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RETROSPECTIVE INVESTIGATION OF PYRIMETHAMINE-SULFADOXINE RESISTANCE INDICATORS IN FALCIPARUM-MALARIA POSITIVE BLOOD SAMPLES FROM SOUTH-WESTERN SAUDI ARABIA

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Abstract

The efficacy of Sulphadoxine/Pyrimethamine (SP) in *Plasmodium falciparum* malaria treatment was increasingly compromised by development of parasites' resistance. Saudi Arabia shifted to new combinations including Artesunat compound during the last decade. We investigated the occurrence of mutations in *P. falciparum* dihydrofolate reductase (*Pfdhfr*) N51I, C59R and S108N and *P. falciparum* dihyropteroate synthetase (*Pfdhps*) A437G and K540D as major indicators of SP resistance in stored DNA extracts of 41 *P. falciparum* infected specimens collected from KSA southern endemic regions between 2012 and 2014.

Analysis of alleles' polymorphisms by Nested-PCR-RFLP showed that 68%, 7%, and 24% of samples carried parasites with *Pfdhfr* 51I, 59R, and 108N mutant type alleles, respectively. Only one isolate's genotype shared both mutations 51I and 108N. All parasites conserved wild type alleles at codons 437 and 540 of *Pfdhps* gene.

Key words: *Falciparum* Malaria, Sulphadoxine/Pyrimethamine, Resistance, Nested PCR-RFLP.

Introduction

Five species of protozoan parasites belonging to the genus of *Plasmodium* are known to cause human malaria; *P. falciparum, P. vivax*, *P. malariae*, *P. ovale*, and the only zoonotic *P. knowlesi* species (Wesolowski *et al*, 2015). *P. falciparum* is the causative agent of malignant malaria, being the most lethal one. It is estimated that 90% of deaths in 2012 were in the African region, followed by the South-East Asia Region; 7% and Eastern Mediterranean Region; 3% (WHO, 2013). In Saudi Arabia, malaria is especially endemic in the lowlands of the Aseer region in the southwestern province and particularly in its southern part of Jazan, and *P. falciparum* accounts for more than 85% of reported cases (Abdoon and Alshahrani, 2003). Except for the central part which is free of malaria, the rest of the country includes malarious areas with an epidemiological aspect highly affected by the control measures (Al-Seghayer *et al,* 1999). Reported malaria cases decreased along the years, in 2012, a total of 4000 confirmed cases were reported, compared to 15,666 cases in 1990; it is obvious that the ministry of health has taken the initiative for strengthening malaria control activities in the country (WHO, 2013). Moreover, antimalarial drug resistance must be highly considered as some pilgrims come from malaria drug resistance areas (Khan *et al*, 2002). In addition to the imported and border malaria, the expected resistance to insecticides currently in use is a major technical problem facing malaria control program in KSA (Coleman *et al*, 2014).

A number of drugs were used to treat or prevent malaria and most of them were developed almost 30 years ago (Cravo *et al*, 2006). Chloroquine was the mainstay of malaria control efforts until the development of resistance to the drug (Marr *et al*, 2003). *P. falciparum* from Sub-Saharan Africa started to show resistance to this drug in late 1950s which was followed in other parts of the world (Sharma, 2005). Saudi Arabia, initially considered one of the few areas where *P. falciparum* remained sensitive to chloroquine, the number of cases and response to therapy were noted to have changed early in

the season of 1997-1998. More cases were reported, and chloroquine failure was noted frequently (Al-Rajhi *et al,* 1999; Kinsara *et al,* 1997; Al-Arishi *et al,* 2001; Al-Harthi, 2007, Hussain *et al,* 2011; El-Bahnasawy *et al,* 2010, 2011), but the main evidence comes from annual random screening conducted by the Ministry of Health. Results of this scree-ning indicated that the endemic *P. falciparum* strains that are resistant to chloroquine by *in vitro* test steadily increased in the Jazan region between 1986 and 1998 (WHO, 2002). The combination of sulfadoxine and pyrime-thamine (SP), known commercially as Fansidar was frequently used in the last decade for treatment for uncomplicated chloroquine resistant *P. falciparum* cases in Saudi Arabia. Several African countries had switched to SP as first-line treatment, in others it remained the second-line choice (Duraisingh *et al*, 1998). Certain countries in Africa, Asia and South America reported that *P. falciparum* parasites seem to develop SP resistance faster than with chloroquine. Drug resistance can be conferred by several mechanisms and reflects genetic mutations or polymorphisms in the parasite population. Drug-resistance parasites had a selective advantage over the drug-sensitive parasites in the presence of drug and would be preferentially transmitted. Major factors in the development and spread of drug resistance were the use of sub-therapeutic doses of drugs or not completing treatment regimen that led to a continued emergence of parasites that tolerate drug higher doses (Shunmay *et al*, 2004).

In this study, a retrospective investigation was carried out on incidence of mutations on *Pfdhfr* and *Pfdhps* genes related to pyrimethamine/sulfadoxine (Fansidar®) resistance of *P. falciparum* parasites in Saudi Arabia endemic regions during the transition of malaria treatment to artemisinin-based combination therapy (ACT).

Material and Methods

Blood samples collection and storage: The study was carried out on blood specimens collected from patients that visited different health care centres in South-Western region of Saudi Arabia between 2012 & 2014. A total of 41 whole blood samples, microscopically confirmed as infected with *P. falciparum* were collected in EDTA-treated test tubes and kept at 4°C for future molecular analysis. Five *P. falciparum* reference strains; K1, HB3, 3D7, 7G8 and TM6 collected from *in vitro* cultures as dry spots on Whatman FTA filter cards, gifted by Liverpool School of Tropical Medicine, were used as controls. A malaria negative whole blood sample collected from a healthy individual in EDTA tube was used as control in all reactions.

DNA templates preparation: Genomic DNA templates were extracted from EDTA-preserved whole blood samples by a commercial chromatographic kit using centrifugation method (QIAmp DNA Blood Mini Kit, Qiagen) following the protocol of the manufacturer. Extracted whole genomic DNA samples were quantified by spectrophotometry and the purity was determined as 280/260 OD ratio. DNA templates were prepared from sensitive and resistant *P. falciparum* reference strains dried culture spots on Whatman-FTA cards using Whatman-FTA® Reagent and TE buffer as recommended by the manufacturer.

Nested-PCR-RFLP: Nested-PCR assays were conducted to amplify DNA regions flanking the codons 51, 59 and 108 of *dhfr*, and 437 & 540 of *dhps* genes of *P. falciparum* parasites known as markers of sensitivity/resistance to pyrimethamine/sulfadoxine. Oligonucleotides' sequences were used (Tab. 1). Both, first (NestI) and second (Nest-II) polymerase chain reactions were carried out in total volume of 25µl and in duplicate using a commercial pre-formulated 2×Reddy Mix™ Master Mix (AB gene, UK). NestI reactions were conducted using 1-2.5µl of whole genomic extracted DNA specimens, primers to a final concentration of 1.5µg/ml each and 12.5µl of 2×Master Mix. NestII reactions were carried out using 2µl tem-

plates taken from NestI PCR products with primers at a final concentration of 1.5µg/ml each and 12.5µl of 2×Master Mix. PCR conditions and times were adopted from literature with some modifications.

Restriction enzyme digestion of Nested-PCR products was performed to determine the presence of polymorphism in the studied codons. Site-specific restriction enzymes were used to digest the Nested-PCR amplicons. Five different restriction enzymes were used (Tab. 2), namely *TSP509*I (for codon 51 *dhfr*), *Xmn*I (59 *dhfr*), *Alu*I (108 *dhfr*), *Ava*II (437 *dhps*) and *Fok*I (540 *dhps*). 15 µl of Nested-PCR products were incubated with restriction enzymes according to manufacturer's recommended time and temperature in 30µl final reaction volume.

Separation of Nested-PCR-RFLP products: The RFLP digests were separated on basis of their sizes by electrophoresis alongside with 10µl of undigested Nested-PCR products onto 2% agarose gels and stained with EtBr. Molecular size of visualized DNA ba-nds was determined by extrapolation to the DNA ladder. Allele's polymorphisms (wild or mutant) were determined for each sample and studied codon in basis of digestion pattern obtained in each experiment (Tab. 2).

Results

Presence of *P. falciparum* in the 41 whole blood samples collected from the patients that visited different health care centres in South-Western region of Saudi Arabia between 2012 & 2014 was confirmed by microscopic examination of Giemsa stained blood films by at least two expert microscopists in the health care centres where the samples were received and in the laboratory. Cycling conditions of NestI and NestII PCR assays used for the investigation of the most reported alleles polymorphisms on *P. falciparum dhfr* and *dhps* genes known as markers of resistance to pyrimethamine/sulfadoxine, were slightly optimized using gradient PCR system.

The results obtained by the interpretation of PCR-RFLP separated products patterns concerning the distribution of wild/mutant alleles of *dhfr* N51I, C59R and S108N and *dhps* A437G and K540D in *P. falciparum* parasites infecting the 41 malaria cases are shown on table 3. 28/41 (68%) samples carried parasites with mutant allele type at *Pfdhfr* N51I codon. 3/41 (7%) samples carried *P. falciparum* with mutation at *Pfdhfr* codon C59R. Concerning alleles' polymorphism at codon S108N, 10/41 (24%) were found to be carrying *P. falciparum* with mutations. Only one sample contained parasites showing simultaneous mutation at *Pfdhfr* codons 51 & 108. The investigation of codons A437G & K540D on *Pfdhps* gene revealed that all samples contained only parasites with wild allelic form of both codons (0% mutants) (Tab. 3).

On the other hand, no amplified products were obtained with the negative control DNA in all experiments.

Table 1: Oligonucleotides' sequences utilized in this study

Primer	Nucleotide sequence (5'-3')	Nts
	Pf.dhfr (codons 51, 59 & 108)	
M1	TTTATGATGGAACAAGTCTGC	21
M5	AGTATATACATCGCTAACAGA	21
M3	TTTATGATGGAACAAGTCTGCGACGTT	27
F/(F1)	AAATTCTTGATAAACAACGGAACCTTTTA	29
F	GAAATGTAATTCCCTAGATATGGAATATT	29
M4	TTAATTTCCCAAGTAAAACTATTAGAGCTTC	31
	Pf.dhps (codons 437 & 540)	
R2	AACCTAAACGTGCTGTTCAA	20
R/(R1)	AATTGTGTGATTTGTCCACAA	21
K	TGCTAGTGTTATAGATATAGGATGAGCATC	30
K/(K1)	CTATAACGAGGTATTGCATTTAATGCAAGAA	31

Table 2: Sets of Primers and restriction enzymes used in PCR-RFLP assays with expected alleles' polymorphism patterns for wild and mutant codons of interest onto *Pfdhfr* and *Pfdhps* genes.

Gene	Codon	NestI	Nest II	PCR(bp)	Rest. enzyme	Cleaving sites	RFLP (bp)	
							Wild	Mutant
Pfdhfr	51	M1,M5	M3,F	522	*Tsp*509I	5'..▼AATT.. 3' 3'..TTAA▲.. 5'	218,120	154
	59	M1,M5	F,M4	326	*Xmn*I	5'..GAANN▼NNTTC..3' 3'..CTTNN▲NNAAG..5'	189,137	163,137,26
	108	M1,M5	F,M4	326	*Alu*I	5'..AG▼CT.. 3' 3'..TC▲GA.. 5'	180,119	299
Pfdhps	437	R2,R	K,K1	438	*Ava*II	5'..G▼G(A/T)CC..3' 3'..CC(T/A)G▲G..5'	438	404
	540	R2,R	K,K1	438	*Fok* I	5'..GGATG$(N)_9$▼..3' 3'..CCTAC$(N)_{13}$▲..5'	405	320,85

Table 3: Alleles' polymorphism onto *dhfr* and *dhps P. falciparum* SP resistance markers among samples.

Genotype	Codons on *Pfdhfr*			Codons on *Pfdhps*	
	N51I	C59R	S108N	A437G	K540D
Wild type (W)	12 (29%)	38 (93%)	30 (73%)	41 (100%)	41 (100%)
Mutant type (M)	28 (68%)	3 (7%)	10 (24%)	0 (0%)	0 (0%)
Simultaneous (W+M)	1	0	1	0	0

Discussion

Despite intensive efforts for its control, human malaria remains the most important parasitic disease worldwide. In Saudi Arabia, especially in the south where infections by *P. falciparum* were common (Coleman *et al*, 2014) resistance to anti-malarial drugs is one of the important emerging problems affecting malaria control and elimination program. Parasites drug resistance is acquired through different mechanisms reflected by genetic mutations that have been largely studied to proof their significant relationship with the decrease of *Plasmodium* populations' sensitivity to anti-malarial compounds in use (Shunmay *et al*, 2004). Thus, several researchers concentrated their investigations on punctual mutations affecting specific genes of *P. falciparum*. Some authors established a strong association of punctual mutations affecting *Pfdhfr* and *Pfdhps* genes with parasites' resistance to pyrimethamine/sulfadoxine antimalarial drug (Wongsrichanalai *et al*, 2002; Talisuna *et al*, 2004). In the present study, 41 *P. falciparum* infected whole blood samples from South-Western Saudi Arabia patients were investigated for allelic polymorphisms of codons N51I, C59R and S108N of *Pfdhfr* and codons A437G and K540D of *Pfdhps* genes were considered as resistance indicators to pyrimethamine/sulfadoxine anti-malarial drug (Wongsrichanalai *et al*, 2002; Tarnchompoo *et al*, 2002; Ngo *et al*, 2003) by Nested-PCR-RFLP using primers with adapted enzymes sites (Sendagire *et al*, 2005). Duraisingh *et al.* (1998) reported that PCR primers tolerated several mismatches in their 3' end, while a reasonable sensitivity maintained, which greatly increased along with the specificity of the technique when the nested approach is used.

The result showed that 68% of the samples carried parasites with *Pfdhfr* 51I mutation, 7% with *Pfdhfr* 59R mutation, & 24% samples with *Pfdhfr* 108N mutant codon. Despite relatively deployment of pyrimethamine/sulfadoxine for extensive malaria treatment in southern Saudi Arabia endemic region, parasites with punctual mutations on *Pfdhfr* gene were shown, particularly, the mutation 108N of *Pfdhfr* gene, a major determinant of pyrimethamine resistance (Zho-

ng *et al*, 2008), had already reached a 24% among parasites population at that time. The presence of only 3 specimens with mutant type allele at codon 59 of *Pfdhfr* gene in all samples agreed with Al-Harthi (2007). Furthermore, when compared to similar studies carried out in African areas at that time, no appearance of mutation at this site was found in Sudan, although it was found in Tanzania with an incidence of 48.8% even at earlier years (Mbugi *et al*, 2006). During a study in provinces of south-eastern Iran, only 11.3% of samples carried the mutant allele (Heidari *et al*, 2007). In Suriname, South America, Peek *et al.* (2005) reported almost similar grade of mutations at *Pdhfr* codon 59 to those in Saudi Arabia southern region in specimens collected before 2007 and in this study. Contreras *et al*. (2002) in Venezuela reported a 0% *Pdhfr* 59R mutations among *P. falciparum* parasites collected between 1998 and 2000. Particularly, no mutations were detected in all the tested samples reflecting amino acid changes from alanine to glycine at codon 437 and from lysine to glutamine at codon 540 of *pfdhps* gene considered as determinant for sulfadoxine resistance occurrence, differently to other areas worldwide where such mutations were frequent in *P. falciparum* populations after introduction of Fansidar$^{(R)}$ as a routine treatment for malaria patients. Zhong *et al.* (2008) reported a mutations incidence of 90% & 100% for *Pfdhps* A437G & K540E, respectively.

Generally speaking, *Plasmodium falciparum* or malignant malaria is not only a public health in Saudi Arabia but also in all the neighbouring countries at risk of malaria transmission and introduced with visitors and immigrants employees. Moreover, one must take into consideration that malaria apart from its *Anopheles* vector(s), people can get malaria if they come into contact with infected blood as in blood transfusion or needle-stick injury also nosocomial and congenital malaria was reported (Saleh *et al*, 2016).

Conclusion

The present outcome findings suggested that although relatively the low prevalences of SP resistance markers were present, which would be sufficient to predict the progress of SP resistance development in the region, and the lack of licensed malaria vaccines of proven efficacy and consequently, the need of the introduction of new treatment lines.

Besides, the spread of insecticide-resistant strains of the mosquito, the feasible control measures of *Anopheles* vector(s) of malaria is a must.

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