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Full Length Research Paper

Prevalence of Sulfadoxine–pyrimethamine resistance-associated mutations in *dhfr* and *dhps* gene of *Plasmodium falciparum* isolates collected in Pala west region of Chad

ISSA Mahamat Souleymane[1*], Offianan Andre TOURE[2], DJIMADOUM Mbanga[3], KERAH Hinzoumbé Clément[1], AKO Aristide Berenger[2], COULIBALY Baba[2], MBAITOLOUM Modobé Denis[1], DJOUMBE Ephraim[1], TCHONFIENE Passiri[4], DJIMRASSENGAR Honoré[5], V. YAMEOGO Jean Marie[5], BOUZID Samir[6], RINGWALD Pascal[7], DOSSO Mireille[8], DJAMAN Allico Joséph[9]

[1]Chad National Malaria Control Programme (NMCP); [2]Malariology Unit/ Parasitology and Mycology Department, Institut Pasteur of Côte d'Ivoire; [3]University of N'Djaména, Faculty of Medicine, NCBT;[4]Emergency service of Pala Hospital; [5]WHO / Chad;[6]PALAT / UNDP;[7]GMP / WHO, Geneva, Switzerland;[8]University of Félix Houphouët Boigny, Abidjan - Institute Pastor of Côte d'Ivoire; [9]University of Félix Houphouët Boigny, Abidjan, Department of Biochemistry- Institut Pasteur of Côte d'Ivoire.

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Sulfadoxine-Pyrimethamine (SP) is the recommended drug for Intermittent Preventive Therapy (IPT) in pregnant women in Chad since 2005. There are few published reports of mutations in dihydropteroate synthetase (*dhps*) and dihydrofolate reductase (*dhfr*) genes in *P.falciparum* populations in Chad. This study was assessed to determine prevalence of *dhfr* and *dhps* polymorphisms in *P. falciparum* isolates collected in Pala west region of Chad. *P. falciparum* isolates were collected from children under five years with a confirmed diagnosis of malaria at Pala hospital in the western region of Chad. The parasite DNA was extracted from the blood sampled on Whatman® filter paper by the Qiagen DNA Mini Kit method (Qiagen, Valencia.CA) according to the manufacturer's instructions. *dhfr* and *dhps* gene were amplified using nested PCR and subsequently sequenced. A total of 30 isolates were sequenced and analyzed. The prevalence of the Asn-51-Ile, Cys-59-Arg and Ser-108-Asn mutations of the *dhfr* gene were 23.3 %, 30% and 33.3%, respectively. The mutations Ala-16-Val, Ile-16-Leu and Ser-108-Thr were not detected. Ser-436-Ala, Ala-437-Gly mutations of the *dhps* gene were found in 23.3% and 16.7%. Lys-540-Glu, Ala-581-Gly, Ala-613-Ser mutations and Phe-436 codon were not observed. The wild type *dhfr* and *dhps* isolates represented 53.3% and 66.7% of the analyzed sequences. The triple mutant *dhfr* and double mutant *dhps* were respectively 3.33% and 6.7%.This study provide baseline prevalence dhfr and dhps polymorphisms in Chad. Deployment of SP for IPTp should be encouraged in Chad, and the corresponding resistance markers be regularly monitored.

Key words: Malaria, Sulfadoxine-Pyriméthamine, Pala/Chad.

INTRODUCTION

Malaria remains an important cause of morbidity and mortality worldwide, in particular in sub-Saharan Africa (WHO, 2016). Children under five years of age and pregnant women, are more at risk of developing clinical malaria.

The World Health Organization (WHO) currently recommends

Corresponding Author's E-mail: issa.mahamatsoul@gmail.com
Tel: (+225) 87420247/57904950

IPTp with Sulfadoxine-pyrimethamine (IPTp- SP) in areas with moderate to high malaria transmission in Africa at each scheduled antenatal care (ANC) visit, starting as early as possible in the second trimester until the time of delivery, with doses given at least 1 month apart, so that women receive at least three doses of SP during pregnancy (WHO, 2014). This recommendation has been adopted and implemented in Chad since 2005.

Important progress in the control of malaria in pregnancy has been made by vector control with long-lasting insecticide-treated bednets (LLIN) and the use of intermittent preventive treatment during pregnancy (IPTp) in areas with moderate to high malaria transmission. However, growing resistance to SP threatens the effectiveness of IPTp-SP (Nnaemeka et al., 2012; Mockehaupt et al., 2008). Accumulation of mutations in dihydrofolate reductase (*dhfr*) and dihydropteroate synthetase (*dhps*) is strongly associated with sulfadoxine-pyrimethamine (SP) treatment failure. Mutations in *dhfr* confer resistance to pyrimethamine while mutations in *dhps* confer resistance to sulphadoxine and other sulpha drugs. There are variations in SP mutations. The presence of three mutations in the dhfr gene (codons 51, 59, and 108) and two mutations in the dhps gene (codons 437 and 540), together referred to as the “quintuple mutation,” has been strongly associated with potential resistance in sub- Saharan Africa (Bwijo et al.,2003; Roper et al., 2003).

In Africa, previous studies showed high frequencies of mutations to confer resistance to SP (Anthony et al., 2015; Elena et al., 2015; Naomi et al., 2015). Data related to mutations in dihydropteroate synthetase (*dhps*) and dihydrofolate reductase (*dhfr*) genes in *P. falciparum* populations in Chad are lacking. The prevalence of Sulfadoxine–pyrimethamine resistance-associated mutations in *dhfr* and *dhps* gene in parasite populations in Chad is currently unknown. The present study investigated the prevalence of relevant *dhfr* and *dhps* gene polymorphisms in parasite isolates from the western region of Chad in order to fill the gap in SP resistance surveillance data in Chad. Results from this study will enable the MPH and malaria control institutions to have a database SP for the first time.

METHODS

Study area, subjects and sample collection

Samples were collected at regional hospital of Pala from November to December 2015. Pala is located in the south of the country in the Mayo-Kebbi West Region (9 ° 21'00 "N; 14°58"W 00). Malaria prevalence reaches 43.8% in this region.

In the study site, malaria transmission occurs from July to December during the raining season. The majority of malaria cases in the area is caused by *P. falciparum*, while *Anopheles gambiae*s.s. and to a lesser extent *Anopheles funestus* are the major vectors. The key malaria control interventions in the district include the use of LLNIs, malaria case management with ACTs and IPTp-SP.

Samples collection

Samples were collected from children coming for visit at the Hospital with signs and symptoms of malaria microscopically confirmed. Three drops of 50 µL of blood were collected onto a Whatman® 3 MM filter paper on the day of visit (D0), after obtaining a written informed consent from children parents or legal guardians. The collected blood was dried at room temperature and then put into a zipped pouch containing silicate desiccant to keep sample away from moisture. Samples were sent to Institut Pasteur of Côte d’Ivoire for molecular biology analyses.

DNA isolation and PCR amplifications

Parasite genomic DNA was isolated from the DBS (dot blood samples) with Qiagen DNA Mini Kit (Qiagen, Valencia.CA) according to the manufacturer's instructions. The procedure included three steps. Briefly, for the first steporlysis step, a volume of 180µl of ATL buffer and 20 µL of proteinase K were added to the sample already put into a 1.5 µL Tube. The tube was then placed onto a heating block at 56 ° C for one hour at 900 rpm. A volume of 200 µL of AL buffer was added to the tube which was heated at 70 ° C for 10 min at 900 rpm. During the washing step, 500 µL Buffer AW1 were added, and centrifuged at 8000 rpm for 1 min. A volume of 500 µL of Buffer AW2 was then added and centrifuged at 8000 rpm for 1 min. The tube was centrifuged at high speed (14.000 rpm) for 3 min. In the last step, a volume of 20 µL of AE Buffer were added. The tube was incubated at 15-25 ° C for 1 min and then centrifuged at high speed (14.000 rpm) for another 1 min in order to collect the DNA. The extracted DNA was stored at -20 ° C.

For the *dhfr* gene to be amplified by nested-PCR, an aliquot of 3 µL of DNA extract was added to a 22 µL master mix comprising, for the first round, 12 µL of pure water (Promega), 2.5 µL of 10 µM primers (M1:5'-TTTATGATGGAACAAGTCTGC-3' and M7: 3'-CTAGTATATACATCGCTAACA-5’), 5 µL of Master Mix (Solis Biodyne, Letonie). For the second round of amplification, 2 µL of PCR product was added to a 48 µL mix, that is 28 µL of pure water (Promega), 5 µL of each primer (10 µM), 10 µL of Master Mix (Solis Biodyne,

Letonie). Here, the primers used were the following M9: 5'-CTGGAAAAAATACATCACATTCATATG-3' and M3: 3'-TGATGGAACAAGTCTGCGACGTT-5') both of the PCR rounds were performed according to the following program: 95°C for 5 min; 35 times the cycle of 95°C for 40 sec, 45°C for 40 sec, 72°C for 1 min. The final extension was run at 72°C for 10 min. Amplification solution was conducted likewise for *dhps* gene, except for the first round primers (N1: 5'-GATTCTTTTTCAGATGGAGG-3' and N2: 3'-TTCCTCATGTAATTCATCTGA-5') and for the second round primers (R2: 5'-AACCTAAACGTGCTGTTCAA-3'; R/: 3-'AATTGTGTGATTTGTCCACAA-5'). The first and second PCR rounds were programed with 95°C (5 min); 35 cycles of 95°C (40 sec), 56°C (40 sec), and 72°C (2 min); and final extension at 72°C (15 min). A 1.5 % agarose gel was prepared and an electrophoresis was run to check for a 594 bp fragment length for *dhfr* gene, or a 771 bp fragment length corresponding to *dhps* gene.

Sequencing and alignment

Nested-PCR products were sent to GENEWIZ (United Kingdom) for Sanger sequencing. Fasta and ab1 files were received back and underwent quality control before alignment with BioEdit v. 7.0.9.1.against the corresponding reference sequences of *P. falciparum* 3D7 (PlasmoDB : PF3D7_0417200 pour *dhfr* ; PF3D7_0810800 pour *dhps*).

Ethics

The procedures followed were in accordance with the ethical standards of Helsinki Declaration. Consent form was signed by parent or legal guardian of children included in the study.
Approval was given by the Ministry of Public Health under ethical clearance N° 2192/PR/PM/MSP/SE/SG/DGAS/DSPELM/DMTNT/PNLP/15 and by WHO ERC.

RESULTS

Table 1 indicates that, overall, the frequencies of wild alleles predominate over the frequency of mutant alleles. These values are two-fold, the frequencies of wild-type alleles at the *dhfr* gene 70% (for Cys-59) and 66.7% (for Ser-108) against Asn-108 (33.3%) and Arg-59 (30%) for key mutations related to resistance to Pyrimethamine from *P. falciparum*. Similarly, for the *dhps* gene, the frequencies of the wild-type alleles Ser-436 (76.7%) and Ala-437 (83.3%) predominated over those of the Ala-436 alleles (23.3 %) and Gly-437 (16. 7 %) (Table 1).

Seven (7) genotypes were identified, including the wild type ($N_{51}C_{59}S_{108}$) of 53.3% (16/30) and the triple mutant ($\underline{\mathbf{I}}_{51}\underline{\mathbf{R}}_{59}\underline{\mathbf{N}}_{108}$) with a frequency of 3.3% (1/30). The results indicate that the triple mutant genotype is rare. At the level of the *dhps* gene, among the 4 genotypes found, the double *dhps* mutants ($\underline{\mathbf{A}}_{436}\underline{\mathbf{G}}_{437}$) was represented at 6.7% (2/30) while the wild type genotype ($S_{43}6A_{437}$) have a predominant frequency of 66.7%. (Table 2).
In this section, a haplotype is defined as a combination of genotypes across the two genes. Thus, twelve haplotypic combinations were described, with the combinations $\underline{\mathbf{I}}_{51}\underline{\mathbf{R}}_{59}\underline{\mathbf{N}}_{108}$ / $\underline{\mathbf{A}}_{436}A_{437}$ represented at 3.3%, i.e. 1 over($N_{51}C_{59}S_{108}$ / $S_{436}A_{437}$) having a frequency of 33.3% (i.e. 10/30). Moreover, this latter frequency also corresponds to the cumulative frequencies of all the genotypes carrying two mutations (Table 3).

DISCUSSION

The Chadian national malaria policy recommends the use of the SP in IPTp with the goal of reducing maternal and infant mortality. In fact, the SP IPTp strategy was evidenced to protect pregnant women against malaria with benefits consisting in reduced LBW, abortion, premature birth, perinatal death, and maternal mortality (Menendez et al., 2010; WHO-MPA, 2012). The strategy consists in a free administration of three doses of SP to any pregnant women in visit for antenatal care during the second and the third trimester of pregnancy. Unfortunately, this strategy covers a very low rate, 15. 3% in TPIp1 and 13. 6% in IPTp2, and no information for the IPTp3. This coverage is quite insufficient and irregular to decide the use SP by medical districts. This result does not correlate with the objectives of the strategic plan 2006 - 2018 to fight against malaria in Chad which recommended a cover rate of 80% for SP (Anonymous, 2014). More, SP resistance is reported to continuously increase and subsequently reduce protection in areas where SP resistance is high (Gosling et al., 2009; Griffin et al., 2010; Harrington et al., 2011).
This study conducted in Pala where SP is used for IPTp demonstrated low prevalence of *dhfr* and *dhps* resistance mutations.
Considering polymorphisms in *dhfr* gene, codon ser108asn is likely to play a key role in pyrimethamine resistance; with mutations at Ile-51, Arg-59 and Leu-164 modulating the level of resistance (Plowe et al., 1997; Tsumori et al, 2011). The present study indicates a predominance of wild-type alleles Ser-108 (76.7%), Cys-59 (70%) and Asn-51 (76.7 %). The *dhfr* triple mutation Ile-51, Arg-59, Asn-108 was shown to be associated with SP treatment failure, regardless *pfdhps* genotype. Here, we report a low level of parasites with the triple mutant genotype $\underline{\mathbf{I}}_{51}\underline{\mathbf{R}}_{59}\underline{\mathbf{N}}_{108}$(3.33%) compared to the wild type

Table 1. Frequency of mutant alleles of the *pfdhfr* and *pfdhps* genes.

gènes	Mutations	Alleles	Frequences (%)
pfdhfr	Asn-51-Ile	**Ile**	7/30 (23.3)
		Asn	23/0 (76.7)
	Cys-59-Arg	**Arg**	9/30 (30)
		Cys	21/30 (70)
	Ser-108-Asn	**Asn**	10/30 (33.3)
		Ser	20/30 (66.7)
pfdhps	Ser-436-Ala	**Ala**	7/30 (23.3)
		Ser	23/30 (76.7)
	Ala-437-Gly	**Gly**	5/30 (16.7)
		Ala	25/30 (83.3)

Table 2. Prevalence of mutant genotypes of the *pfdhfr* and *pfdhps* genes.

Genes	**Mutations**	**Genotypes**	**Frequence (%)**
dhfr (N=30)	Sauvage	$N_{51}C_{59}S_{108}$	16 (53,33)
	Simple mutant	$\mathbf{I}_{51}C_{59}S_{108}$	1 (3,33)
		$N_{51}C_{59}\mathbf{N}_{108}$	2 (7)
	Double mutant	$\mathbf{I}_{51}C_{59}\mathbf{N}_{108}$	2 (7)
		$\mathbf{I}_{51}\mathbf{R}_{59}S_{108}$	3 (10)
		$N_{51}\mathbf{R}_{59}\mathbf{N}_{108}$	5 (17)
	Triple mutant	$\mathbf{I}_{51}\mathbf{R}_{59}\mathbf{N}_{108}$	1 (3,33)
dhps (N=30)	Sauvage	$S_{436}A_{437}$	20 (66,7)
	Simple mutant	$S_{436}\mathbf{G}_{437}$	3 (10)
		$\mathbf{A}_{436}A_{437}$	5 (16,7)
	Double mutant	$\mathbf{A}_{436}\mathbf{G}_{437}$	2 (6,7)

$N_{51}C_{59}S_{108}$ (53.3%). This low prevalence of the triple *dhfr* indicates that in Pala SP continue to be efficient and should be kept for IPTp. At first sight, the low prevalence of the triple *dhfr* mutation seems surprising since SP was accessible more than a decade ago in Chad. This may suggest a low level of SP pressure favored by an access to ACT treatment. However, studies related to SP resistance conducted in neighboring Republic of Congo

Table 3. Combination of the *pfdhfr* and *pfdhps* genotypes.

Nomber of mutations	Genotype *dhfrldhps*	N=30	frequence (%)
4	**I**$_{51}$**R**$_{59}$**N**$_{108}$/**A**$_{436}$A$_{437}$	1	3.3
3	**I**$_{51}$**R**$_{59}$S$_{108}$/A$_{436}$**G**$_{437}$	1	3.3
3	N$_{51}$**R**$_{59}$**N**$_{108}$/S$_{436}$**G**$_{437}$	1	3.3
2	**I**$_{51}$C$_{59}$**N**$_{108}$/S$_{436}$A$_{437}$	2	6.7
2	**I**$_{51}$C$_{59}$S$_{108}$/S$_{436}$**G**$_{437}$	1	3.3
2	**I**$_{51}$**R**$_{59}$S$_{108}$/S$_{436}$A$_{437}$	2	6.7
2	N$_{51}$**R**$_{59}$**N**$_{108}$/S$_{436}$A$_{437}$	4	13.3
2	N$_{51}$C$_{59}$S$_{108}$/**A**$_{436}$**G**$_{437}$	1	3.3
1	N$_{51}$C$_{59}$S$_{108}$/S$_{436}$**G**$_{437}$	1	3,3
1	N$_{51}$C$_{59}$**N**$_{108}$/S$_{436}$A$_{437}$	2	6.7
0	N$_{51}$C$_{59}$S$_{108}$/S$_{436}$A$_{437}$	10	33.3
0	N$_{51}$C$_{59}$S$_{108}$/A$_{436}$A$_{437}$	4	13.3

(Nsimba et al., 2005; Ndounga et*al*., 2007; Koukouikila-Koussounda et al., 2015; Tsumori et al., 2011) showed high frequencies of *dhfr* point mutations at codons 51, 59 and 108 while the mutation at codon 164 was absent.

Analysis of the polymorphisms in *dhps* also showed low prevalence of the mutation at codon 437 and 436. The low frequency of double mutant **AG** (6.7%) could result from a low drug pressure to sulfadoxine. No isolate have the *dhps* double mutation at position 437 and 540, commonly associated with sulfadoxine resistance (Plowe et al., 2004; Kublin et al., 2002). The 437 mutation, which usually occurs first in the progressive selection of resistant parasites was observed in 3 isolates (10%). Such mutation, alone or combined with the K540E, has been associated with treatment failure with SP (Pearce et al., 2009). Nevertheless, in Pala, the A437G prevalence was low compared to that found in Nigeria (Oguike et al., 2016) a bordering country of Chad.

In addition, the wild-type allele of the *pfdhps* / *pfdhfr* combination, i.e genotype N$_{51}$C$_{59}$S$_{108}$/ S$_{436}$A$_{437}$, was predominant in the study population. Quintuple mutations I51R59N108 / **A**$_{436}$**G**$_{437}$ were not observed.

In high-resistance countries, the triple mutant dhfr and double mutant dhps genotype were observed at high prevalence, leading to the withdrawal of SP (Elifaged et al., 2013; Jeremiah et al., 2017; Esmée et al., 2017).

CONCLUSION

This study showed higher prevalence of susceptible parasites than those harboring mutations in the study area. However, further studies are needed to strengthen these data. Thus, the deployment of SP for IPTp should be encouraged, and the corresponding resistance markers be regularly monitored.

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Conflict of interest

The authors state that there is no conflict of interest.
DH and PR are staff members of the World Health Organization. DH and PR alone are responsible for the views expressed in this publication and they do not necessarily represent the decisions, policy or views of the World Health Organization.

ABBREVIATIONS

ASAQ: Artesunate-Amodiaquine; SP: Sulfadoxine-Pyrimethamine; AL: Artemether-lumefantrine; TBS: Thick blood smear ; NPME: National Program for Malaria Eradication in Chad; MPH: Ministry of Public Health; DS: Sanitary District; SNPs: Single nucleotide polymorphisms; IPCI: Institut Pasteur of Côte d'Ivoire; NCBT: National Center for Blood Transfusion; WHO: World Health Organization; PALAT: Support Project for Malaria Control in Chad; UNDP: United Nations Development Program; GMP: Global Malaria Program; ACT: Artemisinin-based Combination Therapy; ACPR: Adequate Clinical and Parasitological Response; LPF: Late Parasitological Failure; LCF: Late Clinical Failure; ETF: Early Therapeutic Failure; LPV: Lost Patient View; PRS: Patient Removed from Study; MPD: Mean Parasite Density; DP: Parasite Density; CI: Confidence Index; Ds: standard deviation.

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