Molecular markers associated with resistance to commonly used antimalarial drugs among *Plasmodium falciparum* isolates from a malaria-endemic area in Taiz governorate—Yemen during the transmission season

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**Abstract**

Since 2005, artemisinin (AS) plus sulfadoxine/pyrimethamine (SP) combination has been adopted as the first-line treatment for uncomplicated malaria in Yemen in response to the high level of *Plasmodium falciparum* resistance to chloroquine (CQ). Therefore, the aim of the present study was to determine the frequency distribution of molecular markers associated with resistance to CQ and AS plus SP combination among *P. falciparum* isolates from a malaria-endemic area in Taiz governorate, Yemen. Fifty *P. falciparum* isolates were collected during a cross-sectional study in Mawza district, Taiz, in the period from October 2013 to April 2014. The isolates were investigated for drug resistance-associated molecular markers in five genes, including *P. falciparum* CQ resistance transporter (*pfCRT*) 76T and *P. falciparum* multidrug resistance 1 (*pfmdr1*) 86Y as markers of resistance to CQ, mutations in the Kelch 13 (*K13*) propeller domain for resistance to AS, and *P. falciparum* dihydrofolate reductase (*pfDhfr*) and *P. falciparum* dihydropteroate synthase (*pfDhps*) genes for resistance to SP. Nested polymerase chain reaction was used to amplify target genes in DNA extracts of the isolates followed by restriction fragment length polymorphism for detecting 76T and 86Y mutations in *pfCRT* and *pfmdr1*, respectively, and by DNA sequencing for detecting mutations in *K13*, *pfDhfr* and *pfDhps*. All the investigated isolates from Mawza district were harboring the 76T mutant and the 86Y wild-type alleles. The 511/108N double mutant allele was found in 2.2% (1/45) of the isolates; however, no mutations were detected at codons 436, 437, 540, 581 and 613 of *pfDhps*. All *P. falciparum* isolates that were successfully sequenced (n = 47) showed the Y493, R539, I543 and C580 wild-type alleles. In conclusion, the 76T mutant allele is fixed in the study area about six years after the official withdrawal of CQ, possibly indicating its over-the-counter availability and continued use as a self-medication in the study area. However, the almost predominant wild-type alleles of the genes associated with resistance to AS and SP among *P. falciparum* isolates in the present study indicates the sustained efficacy of the currently adopted first-line treatment of AS plus SP in the study area.

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1. Introduction

In Yemen, malaria is one of the leading infectious diseases from the public health perspective, where about 25.0% of the popula-
sites covering the disease epidemiologic strata in the period from 2002 to 2005, the Yemeni National Malaria Control Program (NMCP) revisited the national antimalarial treatment policy to artemisinin-based combination therapies (ACTs) in 2005, which was officially endorsed in 2009 (National Malaria Control Program, 2010). The new policy includes artemesin plus SP and artemether-lumefantrine (AL) as first- and second-line treatments for uncomplicated malaria, respectively. However, CQ is still prescribed by physicians or bought by patients without medical consultation in Yemen (Bashrahil et al., 2010). Such practices exacerbate the emergence and spread of resistance to the drug (Escalante et al., 2009).

In Yemen, a few studies have been published on the efficacy of SP alone or in combination with AS. For instance, the NMCP reported an efficacy of 95–100% for SP in eight trials conducted in sentinel sites in endemic areas in the period between 2002 and 2005 (National Malaria Control Program, 2010). Al-Kabsi et al. (2009) also reported the absence of treatment failure of falciparum malaria with SP in Tihama region. Since then, the effectiveness of the currently recommended first- and second-line treatments has been shown in eight drug efficacy trials of AS plus SP and AL for the treatment of uncomplicated falciparum malaria in the period from 2009 to 2013 (Adeel et al., 2015; National Malaria Control Program, 2013).

It is noteworthy that in vivo drug efficacy trials measure the combined effect of both drug partners and do not differentiate between the efficacy of AS and its partner. Failure of the AS partner increases the risk of the emergence of AS-resistant parasites (World Health Organization, 2015). Therefore, surveillance of the molecular markers associated with resistance to antimalarial drug partners can be a useful tool for detecting and monitoring the emergence of resistant Plasmodium strains in a geographic region before treatment failure becomes clinically evident (Hastings et al., 2002; Modzrynska, 2011). The mutation at codon 76 of the P. falciparum CQ resistance transporter (pfCRT) gene, which results in a change of the amino acid lysine into threonine (K76T), is the key molecular marker of resistance to CQ (Djimdé et al., 2001; Fidock et al., 2000). On the other hand, the mutation at codon 86 of the P. falciparum multidrug resistance 1 gene (pfMDR1), which results in a change of the amino acid asparagine into tyrosine (N86Y), has been partially associated with resistance to CQ (Adagu and Warhurst, 2001; Babiker et al., 2001; Djimdé et al., 2001; Mu et al., 2003). Moreover, Picot et al. (2009) concluded the role of pfmdr1 86Y in CQ and amodiaquine treatment failure in a meta-analysis of 29 studies. The pfmdr1 86Y has also been associated with in vitro resistance to quinine among Thai P. falciparum isolates (Poyomtip et al., 2012). In contrast, the pfmdr1 86Y has been associated with in vitro susceptibility of P. falciparum to other antimalarial drugs such as mefloquine and artesinin (Duraisingham et al., 2000a,b), whereas the pfmdr1 N86 wild-type allele has been linked to a declined susceptibility of P. falciparum to AL (Venketesan et al., 2014). In the context of resistance to SP, several mutations in P. falciparum dihydrofolate reductase (pfDHFR) and dihydropteroate synthase (pfDHS) genes have been associated with resistance to pyrimethamine and sulfadoxine, respectively (Kublin et al., 2002; Peterson et al., 1988; Wang et al., 1997). Recently, mutations in the Kelch 13 (K13) propeller domain have been associated with resistance to artesinin (Ariey et al., 2014).

Although in vivo efficacy trials indicate that ACTs are still efficacious in Yemen (Adeel et al., 2015; National Malaria Control Program, 2013), detection of molecular markers associated with resistance to AS and SP is necessary as an early warning system for the evolution of drug resistance to their partner drugs. In addition, detection of the pfCRT 76T may demonstrate the trend in resistance to CQ after replacing CQ with AS plus SP combination. Thus, the present study aimed to detect mutations at the codons 76 and 86 of pfCRT and pfmdr1, respectively, as well as in K13, pfDHFR and pfDHS among P. falciparum isolates from Mawza district, Taiz governorate.

2. Methods

2.1. Parasite isolates and ethical considerations

Fifty microscopy-confirmed P. falciparum isolates were collected through a cross-sectional survey carried out in Mawza in the period from October 2013 to April 2014 during the season of malaria transmission (Alareqi et al., 2016). Mawza is a malaria-endemic area with a high-intensity seasonal transmission located in Taiz, west of Yemen (Fig. 1). The study protocol was approved by the Ethics Committee of the University of Science and Technology, Sana’a, Yemen. In addition, written informed consent was obtained from all study participants and/or their guardians before sample collection.

2.2. Sample processing, DNA extraction and molecular detection of parasite species

Three to five drops of blood obtained by fingerprick were blotched onto Whatman® 3MM filter papers (Whatman International Ltd., Maidstone, UK), which were labeled with the participant’s name, number and date of collection. The filter papers were then air-dried and stored in separate, sealed plastic bags at room temperature until DNA extraction. Genomic DNA was extracted from the dried blood spots using DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions and kept at −20°C until use. P. falciparum infection was confirmed using a nested polymerase chain reaction (PCR) targeting the small subunit ribosomal RNA gene as described previously (Singh et al., 1999). Nuclease-free water was used as a negative control. PCR products were resolved on 1.5% agarose gels stained with SYBR® Safe DNA Gel Stain (Invitrogen, CA, USA).

2.3. Molecular detection of pfCRT 76T and pfMDR1 86Y mutations

Nested PCR, as reported previously by Djimdé et al. (2001) and Plowe (2002), was performed to amplify pfCRT and pfMDR1 followed by restriction fragment length polymorphism (RFLP) to detect the 76T and 86Y mutations, respectively. Second-round PCR products were separated and analyzed by electrophoresis on 2.0% agarose gels stained with SYBR® Safe DNA Gel Stain (Invitrogen, CA, USA).

An aliquot (8 μL) of the pfCRT PCR product (145 bp) was subjected to digestion with two units of the restriction enzyme Apol (New England Biolabs, Hitchin, UK) and incubated for 20 min at 37°C. Apol digests the pfCRT K76 wild-type allele into 99 bp and 46 bp fragments but not the 76T mutant allele. The pfMDR1 PCR product (521 bp) was digested with AluI restriction enzyme by incubation at 37°C for 19 h with one unit of each the restriction enzyme. AluI digests the pfMDR1 86Y mutant allele into 299 bp and 222 bp fragments but not the pfMDR1 N86 wild-type allele. The digested products were resolved on 3.0% agarose gels stained with SYBR® Safe DNA Gel Stain (Invitrogen, CA, USA). Results were read with the Gel Doc™ XR+ System (Bio–Rad Laboratories, USA).

2.4. Amplification of K13, pfDHFR and pfDHS genes

The K13-propeller gene (849 bp) was amplified using a nested PCR protocol described by Ariey et al. (2014). However, the pfDHFR and pfDHS were amplified using nested PCR protocols described by Tinto et al. (2007) and Pearce et al. (2003), respectively.
2.5. DNA sequencing and analysis

Each amplicon of \( K13 \), \( pfdhfr \) and \( pfdhps \) genes was subjected to gel purification and nucleotide sequencing in both directions using the primers of the second-round PCR in a commercial laboratory (MyTACG Bioscience Enterprise, Malaysia). Forward and reverse sequences were used for creating a consensus sequence for each isolate. The consensus sequences of each gene were multiple-aligned with a reference sequence of the gene retrieved from the GeneBank database using BioEdit Sequence Alignment Editor (Hall, 1999), version 7.1.9. The GeneBank accession numbers of the \( K13 \), \( pfdhfr \) and \( pfdhps \) reference sequences were XM_0013350122.1, XM_0013351443.1 and XM_001349382.1, respectively. The nucleotide sequences of the multiple alignments for each gene were translated into their corresponding amino acid sequences and manually analyzed for mutations at the specified codons. Molecular markers associated with resistance to artemisinin were investigated by analyzing mutations at four codons of \( K13 \); namely, Y493H, R539T, I543T and C580Y. On the other hand, those associated with resistance to SP were investigated by examining four codons of \( pfdhfr \) (N51I, C59R, S108N and I164L) and five codons of \( pfdhps \) (S436A, A437G, K540E, A581G and A613T).

3. Results

3.1. Characteristics of parasite isolates

Fifty \( P. falciparum \) isolates detected by microscopic examination were used for the detection of antimalarial drug resistance-associated molecular markers. The median asexual parasite density was 400 parasites/µL of blood with the range of 80–84,000 parasites/µL, whereas gametocytes were detected in 16.0% (8/50) of the isolates.

3.2. Frequency distribution of \( pfCRT \), \( pfmdr1 \), \( K13 \), \( pfdhfr \) and \( pfdhps \) mutant alleles

All of the 50 investigated isolates were found to harbor the \( pfCRT \) 76T mutant allele. In contrast, all the isolates showed the \( pfmdr1 \) N86 wild-type allele. With regards to the \( K13 \) propeller gene, all the 47 isolates (94.0%) successfully sequenced and analyzed for the codons associated with resistance to artemisinin were found to harbor the \( K13 \ Y493, R539, I543 \) and C580 wild-type alleles. Of the 45 isolates (90.0%) successfully sequenced and analyzed for the \( pfdhfr \) and \( pfdhps \) codons associated with resistance to SP, one isolate (2.2%) was found to harbor the \( pfdhfr \) 51I/108N double mutant allele, whereas the N51, C59, S108 and I164 wild-type alleles were harbored by the rest of isolates. On the other hand, all the isolates successfully sequenced for \( pfdhps \) showed the S436, A437, K540, A581 and A613 wild-type alleles. Two \( pfmdr1 \) genotypes of \( P. falciparum \) were identified: the double mutant haplotype ([CNI]) in one isolate and the wild-type haplotype ([NCS]) in 44 isolates; however, a single wild-type haplotype ([SAKA]) was identified for \( pfdhps \) in the 45 isolates (Table 1).

4. Discussion

The \( pfCRT \) 76T mutation has been associated with resistance to CQ and is considered as a useful marker for the field surveillance of resistance to the drug (Djimdé et al., 2001; Wellem and Plowe, 2001; White, 2004; Wongsrichanalai et al., 2002). Saturation of all investigated \( P. falciparum \) isolates with the \( pfCRT \) 76T mutant allele reflects the high level of resistance to CQ in Mawza district, west of Taiz. Despite being officially withdrawn since several years (National Malaria Control Program, 2010), the high frequency of this key mutation of resistance to CQ could be possibly attributed to the drug pressure as a result of its continued self-medication use or prescription, its cheap price and over-the-counter availability in several areas in the country. For instance, Bashrahil et al. (2010) reported that CQ is the most frequently prescribed antimalarial by general practitioners after the new ACT policy in Mukalla, an urban city in Hadhramout governorate. Therefore, CQ prescribing may be practiced more frequently by general practitioners in rural areas, including that of the present study. Poor knowledge of physicians about the ACT policy may be one among several reasons for continued prescription of the drug (Bin Ghouth, 2013).

The frequency of the \( pfCRT \) 76T among parasite isolates from Mawza is higher than that reported by a recent study in Taiz (Al-Hamidi et al., 2013), where 50.9% (55/108) of parasite isolates were harboring the mutant allele. It is also higher than those reported from different geographically-dispersed, malaria-endemic areas in the country, being 85.2–90.5% in Hodeidah governorate, west of Yemen (Abdul-Ghani et al., 2013; Al-Mekhlaifi
et al., 2011) and 73.9% in Hadhramout governorate, east of Yemen (Bamaga et al., 2015b) in its pure and mixed-type alleles. However, it is comparable to the pfcr 76T frequency of 98.0% reported among P. falciparum isolates from Lahj governorate, south of Yemen (Mubjier et al., 2011).

The fixed level of pfcr 76T among P. falciparum isolates reflects the continued use of CQ in the study area as a result of its incomplete withdrawal that provides a sustained drug pressure on the parasite population. In this context, high frequencies of 98.7–100% have been reported for the mutant allele among Ugandan isolates of P. falciparum several years after incomplete CQ withdrawal (Kiwuwa et al., 2013; Nsobya et al., 2010). However, it is difficult to compare the situations between Yemen and Uganda because this requires the consideration of a variety of factors, including transmission intensity as well as CQ availability/use in either country. For instance, theoretical models proved an indirect role of the intensity of malaria transmission on the evolution and spread of drug resistance (Hastings and Watkins, 2005; Talisuna et al., 2006).

On the other hand, the fixation of the mutant allele among parasite isolates in the present study is in contrast to its decline after CQ withdrawal in a number of African countries. Several previous reports showed a significant pfcr 76T decline after CQ withdrawal in Malawi (Kublin et al., 2003; Laufer et al., 2006), Tanzania (Alifrangis et al., 2009; Kamugisha et al., 2012a; Malmberg et al., 2013; Mohammed et al., 2013), Kenya (Mang’era et al., 2012; Mwai et al., 2009), Mozambique (Thomsen et al., 2013) and Ethiopia (Mekonnen et al., 2014).

The complete absence of the pfmdr1 86Y mutant allele among P. falciparum isolates from Mawza, Taiz is discordant with the low pfmdr1 86Y frequency of 16.7% that has been recently reported among parasite isolates from Taiz (Al-Hamidi et al., 2013) and Hadhramout (Bamaga et al., 2015b). The absence of this mutant allele could be attributed to the widespread use of AL as a second-line treatment within ACTs; possibly selecting for the pfmdr1 86Y wild-type allele. In this context, several studies suggested that AL contributes to the selection of pfmdr1 86Y wild-type allele among parasite isolates in Africa (Happi et al., 2009; Kamugisha et al., 2012b; Malmberg et al., 2013; Raman et al., 2011; Sisowath et al., 2005; Thomsen et al., 2011, 2013). Moreover, pooled analysis of 31 clinical efficacy studies identified pfmdr1 86Y as an independent predictor of recrudescence in patients treated with AL and recommended its routine detection for monitoring resistance to the combination (Venkatesan et al., 2014). However, lack of baseline surveys for the molecular markers of resistance before adopting AL as a second-line treatment for uncomplicated falciparum malaria in Yemen makes it difficult to assume that the high frequency of the wild-type allele is due to AL pressure. Moreover, the possible role of the pfmdr1 N86 wild-type allele as a predictor of resistance to AL among Yemeni isolates of P. falciparum has yet to be established.

Concerns are increasingly raised about the emergence and spread of artemisinin-resistant P. falciparum in Southeast Asia (Dondorp et al., 2009; Phyo et al., 2012). Recently, a strong association between K13-propeller mutations and delayed clearance of P. falciparum parasites after treatment with artemisinin has been documented in Southeast Asia (Ariey et al., 2014; Ashley et al., 2014). Although their association with resistance to artemisinin has not been validated outside Southeast Asia, K13 mutations have also been reported from a number of countries in sub-Saharan Africa (Kamau et al., 2014). Thus far, there are no previous reports on K13-propeller gene marker from the study area. In the present study, none of the investigated isolates showed mutations in the K13 sequences analyzed. This finding supports those reported by Adeel et al. (2015) on the effectiveness of the ACTs adopted for malaria treatment by the NMCP against uncomplicated falciparum malaria in Yemen.

Resistance to non-artesminisin partners within ACTs is alarming, possibly canceling the goal of the combination therapy and increasing the risk of emergence and spread of resistance to artemisinin derivatives (World Health Organization, 2015). In the present study, all pfdfhr and pfdfsps alleles were of the wild-type except for one isolate showing the pfdfsps 511/108N double mutant allele. This frequency is lower than those reported from Taiz, Hodeideh, Dhamar and Hadhramout governorates (Al-Hamidi et al., 2013; Bamaga et al., 2015a) as well as those reported from Saudi Arabia (Djem et al., 2012). The pfdfsps 589R mutant allele was not detected in parasite isolates from Mawza, which is consistent with that reported by Al-Hamidi et al. (2013) among parasite isolates from Taiz, Hodeideh and Dhamar. In contrast, low frequencies of 5.0% (5/99) and 0.8% (1/102) of the pfdfsps 589R mutant allele were reported from Lahj (Mubjer et al., 2011) and Hadhramout (Bamaga et al., 2015a), respectively.

It is noteworthy that the emergence of resistance to antimalarial drugs is multi-factorial, including the heterogeneous epidemiology of malaria in Yemen that has been classified into three strata

<table>
<thead>
<tr>
<th>Gene</th>
<th>Codon</th>
<th>Amino acid</th>
<th>Wild-type/Mutant</th>
<th>Wild-type n (%)</th>
<th>Mutant n (%)</th>
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<tr>
<td></td>
<td>539</td>
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<td>47 (100)</td>
<td>0 (0.0)</td>
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<td></td>
<td>543</td>
<td>I/T</td>
<td>47 (100)</td>
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<td></td>
<td>580</td>
<td>C/Y</td>
<td>47 (100)</td>
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<tr>
<td>pfdfhr</td>
<td>51</td>
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<td>44 (97.8)</td>
<td>1 (2.2)</td>
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<td>1 (2.2)</td>
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<td>613</td>
<td>A/S</td>
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different in altitude, seasonality and intensity of transmission (National Malaria Control Program, 2010). The impact of intensity of transmission and seasonality on the emergence of antimalarial drug resistance has been well documented (Babiker, 2009; Babiker et al., 2005; Esclante et al., 2005). In general, all studies conducted in Yemen so far have not reported the quadruple pf 

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sample Mawza programs in antimalarial 51I/59R/108N/164L transmission


