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Mutant *Plasmodium falciparum* chloroquine resistance transporter in Hodeidah, Yemen: Association with parasitologic indices and treatment-seeking behaviors

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ABSTRACT

Malaria still represents a major health problem in Yemen, particularly in Hodeidah, despite continuing efforts to eliminate it. With the absence of clinically proven vaccines, chemotherapy with antimalarials is still greatly needed. Chloroquine (CQ) has been popular as the drug of choice for malaria control. However, *Plasmodium falciparum* resistance to CQ has been one of the main obstacles in malaria control and elimination. Although CQ is no longer the recommended antimalarial chemotherapy, it has remained the number one over-the-counter antimalarial drug in many endemic areas, including Yemen, and is still used for self-medication. In addition, promising reports on CQ efficacy reversal in many African countries brought it again into the scene. This has led to a growing interest in the possibility of its re-introduction, particularly with the concerns raised about the parasite resistance to artemisinin-based combination therapies. Therefore, the present study aimed at analyzing the CQ-associated *pfcrt* 76T mutation in *P. falciparum* isolates from patients with uncomplicated falciparum malaria in Hodeidah, west of Yemen. The association of treatment-seeking behaviors and antimalarial drug use with the *pfcrt* 76T mutant allele was also studied. It was revealed that there is still a sustained high frequency of this molecular marker among parasite isolates associated with younger age, decreased parasite density and the presence of gametocytes in blood. Delay in seeking treatment and frequent use of antimalarials were the behaviors significantly associated with the presence of the *pfcrt* 76T mutant allele among patients reporting a history of malaria treatment.

1. Introduction

The evolution and spread of *Plasmodium falciparum* antimalarial drug resistance, particularly to chloroquine (CQ) and sulfadoxine-pyrimethamine (SP), have become a public health concern and led to a significant resurgence of disease morbidity and mortality (Greenwood and Mutabingwa, 2002; Abdul-Ghani et al., 2013). *P. falciparum* has an overwhelming potential to develop resistance to any drug upon wider introduction, including artemisinin-based combination therapies (ACTs) (Wongsrichanalai and Meshnick, 2008; Carrara et al., 2009). Evolution of one or more point mutations in key protein-encoding genes is the principal strategy adopted by *P. falciparum* to overcome drug challenge, and their identification helps in understanding the molecular basis of resistance and clearance of drug-resistant parasites (Sutherland et al., 2002; Djimdé et al., 2003; Hyde, 2005, 2007). Both *in vitro* and *in vivo* methods for measuring drug-resistant malaria are not well-suited for surveillance studies (Plowe et al., 1997). Molecular markers can be effectively used for surveillance and are replacing *in vitro* and *in vivo* tests in many instances, including detection of emerging resistance in a given population even before treatment failure becomes clinically evident, follow-up of the fate of a resistant allele after drug withdrawal and monitoring resistance to a drug partner in a combination therapy (Hastings et al., 2002; Modrzynska, 2010).

P. falciparum chloroquine resistance transporter (*pfcrt*), a gene situated on chromosome 7 encoding the PfCRT in the vacuolar membrane, is the primary determinant of CQ resistance (Fidock et al., 2000; Ecker et al., 2012). Out of all mutations in PfCRT, the mutation resulting in the amino acid change from lysine to threonine at codon 76 (K76T) is essential for CQ resistance; being a marker of CQ resistance *in vitro* and associated with a significantly increased risk of CQ treatment failure *in vivo* (Wongsrichanalai et al., 2002; Djimdé et al., 2003; Hyde, 2005; Lakshmanan et al.,

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Fig. 1. Map of the Arabian Peninsula showing Yemen and the location of Hodeidah governorate.

2005; Tagelsir et al., 2006; Valderramos and Fidock, 2006). In addition, Cooper et al. (2005) suggested the possible effect of *pfcrt* mutations on the susceptibility of *P. falciparum* to the partner drugs of ACTs. For instance, *pfcrt* mutations have been associated with altered responsiveness to many antimalarial drugs, including amodiaquine, halofantrine and possibly mefloquine (Johnson et al., 2004). Therefore, the role of *pfcrt* 76T in affecting the efficacy of the partner drugs used in such combinations could not be ruled out.

Because CQ pressure is not as intensive as in the last decades, *pfcrt* K76T could be very useful in showing CQ resistance trends at its genetic level. There are several promising reports showing a decrease in the prevalence of resistant genotypes after withdrawal of CQ (Kublin et al., 2003; Mita et al., 2003; Wang et al., 2005; Laufer et al., 2006; Hyde, 2007; Mwai et al., 2009; Frosch et al., 2011; Wurtz et al., 2012; Gharbi et al., 2013). It has been suggested that CQ could be potentially re-circulated in the future as a part of antimalarials arsenal with a rotation period of decades (Ekland and Fidock, 2007). The possibility to use CQ-based combinations, possibly limited to targeted populations, has been suggested in Africa in the future (Frosch et al., 2011). Ursing et al. (2007) also suggested that, even in the presence of CQ resistance, a change in the dosing regimen restores efficacy as evidenced in Guinea-Bissau.

Although CQ is no longer the recommended antimalarial chemotherapy, it is still widely used as an over-the-counter antimalarial drug in many endemic areas for self-medication of malaria (Oster et al., 2010). CQ is not recommended as a treatment for uncomplicated malaria in Yemen, but it is still prescribed and purchased over-the-counter due to its cheap price (Al-Mekhlafi et al., 2011). The national malaria treatment policy was revised in 2005 recommending artesunate plus SPand artemether-lumefantrine (AL) as the first-line and the second-line ACTs, respectively, for the treatment of uncomplicated falciparum malaria (NMCP, 2010). *In vitro* and *in vivo* drug resistance studies in Hodeidah are few (Al-Maktari et al., 2003; Al-Shamahy et al., 2007; Al-Kabsi et al., 2009; NMCP, 2010), with only one study of the *pfcrt* K76T mutation (Al-Mekhlafi et al., 2011) being published. There is a need to frequently monitor the changes in the frequency of CQ-resistant genotypes through the detection of *pfcrt* 76T mutation for the possible re-introduction of the drug. Therefore, the present study aimed at monitoring the *pfcrt* 76T mutation in *P. falciparum* isolates from patients with uncomplicated falciparum malaria in Hodeidah, west of Yemen. The association of treatment-seeking behaviors and antimalarial drug use with the *pfcrt* 76T mutant allele was also studied.

2. Subjects and methods

2.1. Study design, setting and population

The present study was a cross-sectional study carried out in Hodeidah governorate, in the western region of Yemen on the Red Sea, in the period from November 2012 to January 2013 (Fig. 1). Based on a prevalence of 16% of *P. falciparum* among febrile patients in Hodeidah (Al-Mekhlafi et al., 2009), a minimum sample size was 207 at a 95% level of confidence and 5% precision. However, several hundreds of febrile patients seeking healthcare in different governmental and private health centers in Hodeidah were screened for falciparum malaria. All 90 out of all examined patients with confirmed falciparum malaria were included in the study of *pfcrt* 76 alleles.

2.2. Data and blood sample collection

The protocol of the study was approved by the Research Ethics Committee of the Medical Research Institute, Alexandria University-Egypt and by Sana'a University-Yemen. Informed consent was obtained from either the patient or legal guardian before data and sample collection. Data regarding sex, age, residence, previous history of malaria and previous malaria treatment as well as data about treatment-seeking behaviors and antimalarial drug use were collected from patients through a pre-designed structured questionnaire. Blood drops were collected by finger prick onto clean glass slides to make thick and thin blood smears, and blotted onto Whatman® 3MM filter papers (Whatman International Ltd., Maidstone, UK). Filter papers were air-dried, placed in individual,

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