Distribution of Drug Resistance Genotypes in *Plasmodium falciparum* in an Area of Limited Parasite Diversity in Saudi Arabia


Department of Biochemistry, Faculty of Medicine, Sultan Qaboos University, Muscat, Oman; National Center for Training and Research, Ministry of Health, Jazan, Saudi Arabia; Department of Biological and Medical Research, King Faisal Specialist Hospital and Research Center, Riyadh, Saudi Arabia; Institute of Immunology and Infection Research, School of Biological Sciences, University of Edinburgh, Edinburgh, United Kingdom

Abstract. Two hundred and three *Plasmodium falciparum* isolates from Jazan area, southwest Saudi Arabia, were typed for *Pfcr*, *Pfmdr1*, *dhps*, and *dhfr* mutations associated with resistance to chloroquine, mefloquine, halofantrine, artemisinin, sulfadoxine-pyrimethamine, and the neutral polymorphic gene *Pfg377*. A large proportion (33%) of isolates harbored double mutant *dhfr* genotype (51L,59C,108N). However, only one isolate contained mutation *dhps*-437G. For *Pfcr*, almost all examined isolates (163, 99%) harbored the mutant genotype (72C,73V,741,75E,76T), whereas only 49 (31%) contained the mutant *Pfmdr1* genotype (86Y,184R,1034S,1042N), 109 (66%) harbored the single mutant genotype (86N,184E,1034S,1042N), and no mutations were seen in codons 1034, 1042, and 1246. Nonetheless, three new single-nucleotide polymorphisms were detected at codons 182, 192, and 102. No differences were seen in distribution of drug resistance genes among Saudis and expatriates. There was a limited multiplicity (5%), mean number of clones (1.05), and two dominant multilocus genotypes among infected individuals in Jazan. A pattern consistent with limited cross-mating and recombination among local parasite was apparent.

INTRODUCTION

The Arabian Peninsula lies at the fringes of malaria endemicity, where successful control efforts have brought local transmission to a halt in many parts of this region. At the same time, limited foci in Yemen and southern Saudi Arabia remain malarious, with a high prevalence of drug-resistant *Plasmodium falciparum* parasites.1–6

In Saudi Arabia, malaria transmission is confined to southwestern regions (the Ascer and Jazan provinces), where *P. falciparum* is the prevailing species and *Anopheles arabiensis* is the main vector.7–11 However, malaria cases are still reported in different parts of the country, mostly brought by travelers from endemic sites in the south or expatriates from outside the country.1,12

Chloroquine was the drug of choice for the treatment of uncomplicated malaria cases for many years. However, the emergence and spread of chloroquine resistance (CQR)13–15 has lead to the introduction of sulfadoxine-pyrimethamine (SP) therapy, which is effective. Nonetheless, recently health authority in Saudi Arabia has adopted artemisinin combination therapy (ACT), consisting of artemisinin plus SP. A recent study has revealed high prevalence of *Pfcr*76T and *Pfmdr1*86Y mutations among *P. falciparum* parasites in Jazan.9 In addition, mutation *dhfr*59R was suspected12; however, no data were presented on codons 51 and 108 or codons 436, 437, 540, and 581 on *dhps*, which are critical markers for pyrimethamine and sulfadoxine resistance and subsequent SP failure.

Here, we have extended the above findings and examined 16 single-nucleotide polymorphisms (SNPs) on *dhfr*, *dhps*, *Pfcr*, and *Pfmdr1* genes implicated in resistance to an array of antimalarial drugs, including SP, chloroquine, amodiaquine, artemisinin, and lumefantrine. Some of these genes have recently been suggested to have an antagonistic selective role.6,17 Therefore, the pattern of response of *P. falciparum* to different combination antimalarial therapy may be influenced by mutations in these genes, and their role in predicting response to combination therapy can be of paramount importance.

MATERIALS AND METHODS

Study subjects. Jazan region, southwest Saudi Arabia, is endemic for malaria, where the vast majority of the cases are caused by *P. falciparum* and *An. arabiensis* is the main vector. Malaria transmission occurs after the rainy season from November to March and the appearance of *Anopheles* mosquitoes. *P. falciparum* in the region was at one time susceptible to chloroquine; however, resistance emerged in the late 1990s14 and mutations associated with CQR have escalated in the region.9 This finding prompted the change of treatment policy to artemisinin-based combination therapies (ACTs).18

The present study examined 203 samples obtained, with informed consent, from microscopy-confirmed *P. falciparum* malaria patients from 11 hospitals and polyclinics in Jazan (Figure 1). The majority of patients (163; 80%) were Saudi; 145 (89%) were residents of Jazan, and 18 (11%) were visitors from other parts of the country. The remaining 40 (20%) were expatriates: 18 (45%) were residents, and 22 (55%) were visitors. Most of the patients (85%) were males. Their ages ranged from 2 months to 80 years, the largest group being between 20 and 29 years (16%).

Finger prick blood samples were spotted onto filter paper (Whatman 3M, Polybags Ltd., Greenford, Middlesex, UK) and individually sealed in plastic envelopes. All patients who participated in the study signed a consent form, and the study was approved by the Ethics Committee at King Khalid University, Abha, Saudi Arabia. *Dhfr*, *dhps*, *Pfcr*, and *Pfmdr1* genes. *P. falciparum* DNA was prepared from filter paper samples of blood as described previously.19 Alleles of the *dhfr*, *dhps*, *Pfcr*, and *Pfmdr1* genes were amplified using two rounds of polymerase chain reaction (PCR) as described previously.1,12,18–22 The amplified fragment of each gene encompasses mutations associated with pyrimethamine resistance (*dhfr*-51, -59, -108, and -164), sulfadoxine resistance (*dhps*-436, -437, -540, and -581), CQR...
Prevalence of alleles of dhfr, dhps, Pfcrt, and Pfmdr and the polymorphic control gene Pfpg377 among 165 P. falciparum isolates from Jazan, southwest Saudi Arabia

<table>
<thead>
<tr>
<th>Allele</th>
<th>N (%)</th>
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<tbody>
<tr>
<td>dhfr alleles</td>
<td>dhps alleles</td>
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<tr>
<td>N</td>
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<tr>
<td>108N</td>
<td>60 (34)</td>
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<tr>
<td>108S</td>
<td>117 (66)</td>
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<tr>
<td>N51</td>
<td>58 (33)</td>
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<tr>
<td>N51</td>
<td>117 (67)</td>
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<tr>
<td>76K</td>
<td>2 (1)</td>
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RESULTS

dhfr and dhps genotypes.

Table 1 and 2 show distribution of dhfr and dhps alleles and corresponding genotypes in Jazan among the 176 and 179 P. falciparum isolates examined for dhfr and dhps, respectively. For dhfr, mutations N51 and 108N were frequent, occurring at prevalences of 33% and 34%, respectively. However, other mutations, at codons 59R and 164L, associated with high-level SP resistance were not seen. Similarly, dhps mutations associated with SP resistance at codons 436, 437, 540, and 581 were not seen, with the exception of mutation 437G, which was detected in one isolate (Tables 1 and 2).

Wild-type dhfr (N51, C59, S108) and dhps (A437, K540) genotypes were predominant, existing at prevalences of 65.90% and 99.44%, respectively. However, single- (N108) and double-mutant (I51, N108) dhfr genotypes were less prevalent at 1.14% and 32.95%, respectively, whereas the triple mutant (I51, R59, N108) associated with high-level pyrimethamine resistance was not seen in the region (Tables 1 and 2).

Pfcrt genotypes. CQR-associated mutations in Pfcrt were extremely high in Jazan area: 162 (99%) of 164 isolates successfully examined harbored the genotype (C72, V73, I74, E75, T90), and only 2 (1%) isolates carried the wild type (C72, V73, M74, X75, Y90) (Tables 1-3).

Pfmdr1 alleles and genotypes. Genotyping of Pfmdr1 was completed for 165 P. falciparum isolates. We found mutations only in codons 86 and 184 and no mutations at codons 1034 and 1042. At codon 86, 118 (69%), 51 (30%), and 2 (1%) isolates harbored the wild-type allele (86N), the resistance allele (86Y), and mixed alleles (86N/86Y), respectively. For codon 184, 159 (96%), and 1 (1%) carried the wild-type allele (184Y), the resistance allele (184F), and mixed alleles (184Y/184F), respectively (Tables 1 and 2). In addition, 0 of 46 isolates examined for codon 1246 were found to harbor a mutant allele, despite the fact that 13 and 38 of these isolates had mutation at codons N86Y and Y184F, respectively.

Furthermore, three new SNPs were detected: T306C in codon 102, T546G in codon 182, and C575G in codon 192. The first two SNPs are synonymous, and the last one is non-synonymous. Mutation T546G was more common, seen among the isolates examined. The multiplicity of infection was defined as the proportion of people who carry more than one allele (genotype) for any of the examined genes, and the minimum number of clones per infection was estimated as the largest number of alleles at any of the examined loci. SPSS program (16.0.0) has been used to calculate odd ratio (OR), \( \chi^2 \) test, and P value.

Table 1

<table>
<thead>
<tr>
<th>Allele</th>
<th>N (%)</th>
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<tbody>
<tr>
<td>dhfr alleles</td>
<td>dhps alleles</td>
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<tr>
<td>Pfcrt alleles</td>
<td>Pfmdr1 alleles</td>
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<tr>
<td>dhfr alleles</td>
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<td>108N</td>
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<td>76K</td>
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five isolates, compared with the other two mutations, which were each found in one isolate. This mutation has recently been reported in some *P. falciparum* isolates in India.31

Among the 163 isolates with complete sequence data, five (3%) isolates carried the wild genotype (N86 Y184 W186 S1034 N1042). However, 108 (65%) and 49 (30%) harbored single- (N86 L87 F184 W186 S1034 N1042) and double-mutant genotypes (Y86 L87 F184 W186 S1034 N1042), respectively (Tables 1–3).

**Distribution of resistance genotypes among Saudis and expatriates.** The majority of isolates (163; 80%) were obtained from Saudis: 145 (89%) were residents of Jazan, and 18 (11%) were Saudi visitors. In addition, 40 (20%) samples were collected from expatriates: 18 (45%) were residents, and 22 (55%) were visitors.

The risk of harboring a resistance strain of *P. falciparum* among Saudi and non-Saudi was equal (OR = 1), with 95% confidence interval (CI) at 0.458–2.182. No significant association was seen between harboring resistance strain and gender ($\chi^2 = 0.79$), living status ($\chi^2 = 0.865$), or nationality ($\chi^2 = 1$ at $P < 0.005$). However, the only mutant allele in *dhps* gene was harbored by an expatriate. Similarly, the wild-type *Pfcrt* allele was only carried by two expatriated visitors (Table 4).

**Pfg377 alleles and parasite diversity.** At least five *Pfg377* alleles, varying in size between 269 and 352 base pairs, were detected. The difference between each of the two closest alleles is approximately 21 base pairs, which reflects variation in the mean number of clones per infection (1.05) was very close to one. In addition, two major haplotypes (multilocus genotypes with identical alleles for all loci), haplotypes 4 and 5, existed at prevalences of 20% and 18%, respectively, among the 93 *P. falciparum* isolates with single-clone infection (Table 3).

**DISCUSSION**

The southwestern region of Saudi Arabia (Jazan) is a major malaria-endemic site, where chloroquine was previously the drug of choice for the treatment of malaria cases. In this area, *P. falciparum* remained sensitive to chloroquine up until the late 1990s, unlike neighboring malaria-endemic sites, such as Yemen and the closest east African countries, where CQR was common. The health authority in Saudi Arabia has lately changed the first line for treatment of malaria to ACT: SP plus artesunate.

Limited studies have examined drug resistance genes in the Jazan area and shown the presence of mutant alleles of some drug resistance genes: *Pfcr1-76T, Pfmdr1-86Y*, and *dhfr-59R*. The present study has extended the above findings and analyzed 16 SNPs in four genes involved in resistance to commonly used antimalarial drugs, *dhfr* and *dhps* mutations associated with SP resistance existed at low prevalence in Jazan; however, mutations in *Pfcr1* and *Pfmdr1* genes linked with chloroquine, amodiaquine, mefloquine, and possibly artemisinin are common, which is in agreement with a recent report from the area.

The low prevalence of *dhfr* and *dhps* implies that the current use of SP in Jazan may be imposing only weak selection on these genes or that there has been insufficient time for selection to leave a molecular signature. Evidence of selection is generally found in *dhfr* earlier than *dhps*; thus, it is likely that we may be seeing the initial phase of SP resistance in Jazan. An additional point that supports this hypothesis is that almost all the resistance *dhfr* genotypes consist of

### Table 2

<table>
<thead>
<tr>
<th>dhfr genotypes</th>
<th>dhps genotypes</th>
<th>Pfcr genotypes</th>
<th>Pfmdr1 genotypes</th>
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<tbody>
<tr>
<td>Wild type (NC) 116 (65.7)</td>
<td>Sensitive (SA) 177 (99.4)</td>
<td>Wild type (CV) 2 (1)</td>
<td>Wild type (NY) 5 (3)</td>
</tr>
<tr>
<td>Single mutant (NC) 2 (1.14)</td>
<td>Single mutant (SG) 1 (0.56)</td>
<td>Triple mutant (CV) 162 (99)</td>
<td>Single mutant (NF) 109 (66)</td>
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<tr>
<td>Double mutants (IC) 58 (33.9)</td>
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<td>Double (Y) 49 (31)</td>
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### Table 3

<table>
<thead>
<tr>
<th>Haplotypic</th>
<th>dhfr</th>
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double mutants (I51,N108) rather than the highly resistance triple mutant (I51, R59, N108) found in Africa and Southeast Asia where SP resistance is well-established. Thus, it is possible that highly resistance genotypes have not spread in this region. A limited study carried out in 2005 in the Jazan area has previously reported the presence of dhfr-59R allele. However, this allele was not detected in the current study. This discrepancy is unexpected, because the dhfr-59R allele is associated with high-level resistance to pyrimethamine. SP has been used in Jazan for sometime as a second line to chloroquine, and the above allele is expected to be under favorable selection in the face of drug pressure. Therefore, the small sample size (N = 19) analyzed by Al-Harthi may not have been representative of the Jazan parasite population, and the two isolates with 59R alleles may have been obtained from expatriates with asymptomatic chronic infection who acquired it outside Jazan; it is known to be capable of lasting for some months. Saudi Arabia is at great risk of imported malaria because of the large number of expatriate workers and the millions of annual visitors from malaria-endemic countries, for Hajj and Umrah. Analysis of microsatellites flanking the Pfcr gene in Jazan will shed light on its genetic background and whether it has evolved locally or not.39

The change from chloroquine to artemisinin + SP as the first-line antimalarial in Jazan is expected to decrease the CQR genotype and restore the chloroquine wild-type allele within the parasite population. This occurrence has been documented consistently after withdrawal of chloroquine in many African countries, such as Malawi, Tanzania, and Kenya. Therefore, it was unexpected to find Pfcr-K76T at fixation. The persistence of the Pfcr-K76T allele suggests an ongoing use of chloroquine or related drugs such as amodiaquine because of the recent shift to ACT, which can lead to selection of the mutant genotype. In addition, chloroquine, although not used for treatment of falciparum malaria, can be used for infections that are thought to be P. vivax but are actually unrecognized mixed infections or misdiagnosed P. falciparum infections. A large number of expatriates from the Indian subcontinent may import P. vivax infection.

Polymorphisms in the Pfmdr1 alleles (N86Y, Y184F, S1034C, N1042D, and D1246Y) may alter parasite responses to many antimalarial drugs, including chloroquine, quinine, mefloquine, and artemisinin. Mutations at codon 86 have been associated with CQR, whereas mutations at codons 184, 1034, 1042, and 1246 have been implicated to varying degrees in resistance to mefloquine and artesunate. An increased association between artesunate-mefloquine failure and a mutation at codon 184 has been seen in P. falciparum parasites in Cambodia. However, it is unlikely that the high frequency of mutations at codon 184 in the Jazan area is linked to mefloquine resistance, because this drug has not been in common use. A possible explanation is that the 184F allele has been driven by the high rate of CQR in the area.

A correlation between CQR and the Pfmdr1-N86Y mutation is well-established, because the Pfmdr1-N86Y mutation in conjunction with the Pfcr-K76T mutation yields enhanced levels of resistance to chloroquine. However, we found fewer parasites with Pfmdr186Y than expected as Pfcr76T reached fixation. Similarly, findings have been reported from other sites, and it has been postulated that Pfmdr1 N86Y mutation may confer a compensatory advantage for coping
with chloroquine pressure, which may vary in different para-
site populations.\textsuperscript{31,52}

It has recently been suggested that some antimalarial
drugs exert opposite directional selection on parasite geno-
types.\textsuperscript{16,53} For example, artemisinin-lumefantrine (AL) and
amodiaquine (AO) exert opposite within-host selective effects on
the Pfmdr1 genes of \textit{P. falciparum}.\textsuperscript{16} Similarly, the \textit{Pfcr}-
K76T mutation may enhance \textit{P. falciparum} susceptibility to
lumefantrine\textsuperscript{37} and thus, increase the benefits of using AL in
areas affected by CQR \textit{P. falciparum} malaria, such as Jazan.
Therefore, the current use of ACT in Jazan may be exerting
a novel pressure on the local parasites to select for alleles
\textit{Pfmdr1} 86N 184F 1246D and \textit{Pfcr}-74M 75N 76K,\textsuperscript{53} some of
which are currently rare in Saudi Arabia; however, the wild-
type \textit{pfmdr1}-1246D is at fixation, and the mutant form was
not seen in neighboring countries such as Iran.\textsuperscript{54,55} Whether
the prevalence of the wild-type \textit{Pfmdr1} mutations will change
under increasing artemisinin pressure will be of considerable
interest in the future. However, currently, the apparent effec-
tiveness of SP should protect artemisinin and reduce effective
selective pressure.

The observed low genetic diversity of the \textit{P. falciparum} in
Jazan area probably reflects the impact of sustained control
efforts, where limited transmission can restrict the gene pool.
This finding is revealed in the low complexity (concurrent gen-
types) of infection, where almost 95% of infected patients
carried a single clone and only 5% are infected with multi-
ple clones. Here, we have examined one polymorphic gene
\textit{pfgd377}; certainly, the addition of more polymorphic markers,
such as \textit{msp}-2, would have increased the rate of multiplicity.
However, this increase is expected to be significantly higher
because of the low transmission level and limited outcrossing
in Jazan, which is evident by the occurrence of linkage disequi-
librium. The level of multiplicity seen in Jazan is lower than
the level in an area of low and seasonal transmission such as
eastern Sudan, where the multiplicity of infection is approxi-
mately 20–40% and the mean number of clones per infection
is 1.3.\textsuperscript{56}

Thus, the markedly low level of diversity seen in Jazan is
rare and seen typically in limited endemic sites such as island
populations in Papua New Guinea and the Solomon Islands.\textsuperscript{57}
The low within-host multiplicity can have profound effect of
evolution of drug resistance. It affects both the strength of
advantageous selection of resistance genotype in the presence
of drug and the disadvantageous selection (fitness cost) in the
absence of resistance.\textsuperscript{58,59} In addition, the low genetic diversity
can result in limited opportunities for crossing and recombin-
ation to build up or break down existing multilocus drug resis-
tance genotypes.\textsuperscript{60} In such areas, mutations associated with
antimalarial drug resistance tend to reach fixation.\textsuperscript{61} Thus, it
may take longer for the dominant \textit{Pfcr} haplotype in Kingdom of
Saudi Arabia to lose its grip in the absence of chloroquine
pressure compared with areas with high transmission such as
Africa. The absence of mutations in \textit{Plfs} in Jazan, which are
already present in east Africa and Yemen, is a possible further
illustration of such a low gene flow.

In summary, the present data provide baseline information
on the prevalence of \textit{P. falciparum} drug resistance-associated
SNPs and highlight the low level of genetic diversity in the
\textit{P. falciparum} population in the Jazan area of western Saudi
Arabia. Surveillance of molecular markers of drug resistance
should be an integral part of the planned malaria eradication

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