine. With wide availability and continuous use, resistance to SP may build up, as happened in some South-east Asian countries, East Africa and South America [30, 31]. Alternative combination therapies are still needed to evaluate the effectiveness and possible harmful side effects of different drugs. Improvement of health personnel having adequate knowledge on drugs from village levels may reduce uncontrolled and inappropriate use of the drug, consequently reducing the chances of selecting SP resistance mutations against malaria.

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REFERENCES

[1] Sharma VP. In: Mahajan RC, ed. “Multi drug resistance in emerging and re-emerging diseases,” Indian National Science Academy 2000; pp. 191-202.
[2] National drug policy on malaria. National vector borne disease control programme, Ministry of Health and Family Welfare, 22-Shamnath Marg,Delhi-110054, 2013.
[3] Malaria situation in India. [accessed Mar 5, 2014].http://nvbdcp.gov.in/Doc/mal-situation-Nov13.pdf
[4] Hyde JE. Trends Parasitol 2005; 21: 494-498.
[5] National drug policy on malaria. [accessed Mar 6, 2014].http://nvbdcp.gov.in/Doc/drug-policy-2010.pdf.
[6] Bzik DJ, Li WB, Horii T, and Inselburg J. Proc Natl Acad Sci USA 1987; 84: 8360-8364.
[7] Triglia T, and Cowman AF. Proc Natl Acad Sci USA 1994; 91: 7149-7153.
[8] Brooks DR, Wang P, Read M, Watkins WM, Sims PF, and Hyde JE. Eur J Biochem 1994; 224: 397-405.
[9] Peterson DS, Walliker D, and Wellems TE. Proc Natl Acad Sci USA 1988; 85: 9114-9118.
[10] Sirawaraporn W, Sirawaraporn R, Cowman AF, Yuthavong Y, and Santi DV. Biochemistry 1990; 29:10779-10785.
[11] Triglia T, Menting JG, Wilson C, Cowman AF. Proc Natl Acad Sci USA 1997; 94: 13944-13949.
[12] Gregson A, and Plowe CV. Pharmacol Rev 2005; 57: 117-145.
[13] Wang P, Read M, Sims PF, and Hyde JE. Mol Microbiol 1997; 23: 979-986.
[14] Triglia T, Wang P, Sims PF, Hyde JE, and Cowman AF. *EMBO J* 1998; 17: 3807-3815.
[15] Plowe CV, Djimde A, Bouare M, Doumbo O, Wellems TE. Am J Trop Med Hyg 1995; 52:565-568.

[16] Kublin JG, Dzinjalamala FK, Kamwendo DD, Malkin EM, Cortese JS, Martino LM, Mukadam RAG, Rogerson SJ, Lescano AG, Molyneux ME, Winstanley PA, Chimpeni P, Taylor TE, Plowe CV. J Infect Dis 2002; 185: 380-388.
[17] Mbugi EV, Mutayoba BM, Malisa AL, Balthazary ST, Nyambo TB, and Mshinda H. Malaria Journal 2006; 5:94.
[18] Duraisingh TM, Curtis J, Warhurst DC. Exp Parasitol 1998; 89:1-8.
[19] Jelinek T, Peyer-Hoffmann G, Mühlberger N, Wichmann O, Wilhelm M, Schmide N, Grobusch MP, Sonnenburg F.von, Gascon J, Laferl H, Hartz C, Alifrangis M, Burchard G, McWhinney P, Schulze M, Kollaritsch H, daCunha S, Beran J, Kern P, Gjørup I, and Cuadros J. Malaria Journal 2002; 1:11.
[20] Jelinek K, Kilian AH, Kabagambe G, and Sonnenburg F.von. AmJ Trop Med Hyg 1999; 61: 463-466.
[21] Lumb V, Das MK, Singh N, Dev V, Wajihullah, and Sharma YD. Antimicrobial Agents and Chemotherapy 2009; 5173-5180.
[22] Basco LK, Tahar R, Keundjian A, and Ringwald P. J Infect Dis 2000; 182: 624-628.
[23] Nzila AM, Nduati E, Mberu EK, Hopkins SC, Monks SA, Winstanley PA, and Watkins WM. J Infect Dis 2000; 181: 2003-2028.
[24] Omar SA, Adagu IS, and Warhurst DC. Trans R Soc Trop Med Hyg 2001; 95: 315-319.
[25] Alifrangis M, Enosse S, Khalil IF, Tarimo DS, Lemnge MM, Thompson R, Bygbjerg IC, and Rønn AM. Am J Trop Med Hyg 2003; 69: 601-606.
[26] Ahmed A, Bararia D, Vinayak S, Yameen M, Biswas S, Dev V, Kumar A, Ansari MA, and Sharma YD. Antimicrob Agents Chemother 2004; 48: 879-889.
[27] Ahmed A, Das MK, Dev V, Saifi MA, Wajihullah, and Sharma YD. Antimicrob Agents Chemother 2006; 50:1546-1549.
[28] Biswas S, Escalante A, Chaiyaroj S, Angkasekwinai P, and Lal AA. Tropical Medicine and International Health 2000; 5(10): 737-743.
[29] Bjorkman A, and Philips-Howard PA. Trans R Soc Trop Med Hyg 1990; 84:177-180.