Analysis of gene mutations involved in chloroquine resistance in *Plasmodium falciparum* parasites isolated from patients in the southwest of Saudi Arabia

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BACKGROUND AND OBJECTIVES: Chloroquine has been the drug of choice for the treatment of malaria for many decades. We aimed to examine the molecular basis of chloroquine resistance among *Plasmodium falciparum* isolates from the southwestern region of Saudi Arabia by analyzing the K76T and N86Y mutations in the PfCRT and PfMDR1 genes, respectively.

RESULTS: Of the 121 samples, 95 and 112 samples could be amplified for PfCRT K76T and PfMDR1 N86Y mutations, respectively. All of the samples amplified for the PfCRT K76T mutation were undigestible by ApoI, suggesting the presence of the K76T mutation. For the PfMDR1 N86Y mutation, 65/109 samples (59.6%) were digestible when treated with ApoI in a pattern, suggestive of the presence of the investigated wild allele (N86). However, 44/109 samples (40.4%) were digestible by ApoI, suggesting the presence of the mutated allele (Y) at position 86. DNA sequencing confirmed these results.

CONCLUSION: Surprisingly, all isolates exhibited the mutated allele at codon 76 (K76T) of PfCRT. However, the mutated mutant allele at codon 86 (N86Y) of PfMDR1 was found in 40.4% of the samples studied. To our knowledge, this is the first study that has investigated the existence of the mutation in the PfMDR1 gene in the country. This study will contribute to the development of new strategies for therapeutic intervention against malaria in Saudi Arabia.
Sudan, India, and Yemen, complicate the control of the disease.9,10 Chloroquine is the drug of choice for the treatment of complicated malaria cases. However, another combination of two drugs (sulfadoxine and pyrimethamine [SP]) is also effective against the disease. The current mainstream view of chloroquine antimalarial action is that chloroquine kills malaria parasites by binding to its target, a nonprotein molecule, ferriprotoporphyrin IX in lysosomes (food vacuoles) to form a toxic complex that lyases the parasites.11 The emergence and spread of chloroquine resistance (CQR) has been a problem for world health.12 In Saudi Arabia, the strategy to treat malaria cases was changed in 2008 by the Saudi health authorities with the introduction of artemisinin combination therapy (ACT) that consists of SP and artesiminin.13 Saudi Arabia is one of the few areas where Pfalciparum remained sensitive to chloroquine until the early 1990s despite the fact that CQR emerged in malaria-endemic countries close to Saudi Arabia, such as Iran, Pakistan, and East Africa where CQR was first reported in the early 1980s.14,35 A recent study examined a limited number of samples from the Jazan region for Pfalciparum chloroquine-resistant transporter (PfCRT) gene and found a CQR rate of 89.5%.16

Over the years, the emergence of drug-resistant parasites has hampered the efforts to control malaria worldwide. Since the late 1950s, reports of CQR have been documented in all endemic areas. Mutations in two genes, namely, PfCRT and Pfalciparum multidrug-resistant gene 1 (PfMDR1) have been implicated in resistance to chloroquine.17 Several point mutations in the PfCRT gene have been shown to correlate with resistance. Of these, only the mutation of lysine to threonine at the 76th position (K76T) in CRT is significantly found in resistant strains of malaria from different endemic areas of the world.18,19 Other mutations observed in the PfMDR1 gene were found to be strongly linked to CQR in various regions.20 Several field studies have related CQR to the mutation of aspargine to tyrosine at the 86th position (N86Y) of the MDR1 protein.21,22 In this study, we examined the presence of K76T and N86Y mutations in PfCRT and PfMDR1, respectively, among Pfalciparum parasites isolated from the southwestern region of Saudi Arabia.

**PATIENTS AND METHODS**

The cases studied in this report were individuals (n=121) suspected of malaria infection who visit the Malaria Center in Aseer and Jazan Provinces. Only malaria cases confirmed with blood microscopic examination were included in this study. Blood samples (nearly 50 µL) were collected by finger-pricking and three drops from each patient were blotted onto Whatman (3 mm) chromatography paper. The spotted blood samples were placed in plastic bags and transported to the laboratory. All patients who participated in this study signed a consent form and the study was approved by the Ethics Committee at King Khalid University, Abha, Saudi Arabia.

DNA was extracted from the spotted blood samples according to the method of Sakihama et al with some modifications.23 Briefly, filter papers containing blood spots were placed into 1.5 mL Eppendorf tubes and 0.5% saponin was added and incubated overnight at 4ºC. One hundred microliters of 5% Chelex-100 solution (Bio-Rad Laboratories, USA) were added to each tube and incubated at 100ºC for 5 minutes in a heating

| Primers | Sequence (5’–3’) | Size (bp) | PCR conditions |
|---------|------------------|-----------|----------------|
| First round | | | |
| PfMDR1 | OMDR1/F | TGGTGAAGATGGTGTTAACAGAGAGAG | 660 | 94ºC, 3 min followed by 45 cycles (94ºC, 30 secs; 56ºC, 30 secs; 68ºC, 30 secs); 68ºC, 3 min |
| PfMDR1 | OMDR1/R | TACCTTCTTAAATATGACACCAAGACAC | 1389 | |
| PfCRT | OCRT/F | CCGTAAAAATTAACACGCAC | | |
| PfCRT | OCRT/R | CGGATGTACAAAATATAGTTAC | | |
| Second round | | | |
| PfMDR1 | IMDR1/F | GTCAACCGT6GATTTTTTATATGACACATTTA | 560 | 94ºC, 3 min followed by 40 cycles (94ºC, 30 secs; 47ºC, 30 secs; 68ºC, 1 min); 64ºC, 3 mins |
| PfMDR1 | IMDR1/R | AAAGATGTAACTCAGATCAAGAGAG | | |
| PfCRT | ICRT/F | TGTGTCATGTTAAAACTTT | 145 | |
| PfCRT | ICRT/R | CAAAACATAAGTTACAATTTTG | | |

**Table 1.** Primers, expected molecular weight of amplicons, and the PCR conditions for genes investigated in this study.
block with vortexing every one minute. The supernatant was transferred to a new tube and stored at 4°C until further processing.

The primers used in this study were originally based on published data. A set of outer and inner primers was synthesized for the amplification of DNA fragments from PfCRT and PfMDR1 genes. Primer sequences, expected molecular weight of amplicons, and the PCR conditions are illustrated in Table 1. For digestions, aliquots of PfCRT and PfMDR1 PCR products were digested with 10 U of the restriction enzyme ApoI (New England Laboratories, USA), according to the manufacturer’s recommendations. All digested products were electrophoretically processed in 2% agarose gels and the bands were visualized with 1 µg/mL ethidium bromide under UV light. DNA sequencing was carried out at the DNA sequencing core facility at the Research Center of King Faisal Specialist Hospital and Research Center (KFSHRC), using BigDye Terminator v3.1 Cycle Sequencing Kit according to the manufacturer’s instructions (BigDye Terminator v3.1 Cycle Sequencing Kit, Applera, USA) and the sequences were analyzed using DNASTAR software.

RESULTS

Analysis of the PfCRT K76T codon
Of the 121 samples examined, 26 (21.5%) were not amplifiable but the remaining 95 samples (78.5%) gave amplification products (Table 2) and the amplicons of expected molecular weight (145 bp) were visualized after nested PCR (Figure 1a). None of the 95 amplified samples (100%) could be digested by ApoI, suggesting the presence of the mutation of interest (76T) that indicates chloroquine resistance (Figure 1b). DNA sequencing of the PCR products of these samples confirmed the results obtained by the PCR/RFLP method (Figure 1c).

Analysis of the PfMDR1 N86Y codon
Of the 121 samples analyzed, 112 samples (92.5%) yielded amplification products (Table 2) and the amplicons of expected molecular weight (560 bp) were visualized after nested PCR (Figure 2a). Of the total
samples examined, 9 (7.5%) were not amplifiable under the conditions used in this study. This suggested that these samples could have been misdiagnosed for malaria or that it could have been a form of malaria caused by a species other than *P. falciparum*. Of the 109 correctly amplified samples, 65 (59.6%) gave 250, 231, and 79 bp bands after ApoI digestion, indicating the presence of the wild-type 86N codon. On the other hand, 44 (40.4%) samples gave 481 and 97 bp bands after ApoI digestion, indicating the presence of the mutated 86Y codon (Figure 2b). The remaining three (of the 112 yielding amplification products) samples showed faint bands that were not appropriate for the digestion assay. DNA sequencing confirmed the results obtained by PCR/RFLP analysis (Figure 2c and 2d).

**DISCUSSION**

At present, there is little information on the association of genetic variation in *P. falciparum* parasites with drug resistance in Saudi Arabia. As many field and laboratory studies have investigated the association of CQR with specific mutations in codon 76 of the PfCRT gene and at codon 86 of the PfMDR1 gene, we aimed to investigate these mutations by PCR-RFLP and DNA sequencing technologies in isolates from the southwestern part of the country. Chloroquine was introduced in the 1940s as the drug of choice for malaria treatment.25 However, parasite resistance to chloroquine appeared after more than 60 years of intensive use. Extended parasite exposure to chloroquine has necessitated the exploration of the characteristics of *P. falciparum* population genetics in relation to resistance to chloroquine.

When the PfCRT gene was identified, many studies confirmed the presence of the K76T mutation in CQR parasites.27 The investigation of another gene, PfMDR1,28 revealed that a mutation at the 86th position was strongly linked to CQR in laboratory clones, even though it is not the sole requirement for CQR.29 Globally, it has been found that replacement of the lysine (a positively charged amino acid) in PfCRT with threonine (an uncharged amino acid) at position 76 confers resistance to chloroquine.30 The very high prevalence of the PfCRT 76T variant in our study is in agreement with the findings of other studies where clinical chloroquine treatment failure was evident.31 A study from Iran reported that PfCRT 76T was found in 99% of the investigated samples.32 In a recent prevalence study reported from Thailand, the PfCRT 76T allele frequency was found in 99.1% of the investigated samples.33 A molecular prevalence survey has shown that the prevalence of the PfCRT 76T mutation was over 90% in Yunnan province, China.34 A study of PfCRT point mutations and the level of CQR in *P. falciparum* isolates imported into Europe from Congo and Tanzania showed that the frequency of the 76T mutated allele was 71.4%.35 The presence of the PfCRT K76T mutation from Papua New Guinea was found to be 92.9%.36 A recent study from the Philippines describing *P. falciparum* isolates from three areas of the country showed that the frequency of the PfCRT 76T mutation was found to be 100% in Kalinga, 80% in Palawan, and 87% in Mindanao.37 Different PfCRT haplotypes have been reported from malarial isolates in endemic areas. The CQR-associated haplotype (amino acids from 72 to 76) detected in this study was the CVIET. This is considered the typical haplotype isolated from Southeast Asia and the African continent.38 Finding the same haplotypes in Saudi Arabia could be attributed to the continuous travel and human migration related to employment, tourism, or religious pilgrimage.39,40

Currently, mutations detected in PfMDR1 have been hypothesized to augment the level of resistance in CQR *P. falciparum* parasites.24,41 In our study, the PfMDR1 86Y allele frequencies were found to be 59.6% and 40.4% for the N86 allele. A similar find-

| Gene  | N   | Wild Allele | Mutant Allele | Mutation (%) |
|-------|-----|-------------|---------------|--------------|
| PfCRT | 95  | 76K (0/95)  | 76T (95/95)   | 100          |
| PfMDR1| 109 | 86N (65/109)| 86Y (44/109)  | 40.4         |
ing was reported from Iran where it was found that PfMDR1 86Y appeared in 72% of the samples in the Sistan-Baluchistan Province. In Thailand, the PfMDR1 86N and 86Y alleles were identified in 75.5% and 20% of samples, respectively. Isolates from three distinct areas of the Philippines showed that the frequency of the PfMDR1 N86Y mutation was 39% in Kalinga town, 35% in Palawan town, and 93% in Mindanao town isolates. The PfMDR1 N86Y mutation was found in 23.1% of the isolates from Southeast Mindanao town isolates. The PfMDR1 N86Y mutation contributes more to CQR in the Babiker and colleagues reported that the PfMDR1 and 20% of samples, respectively. Isolates from three nation was found in 23.1% of the isolates from Southeast Mindanao town isolates. The PfMDR1 N86Y muta-
in Kalinga town, 35% in Palawan town, and 93% in Mindanao town isolates. The PfMDR1 N86Y mutation was found in 23.1% of the isolates from Southeast Mindanao town isolates. The PfMDR1 N86Y mutation was found in 23.1% of the isolates from Southeast Mindanao town isolates.

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binations as tools for surveillance, will help in malaria control, guide national malaria treatment policies, and monitor changes in parasite drug susceptibility follow-
ing changes in malaria drug treatment policy.

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**MUTATIONS IN P. FALCIPARUM**

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