The return of chloroquine-sensitive Plasmodium falciparum parasites in Jazan Region, Southwestern Saudi Arabia over a decade after the adoption of artemisinin-based combination therapy: analysis of genetic mutations in the pfcrt gene

Aymen M. Madkhali
Department of Medical Laboratory Technology, Faculty of Applied Medical Sciences, Jazan University, Jazan, Kingdom of Saudi Arabia
Ahmed A. Abdulhaq
Department of Medical Laboratory Technology, Faculty of Applied Medical Sciences, Jazan University, Jazan, Kingdom of Saudi Arabia
Wahib M. Atroosh
Department of Parasitology, Faculty of Medicine, University of Malaya, Kuala Lumpur 50603 Malaysia
Ahmad Hassn Ghzwani
Medical Research Centre, Jazan University, Jazan, Kingdom of Saudi Arabia
Khalid Ammash Zain
Medical Research Centre, Jazan University, Jazan, Kingdom of Saudi Arabia
Khalid Y. Ghailan
Faculty of Public Health and Tropical Medicine, Jazan University, Jazan, Kingdom of Saudi Arabia
Hassan A. Hamali
Department of Medical Laboratory Technology, Faculty of Applied Medical Sciences, Jazan University, Jazan, Kingdom of Saudi Arabia
Abdullah A. Mobarki
Department of Medical Laboratory Technology, Faculty of Applied Medical Sciences, Jazan University, Jazan, Kingdom of Saudi Arabia
Zaki M. Eisa
Saudi Centre for Disease Prevention and Control, Ministry of Health, Jazan, Kingdom of Saudi Arabia
Yee-Ling Lau
Department of Parasitology, Faculty of Medicine, University of Malaya, Kuala Lumpur 50603 Malaysia
Hesham M. Al-Mekhlafi (halmekhlafi@yahoo.com)
Medical Research Centre, Jazan University, Jazan, Kingdom of Saudi Arabia https://orcid.org/0000-0003-2582-7410

Research Article

Keywords: Chloroquine, drug resistance, infectious diseases, malaria, pfcrt, Plasmodium falciparum, Saudi Arabia

Posted Date: August 16th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-812480/v1

Abstract

This study investigated the polymorphism in the *P. falciparum chloroquine resistance transporter* (*pfcrt*) gene 11 years after chloroquine (CQ) cessation in Jazan region, southwestern Saudi Arabia. Two hundred and thirty-five *P. falciparum* isolates were amplified to detect mutations in the *pfcrt* gene. The *pfcrt* 76**T** molecular marker for CQ resistance was detected in 66.4% (156/235) of the isolates, while the K76 CQ-sensitive wild type was detected in 33.6%. The *pfcrt* 74**I** and *pfcrt* 75**E** point mutations were each found to be present in 56.2% of isolates, while only four isolates (1.7%) were found to carry the *pfcrt* 72**S** mutation. Moreover, four *pfcrt* haplotypes were identified: the CV**IET** triple-allele (56.2%), **S**VME**T** double-allele (1.7%), and CVMN**T** single-allele (8.5%) mutant haplotypes, and the CVMNK wild haplotype (33.6%). The analysis also revealed significant associations between the prevalence of mutant *pfcrt* alleles and haplotypes and the age group, governorate, and nationality of the patients as well as the parasitaemia level ($P < 0.05$). The findings provide evidence of the potential re-emergence of CQ-susceptible *P. falciparum* strains in Jazan region over a decade after CQ discontinuation, with about one third of the isolates analysed carrying the *pfcrt* K76 CQ-sensitive wild allele and the CVMNK ancestral wild haplotype. Although the reintroduction of CQ cannot be recommended at present in Saudi Arabia, these findings support the rationale for a potential future role for CQ in malaria treatment. Therefore, continuous molecular and *in-vitro* monitoring mutations of *pfcrt* polymorphism in Jazan region is highly recommended.

Introduction

Malaria, a mosquito-borne disease transmitted by the bite of the female *Anopheles* mosquito, is a leading cause of morbidity and mortality worldwide, especially in the tropics and subtropics. Despite global success in reducing the malaria burden over the last several decades and the successful elimination of malaria from more than 100 countries, the disease is still endemic in 106 countries (WHO 2020; Feachem et al. 2019). Antimalarial drug resistance has emerged as one of the most critical threats hampering the global efforts to control and eliminate malaria. For many decades, chloroquine (CQ) was used extensively as the first-line treatment for uncomplicated malaria. However, in the late 1950s, CQ-resistant *Plasmodium falciparum* malaria emerged independently in Southeast Asia (Thai–Cambodian border) and in South America (Colombia) (Payne 1987). Subsequently, resistant *P. falciparum* strains spread steadily across different countries throughout Southeast Asia and into India, as well as across South America in the 1960s and 1970s, and then spread to Africa in the late 1970s with confirmed treatment failures reported in Kenya and Tanzania (Fogh et al. 1979; Wellems and Plowe 2001). Yet, despite the increasing spread of CQ resistance worldwide, CQ remained the first-line treatment for malaria until the 2000s.

It is well documented that CQ resistance is associated with specific mutations in polymorphism of the *P. falciparum chloroquine resistance transporter* (*pfcrt*) gene located on chromosome 7 (Roux et al. 2021). The cluster of mutations at codons 72 to 76 of the *pfcrt* gene has been found to be associated with the evolution of three main distinct haplotypes/genotypes: 1) the ancestral CQ-susceptible haplotype, which is found in CQ-sensitive isolates and referred to as $C_{72}V_{73}M_{74}N_{75}K_{76}$; 2) the double mutated haplotype $\mathbf{S}_{72}V_{73}M_{74}N_{75}\mathbf{T}_{76}$, which is widely prevalent in Latin America and Asia; and 3) the triple-mutated haplotype $C_{72}V_{73}\mathbf{I}_{74}\mathbf{E}_{75}\mathbf{T}_{76}$, which predominates in Africa and Southeast Asia (mutated alleles are shown in bold) (Ariey et al. 2006; Awasthi et al. 2012). Moreover, other mutated haplotypes such as $C_{72}V_{73}M_{74}\mathbf{E}_{74}\mathbf{T}_{76}$, $C_{72}V_{73}M_{74}N_{75}\mathbf{T}_{76}$ and $\mathbf{S}_{72}V_{73}\mathbf{I}_{74}\mathbf{E}_{75}\mathbf{T}_{76}$ have been reported in other regions (Awasthi et al. 2012; Atroosh et al. 2016). Studies have also revealed that a point mutation at codon 76 of the *pfcrt* gene, which occurs as a result of lysine amino acid substitution by threonine (wild-type K to mutant T-K76T), is highly associated with CQ resistance and clinical CQ treatment failure (Djimdé et al. 2001; Wicht et al. 2020).

In Saudi Arabia, CQ resistance was first recorded in 1992 when reports on clinical treatment failures increased (Malik et al. 1997; Alrajhi et al. 1999; Al-Arishi et al. 2001). Subsequently, the malaria drug policy in the country was scaled up in 2007 and CQ was replaced with ACT, with artesunate plus SP as a first-line treatment and artemether-lumefanthrine as the second-line for the treatment of uncomplicated falciparum malaria (Madkhali et al. 2020; MOH 2018). Since then, some molecular studies have revealed a high prevalence of *pfcrt* 76**T** mutations in isolates from different areas of the country (Al-Harthi, 2007; Bin Dajem and Al-Qahtani 2010; Bin Dajem et al. 2012). However, those studies only evaluated the mutations at codon 76 of the *pfcrt* gene, so data on mutations at other codons as well as on *pfcrt*-related haplotypes are very limited. Interestingly, some previous studies conducted in Africa reported the re-emergence of CQ-sensitive *P. falciparum* strains several years following the discontinuation of CQ use and the switching of malaria treatment to ACT (Laufer et al. 2006; Dagnogo et al. 2018; Balikagala et al. 2020).

The paucity of data on the status of *pfcrt* point mutations in Saudi Arabia after 2012 and the potential re-emerging sensitivity to CQ motivated the current study to investigate the frequency and distribution of *pfcrt* point mutations in *P. falciparum* isolates in the malaria-endemic Jazan region, 11 years after the ban on the use of CQ to treat uncomplicated falciparum malaria.

Materials And Methods

Study design and area

The current study was a cross-sectional hospital-based study conducted in Jazan region that targeted febrile patients who were suspected to have malaria. Jazan region is located 16° 17' North, 42° 43' East in the southwestern part of Saudi Arabia. The region is bordered by Yemen to the south

and by the Red Sea to the west. The region comprises 17 governorates, extends over a total area of 11671 km² and is home to a population of about 1.4 million (GASTAT 2021).

Topologically speaking, Jazan region can be divided into three different areas: the highlands, as represented by the Faifa mountains (which constitute part of the As Sarawat mountain range) at an elevation of over 2000 m above sea level, the foothills at an elevation of 400–600 m and the coastal plains alongside the Red Sea at an altitude of less than 400 m. The aggregated rainfall varies between less than 100 mm/year on the coastal plains and more than 300 mm/year in the highlands (Lashin and Al Arifi 2012). The region also contains some valleys, as well as a number of streams and dams that act as a source of drinking and irrigation water (MEWA 2019). Malaria is endemic in Jazan region, with a few foci of malaria transmission, and transmission peaks between December and March (Madkhali et al. 2020). *Anopheles arabiensis* is considered the principal malaria vector in the region; however, other species including *An. sergentii*, *An. dthali* and *An. stephensi* have also been recorded (Alahmed et al. 2019; Snow et al. 2013).

Study population

Febrile individuals who presented at selected healthcare facilities in Jazan region and tested positive for malaria between April and December 2018 were invited to participate in the current study, irrespective of their gender, nationality and age. The participants' demographic data (residency, gender, age and nationality) were collected through the use of a semi-structured questionnaire or from the patients' medical records. This was an exploratory descriptive study that did not necessitate the calculation of a power and sample size. However, the required sample size was calculated based on the WHO's guidelines (Lwanga et al. 1991). Accordingly, at the high prevalence of *pfcrt* 76**T** mutation previously reported, a 95% confidence level and a 5% significance level, the calculation yielded a minimum sample size of 138 *P. falciparum* isolates. During the study period, a total of 530 participants were screened for malaria, from whom 250 *P. falciparum* isolates were collected for the molecular examination of point mutations in the *pfcrt* gene. Subsequently, *pfcrt* gene were successfully amplified from 235 of those isolates and used for the data analysis.

Blood sampling and examination

A blood sample of approximately 2–3 ml was obtained from each patient and put into an EDTA tube labelled clearly with the participant's identification number, name, gender and age. Promptly thereafter, both thin and thick blood films/smears were prepared for each blood sample and stained with Giemsa stain in accordance with a standard protocol (WHO 2015). Additionally, dried blood spots were prepared on 3MM Whatman® filter paper and kept in labelled, separate, sealed plastic bag for molecular analysis. The thin and thick stained blood smears were examined microscopically for the presence of *Plasmodium* parasites and identification of species. From the thick blood film, the parasitaemia (also called parasite density) was estimated by counting the asexual stages of the *Plasmodium* parasite against 200 white blood cells (WBCs), under the assumption of an average WBC count per µl of blood of 8000 (WHO 2015; Al-Mekhlafi et al. 2021). Some archived malaria-positive slides for the patients examined during the study period were also obtained from the participating healthcare facilities and re-examined for parasite species and parasite density.

Molecular analysis

Extraction of DNA from dried blood spots was done using a Qiagen blood and tissue kit (QIAGEN, DNeasy® Blood & Tissue Kit, Cat. no. 69506, Germany) following the manufacturer's instructions provided in the kit. The extracted DNA was eluted using 100 µl of AE elution buffer, which contained 10 mM Tris-Cl; 0.5 mM EDTA; at pH 9.0 and was kept at -20°C until used.

Detection of pfcrt point mutations

The genomic DNA of *P. falciparum* was subjected to the polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) technique in order to detect mutations at codons 72 to 76 of the *pfcrt* gene, according to an established protocol (Djimdé et al. 2001). Restriction enzyme (RE) digestion was performed in 20 µl of reaction mixture that included 6–8 µl of PCR product, 1 unit of the specific RE (New England Biolabs Inc., UK) and 1X of the specific buffer provided in the RE kit. The reaction mixture was then incubated for 15–60 minutes at 37°C or 50°C (based on the RE) following the manufacturer's instructions. Then, the mixture was subjected to gel electrophoresis using 4% of TAE-buffered agarose gel stained with Sybr® safe DNA gel stain (Invitrogen, USA). The RE digestion products were visualised under UV light using a Molecular Imager Gel Doc XR System (Bio-Rad, Hercules, CA, USA). The primer sequences, PCR thermal conditions, and RE used for the detection of point mutations of the *pfcrt* gene have been described previously (Atroosh et al. 2016).

The genomic DNA of *P. falciparum* reference strains that were used by the current study as positive controls were provided by the Malaria Research and Reference Reagents Resources Center (MR4, ATCCW, Manassas, VA, USA). The genomic DNA of *P. falciparum* strain Dd2 (MRA-150G) was utilised as a positive control for the *pfcrt* mutated types, while the *P. falciparum* reference strain 3D7 (MRA-102G) was utilised as a positive control for the wild types.

Statistical data analysis

Statistical data analysis was done using IBM SPSS v20 (IBM Corp., NY, USA). Categorical variables, i.e. the *pfcrt* point mutations and haplotypes at the studied codons (dependent variable) and other variables such as gender, nationality and age group were described as frequencies and

proportions. On the other hand, mean ± standard deviation (SD) or median (interquartile range, IQR) were used to describe quantitative variables namely age (years) and parasite density. Associations between the dependent variables and the explanatory variables were examined using the Chi-square test or Fisher's exact test, where appropriate. The significance level of all tests was set at $P < 0.05$.

Results

A total of 235 *P. falciparum*-positive patients aged between 2 and 78 years, with a mean age of 30.5 ± 11.6 years, were included in the current study. The majority of the participants (82.1%) were male and 17.9% were female. As regards residency, 69.8% and 30.2% of the participants were from urban and rural areas, respectively. During the study period, malaria-positive samples were collected from all the governorates of Jazan except Altwal, Alraith, Haroob, Fayfa highlands and Farasan Island. The highest percentage (19.1%) of the isolates was from Baysh governorate followed by Jizan (12.8%) and Alharth (11.5%) governorates. About one third (34%) of the participants were Saudi and 66% (155/235) were non-Saudi, and 44.5% (69/155) of the non-Saudi participants were from Yemen. Table 1 displays the general characteristics of the patients who participated in the study.

Table 1
General characteristics of study participants (n = 235).

Variables	Number	%
Age*	30.5 ± 11.6	-
Age groups		
< 18	34	14.5
18–30	96	40.9
31–40	61	26.0
41–50	30	12.8
≥ 51	14	6.0
Gender		
Male	193	82.1
Female	42	17.9
Nationality		
Saudi Arabia	80	34.0
Yemen	69	29.4
Pakistan	29	12.3
India	21	8.9
Sudan	11	4.7
Egypt	9	3.8
Bangladesh	8	3.4
Ethiopia	5	2.1
Philippine	2	.9
Syria	1	.4
* mean ± SD		

Prevalence and distribution of pfcrt mutations and haplotypes

Table 2 shows the frequency distribution of the *pfcrt* gene point mutations and haplotypes/genotypes in the study sample. Of the 250 isolates subjected to the initial molecular analysis, 235 (94%) were successfully amplified for the *pfcrt* gene. The *pfcrt* 76**T** CQ-resistant mutant type was found in 66.4% (159/235) of the isolates, while 33.6% of the isolates were found to be carrying the K76 CQ-sensitive wild type. The *pfcrt* 74**I** and

pfcrt 75**E** point mutations were each detected in 56.2% of the isolates. On the other hand, isolates were found of all wild types for the *pfcrt* 73 codon, while only four isolates (1.7%) had *pfcrt* 72**S** mutated alleles.

Table 2
Prevalence and distribution of *pfcrt* point mutations and haplotypes for *P. falciparum* isolates from Jazan region (n = 235).

Marker	Type of mutations	Number	%
Pfcrt 72	Wild	231	98.3
	Mutated	4	1.7
Pfcrt 73	Wild	235	100
	Mutated	0	0
Pfcrt 74	Wild	103	43.8
	Mutated	132	56.2
Pfcrt 75	Wild	103	43.8
	Mutated	132	56.2
Pfcrt 76	Wild	79	33.6
	Mutated	156	66.4
CVMNK	Wild	79	33.6
CVMNT	Single	20	8.5
SVMNT	Double	4	1.7
CVIET	Triple	132	56.2
Mutant alleles are bold and underlined.			

With regard to the haplotypes/genotypes, four *pfcrt* 72–76 haplotypes were found to be circulating in the study area. The haplotype identified as having the highest prevalence was the CV**IET** triple-allele mutated haplotype (56.2%), while the wild haplotype, CVMNK was found in 79 isolates (33.6%). In addition, single-allele CVMN**T** and double-allele **S**VME**T** mutated haplotypes were found in 8.5% and 1.7% of the study isolates.

Associations of pfcrt mutations with demographic factors and parasitaemia

Table 3 shows the associations between the *pfcrt* point mutations and related haplotypes and the participants' demographic factors and parasitaemia level. In respect of age, a significantly higher percentage of isolates were found to be carrying the mutated *pfcrt* 76**T** allele among patients aged ≥ 30 years (72.4%, 89/123) as compared to patients aged < 30 years (59.8%, 67/112) ($\chi^2 = 4.128$; df = 1; $P = 0.042$). Also, the percentage of isolates carrying the CVMNK wild haplotype from patients aged < 30 years (40.2%) was significantly higher than the percentage in the isolates from those aged ≥ 30 years (27.6%) ($P = 0.042$).

Table 3
Distribution of *pfcrt* mutant alleles and haplotypes for *P. falciparum* isolates from Jazan region according to demographic factors and parasitaemia (n = 235).

Marker	Age group			Sex			Nationality			Parasitaemia		
	< 30	≥ 30	*P*	Females	Males	*P*	Saudi	Non-Saudi	*P*	Low	Moderate-to-high	*P*
Pfcrt 72S	2 (1.8)	2 (1.6)	0.979†	1 (2.4)	3 (1.6)	0.548†	0 (0.0)	4 (2.6)	0.302†	2 (1.9)	2 (1.5)	0.892†
Pfcrt 74I	57 (50.9)	75 (61.0)	0.120	22 (52.4)	110 (57.0)	0.585	43 (53.8)	89 (57.4)	0.591	66 (64.1)	66 (50.0)	0.031*
Pfcrt 75E	57 (50.9)	75 (61.0)	0.120	22 (52.4)	110 (57.0)	0.585	43 (53.8)	89 (57.4)	0.591	66 (64.1)	66 (50.0)	0.031*
Pfcrt 76T	67 (59.8)	89 (72.4)	0.042*	26 (61.9)	130 (67.4)	0.498	47 (58.8)	109 (70.3)	0.075	73 (70.9)	83 (62.9)	0.198
CVMNK	45 (40.2)	34 (27.6)	0.042*	16 (38.1)	63 (32.6)	0.498	33 (41.2)	46 (29.7)	0.075	30 (29.1)	49 (37.1)	0.198
CVMNT	8 (7.1)	12 (9.8)	0.473	3 (7.1)	17 (8.8)	0.935†	4 (5.0)	16 (10.3)	0.166	5 (4.9)	15 (11.4)	0.076
SVMNT	2 (1.8)	2 (1.6)	0.979†	1 (2.4)	3 (1.6)	0.548†	0 (0.0)	4 (2.6)	0.302†	2 (1.9)	2 (1.5)	0.892†
CVIET	57 (50.9)	75 (61.0)	0.120	22 (52.4)	110 (57.0)	0.585	43 (53.8)	89 (57.4)	0.591	66 (64.1)	66 (50.0)	0.031*

All values are number (%).

Pfcrt 73 was of wild type (V73) and thus, not included in the analysis.

Parasitaemia levels: low (< 1,000 parasites/µl of blood); moderate-to-high (≥ 1,000 parasites/µl of blood).

Mutant alleles are bold and underlined.

*Significant association ($P < 0.05$)

† The difference was examined using Fisher's exact test (otherwise, Chi Square test was used).

In regard to parasite density, the percentages of *pfcrt* 74**I** and *pfcrt* 75**E** mutated alleles were significantly higher ($\chi^2 = 4.657$; df = 1; $P = 0.031$) in isolates from patients with low parasitaemia (64.1%, 66/103) as compared to those from patients with moderate-to-high levels of parasitaemia (50%, 66/132). Moreover, a significantly higher percentage of the CV**IET** mutated haplotype (also known as the classical old-world African/Southeast Asian haplotype) was observed in isolates from patients with low parasitaemia as compared to those from patients with moderate-to-high parasitaemia (64.1%, 50%; $P = 0.031$).

As for nationality, the *pfcrt* 76**T** allele was observed to be present predominantly among patients from Yemen (94.2%) followed by those from Pakistan (65.5%) and then among Saudi nationals (58.8%) (Table 3). Interestingly, higher percentages of isolates carrying the mutated *pfcrt* 76**T** allele and CV**IET** mutated haplotype were found in isolates from non-Saudi patients (70.3% and 57.4%, respectively) as compared to their Saudi counterparts (58.8% and 53.8%, respectively). However, the differences were not statistically significant ($P > 0.05$). In other words, a higher percentage of the wild-type K76 allele was observed in isolates from Saudi (41.2%) as compared to non-Saudi (29.7%) patients.

On the other hand, there were significant variations across governorates in the *pfcrt* point mutations ($\chi^2 = 23.040$; df = 11; $P = 0.017$) and in haplotypes ($\chi^2 = 22.486$; df = 11; $P = 0.021$) (Fig. 1 and Supplementary Table S1). The highest percentage of the mutant *pfcrt* 76**T** allele was reported in isolates from Aldair governorate (95.7%, 22/23) followed by isolates from Baysh (77.8%, 35/45) and Sabya (76.9%, 10/13) governorates, whereas the highest percentage of the *pfcrt* K76 wild allele and the respective CVMNK haplotype were found in isolates from Samtah (63.2%, 12/19) followed by Ahad Almsariha (50%, 2/4) governorate. Also, the percentage of the CV**IET** haplotype was highest in isolates from Aldair (91.3%, 21/23) followed by Dhamad (70%, 7/10) governorate.

Discussion

Given the ongoing challenges associated with the elimination of this serious disease, the current study investigated the frequency and distribution of *pfcrt* gene point mutations in *P. falciparum* isolates collected from Jazan region in southwestern Saudi Arabia, just over a decade after the cessation of CQ use and the adoption of ACT for the treatment of uncomplicated falciparum malaria.

The results revealed that the *pfcrt* 76**T** point mutation (the most important mutation linked with CQ resistance) was present in about two thirds (66.4%) of the examined isolates, whereas about one third (33.6%) of the isolates were found to carry the K76 wild variant. The results also showed a higher percentage of the wild-type K76 allele of the *pfcrt* gene in isolates from Saudi (41.2%) as compared to those from non-Saudi (29.7%) patients, and importantly, there were also some locally transmitted isolates (autochthonous malaria). Therefore, these results indicate the potential return of CQ-sensitive strains in Saudi Arabia.

Although CQ resistance and clinical treatment failures in the country were reported in the early 1990s (Malik et al. 1997), molecular studies on *pfcrt* were not conducted until in the mid-2000s. Prior to those studies, the CQ treatment failure rates varied between 12% and 38% (Alrajhi et al. 1999; Malik et al. 1998; Ghalib et al. 2001). The first molecular study on *pfcrt* gene polymorphism was conducted in 2007 and showed that about 90% of 19 *P. falciparum* isolates from Jazan region carried the *pfcrt* 76**T** mutated allele (Al Harithi et al. 2007). Later, other studies found the 76**T** mutated allele of *pfcrt* in 100% of 121 *P. falciparum* isolates from both the Jazan and Aseer regions (Bin Dajem and Al-Qahtani 2010; Bin Dajem et al. 2011). Likewise, a comprehensive previous study found that 99% of 165 *P. falciparum* isolates from Jazan were carrying the *pfcrt* 76**T** mutated allele (Bin Dajem et al. 2012). A more recent study detected the *pfcrt* 76**T** mutation in only four out of 13 (30.7%) *P. falciparum* isolates from Taif province, about 720 km north of Jazan, while the remainder (eight isolates) were carrying the wild allele (Soliman et al. 2018). However, the results of that study are limited by the very small sample size.

Overall, the withdrawal of CQ and the adoption of ACT treatments in Saudi Arabia in 2007 might explain the re-emergence of CQ-sensitive isolates, as identified by the current study. Yet, there is a dearth of information on the frequency of *pfcrt* point mutations in Saudi Arabia. Therefore, the hypothesis proposed herein that CQ-sensitive strains have reappeared in the country requires further evaluation. Moreover, microsatellite loci analyses are essential to compare the genetic similarity among CQ-susceptible and among CQ-resistant parasites as well as between parasite populations circulating before and after the official banning of CQ as a falciparum malaria chemotherapy in Saudi Arabia.

Interestingly, some previous studies, exclusively from Africa, have demonstrated the return of CQ-sensitive *P. falciparum* strains several years after the cessation of CQ use and have suggested that CQ might once again be effective. In 1994, Malawi was the first country in Africa to withdraw CQ, and 12 years later molecular markers of CQ resistance had completely disappeared and the K76 wild type had reached fixation (Kublin et al. 2003; Frosch et al. 2014). Subsequently, a randomised clinical trial demonstrated the superior efficacy of CQ against uncomplicated falciparum malaria in Malawi, with a cumulative efficacy of 99% as compared to 21% for SP, and also detected the K76 wild type in all isolates (Laufer et al. 2006). Similarly, in 1998, Kenya scaled up its national malaria drug policy and switched from CQ to SP. Subsequently, a study showed increasing rates of the K76 wild type until 2006 (Mwai et al. 2009). However, another study conducted in western Kenya, where CQ use continued despite a national-level policy change, showed that the prevalence of mutant type *pfcrt* 76**T** increased significantly, from 76% in 2001 to 94% in 2007 (Shah et al. 2015). Moreover, the re-emergence of CQ-sensitive falciparum malaria has been reported in other African countries, including Sudan (Bakhiet et al. 2019), Tanzania (Alifrangis et al. 2009; Mohammed et al. 2013), Côte d'Ivoire (Dagnogo et al. 2018) and Uganda (Balikagala et al. 2020).

Looking at those countries geographically closer to Saudi Arabia, the prevalence of the mutant *pfcrt* 76**T** allele in the neighbouring endemic country of Yemen was reported to be as high as 100% several years after switching from CQ to ACT in 2009 (Al-Mekhlafı et al. 2011; Alareqi et al. 2016; Atroosh et al. 2016). Similar findings were reported for Pakistan (Khattak et al. 2013; Khan et al. 2020). These findings are consistent with those of the current study, which showed that the highest percentage of the mutant *pfcrt* 76**T** allele was in the isolates from Yemeni (94.2%) followed by Pakistani (65.5%) patients, as compared to those from other nationalities. Bearing in mind that over 12 million foreign nationals are employed in Saudi Arabia, and that the majority of them are from malaria-endemic countries such as Pakistan, Yemen, Sudan and Ethiopia (US-SABC 2020), and that malaria in Jazan region is mostly imported, with a very low number of locally transmitted cases reported (Al-Mekhlafı et al. 2021), it would be desirable to carry out further studies on a larger number of samples to carefully evaluate *pfcrt* polymorphism according to nationality and source of case.

The findings of the current study also showed that the percentage of CV**IET** triple-mutant haplotype in the studied isolates was 56.2%, while about only one third (33.6%) of the isolates harboured the CVMNK wild haplotype. The only previous study that reported the presence of *pfcrt*-related haplotypes in Saudi Arabia was conducted in Jazan region and showed that almost all the studied isolates (99%; 163/165) harboured the triple-mutant CV**IET** haplotype (Ben Dajem et al. 2012). The reduction in the prevalence of this haplotype in the region is attributed to the re-emergence of the *pfcrt* K76 wild allele reported by the current study. Similar findings have also been reported in some African countries that witnessed a partial return of the *pfcrt* K76 wild allele (Alifrangis et al. 2009). The CV**IET** haplotype which originated in Southeast Asia is the most common haplotype in some African and Middle Eastern countries, including Yemen (Al-Hamidhi et al. 2013), and Sudan (Gadalla et al. 2010), whereas the **S**VMN**T** double mutant haplotype has remained the predominant haplotype in Asian countries close to the Arabian Peninsula, such as Pakistan (Sahar et al. 2015) and Iran (Ursing et al. 2006). These findings are consistent with the current study which found that the **S**VMN**T** haplotype was present only in isolates from Pakistani patients, while the percentage of the CV**IET** haplotype was highest in isolates from Yemeni patients.

The current study also examined the associations of point mutations and haplotypes in the *pfcrt* gene with the participants' demographic factors and parasitaemia. The results showed that the percentage of the *pfcrt* 76**T** mutation and the percentage of the CVMNK wild haplotype was significantly higher in isolates from participants aged ≥ 30 years and < 30 years, respectively. The association between the *pfcrt* 76**T** mutation and the host's age is controversial, where some studies have showed a significant association (May and Meyer 2003; Happi et al. 2006; Al-Mekhlafi et al. 2011) and others have reported no association (Atroosh et al. 2016; Acharya et al. 2018).

As regards a link with parasite density, the current study found that the percentages of the *pfcrt* 74**I** and 75**E** mutations and the CV**IET** mutant haplotype were significantly high in isolates from patients with a low parasitaemia level. Although the reason for these associations is unclear, previous studies reported significant associations of *pfcrt* mutations (particularly 76**T**) with parasitaemia and severity of infection (Al-Mekhlafi et al. 2011; Wélé et al. 2011; Atroosh et al. 2012; Acharya et al. 2018; Cuu et al. 2020). However, another study found no significant association (Mayeng et al. 2007; Atroosh et al. 2016).

In respect of a connection with residency, there was significant variation in the distribution of *pfcrt* polymorphism across the governorates involved in the current study. Almost all of the isolates from Aldair governorate were found to carry the mutant *pfcrt* 76**T** allele and over 90% of them were carrying the CV**IET** haplotype. Aldair borders Yemen to the south, and the highest prevalence of these markers was found in isolates from Yemeni patients. Studies conducted in Yemen have reported a very high prevalence of the *pfcrt* 76**T** mutation, which has reached fixation (100%) in some areas (Al-Mekhlafi et al. 2011; Alareqi et al. 2016; Atroosh et al. 2016). Therefore, taking into account that Jazan region is the smallest region of Saudi Arabia, the reported variation can be attributed to the patients' residency. Nonetheless, the distinct variation in *pfcrt* 76**T** either between countries or within the same country has previously been reported (Bamaga et al. 2015; Shah et al. 2015).

When interpreting the findings of the current study that have been discussed above, it is important to take bear in mind that the study has a few limitations. First, only codons 72 to 76 of the *pfcrt* gene were assessed, while other codons such as 97, 220, 271, 326, 353, 356, and 371 were not included. Indeed, point mutation 76**T** has become a hallmark of CQ resistance worldwide, and is widely used as an epidemiological tool for the monitoring of CQ resistance in large-scale field studies (Djimdé et al. 2001; Roux et al. 2021). Moreover, the cluster of mutations at codons 72 to 76 describes the evolution of the main *pfcrt* genotypes, including the SVMNT ancestral wild haplotype as well as the related mutated haplotypes such as the predominant and widespread CV**IET** haplotype (Awasthi et al. 2012). Second, a small number of blood samples was collected from female as compared to male participants as well as from some governorates and some nationalities. Nonetheless, the current study still provides important data about the distribution of *pfcrt* polymorphism in Jazan region after over a decade of CQ treatment withdrawal.

Conclusion

The current study provides important information on the status of CQ resistance in Jazan region, southwestern Saudi Arabia. The findings revealed a potential return of CQ-susceptible *P. falciparum* strains to the region. While the mutant *pfcrt* 76**T** allele and the triple-mutant haplotype CV**IET** were detected in 66.4% and 56.2% of the examined isolates, respectively, 33.6% of the isolates were found to be carrying the *pfcrt* K76 wild allele and the SVMNT wild haplotype. There is a consensus in the research community globally that ceasing the use of CQ in a region could result in the re-emergence of CQ-sensitive *P. falciparum* strains. This raises the possibility of the reintroduction of this safe and affordable drug, ideally in combination with another antimalarial drug, for malaria treatment. However, further studies to test this hypothesis are required.

Studies on the molecular markers of antimalarial drug resistance in Saudi Arabia are limited. Therefore, the findings from this study have significant implications for the monitoring of antimalarial drug resistance in the region. Consequently, the continuous molecular surveillance using the *pfcrt* 76**T** point mutation as a reliable marker for CQ resistance as well as further *in vitro* and *ex vivo* molecular studies would be highly beneficial. Furthermore, microsatellite loci analyses of larger number of samples set nationally to carefully evaluate *pfcrt* polymorphisms in Saudi Arabia is strongly recommended.

Declarations

Acknowledgements

The authors are thankful to all participants for their voluntary participation in this study. The authors would also like to thank the administrative and laboratory staff from all participating healthcare facilities in Jazan region for their fruitful cooperation and support.

Authors' contributions

HMA AMM and AAA conceived and designed the study. AHG KAZ and HMA conducted the field survey and collection of samples and involved in the laboratory examination of samples. WMA performed the laboratory molecular experiments. WMA and HMA analysed the data. ZME provided logistic support for data collection and fieldwork. YLL provided logistic support for lab work. HMA wrote the paper. KYG AAA HAH AAM ZME and YLL revised the manuscript critically. The manuscript has been approved by all authors prior to submission. All authors approved the final version of the manuscript.

Funding

The work described here was funded by the Research Groups funding programme of the Deanship of Scientific Research, Jazan University (Vector-Borne Diseases Research Group, Grant no. RG-2-1).

Availability of data and material

All relevant data is available in the manuscript.

Code availability

Not applicable.

Declarations

Ethical approval

Ethical approval for the current study was obtained from the Ethics Committee of Jazan University (Ref. no. REC39/6-261). The study was also approved by the Ethics Committee of the Health Affairs Directorate of King Fahd Central Hospital, Jazan (Registry no. 086). Before data collection, the patients, and their guardians in case of children, were informed about the objectives of the study and their role. They were also informed that their participation was entirely voluntary and that they could withdraw at any time without giving any reasons whatsoever and without any consequences in respect of their health care. Then, written signed consent was obtained from the adult participants or from the children's parents or guardians. The patients were treated for malaria at the respective healthcare facilities according to the national malaria drug policy.

Conflict of interest

The authors declare that they have no competing interests.

Consent for publication

All authors have agreed and consented to publish the manuscript.

References

1. Acharya A, Bansal D, Bharti PK, Khan FY, Abusalah S, Elmalik A, ElKhalifa M, Mohapatra PK, Mahanta J, Sehgal R, Singh N, Sultan AA (2018) Molecular surveillance of chloroquine drug resistance markers (*Pfcrt* and *Pfmdr1*) among imported *Plasmodium falciparum* malaria in Qatar. Pathog Glob Health 112:57–62. https://doi.org/10.1080/20477724.2017.1399234.
2. Al Harthi SA (2007) Detection of drug resistance markers for chloroquine and pyrimethamine-sulfadoxine in Jazan area, Saudi Arabia using PCR and restriction digestion. J Egypt Soc Parasitol 37:17–30.
3. Alahmed AM, Munawar K, Khalil SMS, Harbach RE (2019) Assessment and an updated list of the mosquitoes of Saudi Arabia. Parasit Vectors 12:356. https://doi.org/10.1186/s13071-019-3579-4.
4. Alareqi LMQ, Mahdy MAK, Lau YL, Fong MY, Abdul-Ghani R, Mahmud R (2016) 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. Acta Trop 162:174–179. https://doi.org/10.1016/j.actatropica.2016.06.016.
5. Al-Hamidhi S, Mahdy MA, Al-Hashami Z, Al-Farsi H, Al-mekhlafi AM, Idris MA, Beja-Pereira A, Babiker HA (2013) Genetic diversity of *Plasmodium falciparum* and distribution of drug resistance haplotypes in Yemen. Malar J 12:244. https://doi.org/10.1186/1475-2875-12-244.
6. Alifrangis M, Lusingu JP, Mmbando B, Dalgaard MB, Vestergaard LS, Ishengoma D, Khalil IF, Theander TG, Lemnge MM, Bygbjerg IC (2009) Five-year surveillance of molecular markers of *Plasmodium falciparum* antimalarial drug resistance in Korogwe District, Tanzania: accumulation of the 581G mutation in the *P. falciparum* dihydropteroate synthase gene. Am J Trop Med Hyg 80:523–527.
7. Al-Mekhlafi AM, Mahdy MA, Al-Mekhlafi HM, Azazy AA, Fong MY (2011) High frequency of *Plasmodium falciparum* chloroquine resistance marker (pfcrt T76 mutation) in Yemen: an urgent need to re-examine malaria drug policy. Parasit Vectors 4:94. https://doi.org/10.1186/1756-3305-4-94.
8. Al-Mekhlafi HM, Madkhali AM, Ghailan KY, Abdulhaq AA, Ghzwani AH, Zain KA, Atroosh WM, Alshabi A, Khadashi HA, Darraj MA, Eisa ZM (2021) Residual malaria in Jazan region, southwestern Saudi Arabia: the situation, challenges and climatic drivers of autochthonous malaria. Malar J 20:315. https://doi.org/10.1186/s12936-021-03846-4.
9. Alrajhi AA, Rahim I, Akood M, Hazmi M (1999) Chloroquine-resistant *Plasmodium falciparum* cerebral malaria in a chloroquine-susceptible area. J Infect Dis 180:1738–1741.
10. Ariey F, Fandeur T, Durand R, Randrianarivelojosia M, Jambou R, Legrand E, Ekala MT, Bouchier C, Cojean S, Duchemin JB, Robert V, Le Bras J, Mercereau-Puijalon O (2006) Invasion of Africa by a single pfcrt allele of South East Asian type. Malar J 5:34. https://doi.org/10.1186/1475-2875-5-34.

11. Atroosh WM, Al-Mekhlafi HM, Al-Jasari A, Sady H, Dawaki SS, Elyana FN, Al-Areeqi MA, Nasr NA, Abdulsalam AM, Subramaniam LR, Azzani M, Ithoi I, Lau YL, Surin J (2016) Different patterns of pfcrt and pfmdr1 polymorphism in *Plasmodium falciparum* isolates from Tehama region, Yemen. PeerJ 4:e2191. https://doi.org/10.7717/peerj.2191.
12. Atroosh WM, Al-Mekhlafi HM, Mahdy MA, Surin J (2012) The detection of pfcrt and pfmdr1 point mutations as molecular markers of chloroquine drug resistance, Pahang, Malaysia. Malar J 11:251. https://doi.org/10.1186/1475-2875-11-251.
13. Awasthi G, Satya Prasad GB, Das A (2012) Pfcrt haplotypes and the evolutionary history of chloroquine-resistant *Plasmodium falciparum*. Mem Inst Oswaldo Cruz 107:129–134. https://doi.org/10.1590/s0074-02762012000100018.
14. Bakhiet AMA, Abdelraheem MH, Kheir A, Omer S, Gismelseed L, Abdel-Muhsin AA, Naiem A, Al Hosni A, Al Dhuhli A, Al Rubkhi M, Al-Hamidhi S, Gadalla A, Mukhtar M, Sultan AA, Babiker HA (2019) Evolution of *Plasmodium falciparum* drug resistance genes following artemisinin combination therapy in Sudan. Trans R Soc Trop Med Hyg 113:693–700. https://doi.org/10.1093/trstmh/trz059.
15. Balikagala B, Sakurai-Yatsushiro M, Tachibana SI, Ikeda M, Yamauchi M, Katuro OT, Ntege EH, Sekihara M, Fukuda N, Takahashi N, Yatsushiro S, Mori T, Hirai M, Opio W, Obwoya PS, Anywar DA, Auma MA, Palacpac NMQ, Tsuboi T, Odongo-Aginya EI, Kimura E, Ogwang M, Horii T, Mita T (2020) Recovery and stable persistence of chloroquine sensitivity in *Plasmodium falciparum* parasites after its discontinued use in Northern Uganda. Malar J 19:76. https://doi.org/10.1186/s12936-020-03157-0.
16. Bamaga OA, Mahdy MA, Lim YA (2015) Survey of chloroquine-resistant mutations in the *Plasmodium falciparum* pfcrt and pfmdr-1 genes in Hadhramout, Yemen. Acta Trop 149:59–63. https://doi.org/10.1016/j.actatropica.2015.05.013.
17. Bin Dajem SM, Al-Farsi HM, Al-Hashami ZS, Al-Sheikh AA, Al-Qahtani A, Babiker HA (2012) Distribution of drug resistance genotypes in *Plasmodium falciparum* in an area of limited parasite diversity in Saudi Arabia. Am J Trop Med Hyg 86:782–788. https://doi.org/10.4269/ajtmh.2012.11-0520.
18. Bin Dajem SM, Al-Qahtani A (2010) Analysis of gene mutations involved in chloroquine resistance in *Plasmodium falciparum* parasites isolated from patients in the southwest of Saudi Arabia. Ann Saudi Med 30:187–192.
19. Bin Dajem SM, Al-Sheikh AA, Bohol MF, Alhawi M, Al-Ahdal MN, Al-Qahtani A (2011) Detecting mutations in PfCRT and PfMDR1 genes among *Plasmodium falciparum* isolates from Saudi Arabia by pyrosequencing. Parasitol Res 109:291–296. https://doi.org/10.1007/s00436-011-2251-5.
20. Cuu G, Asua V, Tukwasibwe S, Nsobya SL, Nanteza A, Kimuda MP, Mpimbaza A, Rosenthal PJ (2020) Associations between aminoquinoline resistance genotypes and clinical presentations of *Plasmodium falciparum* infection in Uganda. Antimicrob Agents Chemother 64:e00721-20. https://doi.org/10.1128/AAC.00721-20.
21. Dagnogo O, Ako AB, Ouattara L, Dago ND, Coulibaly DN, Touré AO, Djaman JA (2018) Towards a re-emergence of chloroquine sensitivity in Côte d'Ivoire? Malar J 17:413. https://doi.org/10.1186/s12936-018-2551-7.
22. Djimdé A, Doumbo OK, Cortese JF, Kayentao K, Doumbo S, Diourté Y, Coulibaly D, Dicko A, Su XZ, Nomura T, Fidock DA, Wellems TE, Plowe CV (2001) A molecular marker for chloroquine-resistant falciparum malaria. N Engl J Med 344:257–263. https://doi.org/10.1056/NEJM200101253440403.
23. Feachem RGA, Chen I, Akbari O, Bertozzi-Villa A, Bhatt S, Binka F, Boni MF, Buckee C, Dieleman J, Dondorp A, Eapen A, Sekhri Feachem N, Filler S, Gething P, Gosling R, Haakenstad A, Harvard K, Hatefi A, Jamison D, Jones KE, Karema C, Kamwi RN, Lal A, Larson E, Lees M, Lobo NF, Micah AE, Moonen B, Newby G, Ning X, Pate M, Quiñones M, Roh M, Rolfe B, Shanks D, Singh B, Staley K, Tulloch J, Wegbreit J, Woo HJ, Mpanju-Shumbusho W (2019) Malaria eradication within a generation: ambitious, achievable, and necessary. Lancet 394:1056–1112. https://doi.org/10.1016/S0140-6736(19)31139-0.
24. Fogh S, Jepsen S, Effersoe P (1979) Chloroquine-resistant *Plasmodium falciparum* malaria in Kenya. Trans R Soc Trop Med Hyg 73:228–229. https://doi.org/10.1016/0035-9203(79)90220-7
25. Frosch AE, Laufer MK, Mathanga DP, Takala-Harrison S, Skarbinski J, Claassen CW, Dzinjalamala FK, Plowe CV (2014) Return of widespread chloroquine-sensitive *Plasmodium falciparum* to Malawi. J Infect Dis 210:1110–1114. https://doi.org/10.1093/infdis/jiu216.
26. Gadalla NB, Elzaki SE, Mukhtar E, Warhurst DC, El-Sayed B, Sutherland CJ (2010) Dynamics of pfcrt alleles CVMNK and CVIET in chloroquine-treated Sudanese patients infected with *Plasmodium falciparum*. Malar J 9:74. https://doi.org/10.1186/1475-2875-9-74.
27. General Authority for Statistics (GASTAT), Kingdom of Saudi Arabia. Census 2010. Available at: https://www.stats.gov.sa/en/13. Accessed 15 July 2021.
28. Ghalib HW, Al-Ghamdi S, Akood M, Haridi AE, Ageel AA, Abdalla RE (2001) Therapeutic efficacy of chloroquine against uncomplicated, *Plasmodium falciparum* malaria in south-western Saudi Arabia. Ann Trop Med Parasitol 95:773–779. https://doi.org/10.1080/00034980120111127.
29. Happi CT, Gbotosho GO, Folarin OA, Bolaji OM, Sowunmi A, Kyle DE, Milhous W, Wirth DF, Oduola AM (2006) Association between mutations in *Plasmodium falciparum* chloroquine resistance transporter and *Plasmodium falciparum* multidrug resistance 1 genes and in vivo amodiaquine resistance in *Plasmodium falciparum* malaria-infected children in Nigeria. Am J Trop Med Hyg 75:155–161.
30. Khan AQ, Pernaute-Lau L, Khattak AA, Luijcx S, Aydin-Schmidt B, Hussain M, Khan TA, Mufti FU, Morris U (2020) Surveillance of genetic markers associated with *Plasmodium falciparum* resistance to artemisinin-based combination therapy in Pakistan, 2018–2019. Malar J 19:206.

https://doi.org/10.1186/s12936-020-03276-8.

31. Khattak AA, Venkatesan M, Jacob CG, Artimovich EM, Nadeem MF, Nighat F, Hombhanje F, Mita T, Malik SA, Plowe CV (2013) A comprehensive survey of polymorphisms conferring anti-malarial resistance in *Plasmodium falciparum* across Pakistan. Malar J 12:300. https://doi.org/10.1186/1475-2875-12-300.
32. Kublin JG, Cortese JF, Njunju EM, Mukadam RA, Wirima JJ, Kazembe PN, Djimdé AA, Kouriba B, Taylor TE, Plowe CV (2003) Reemergence of chloroquine-sensitive *Plasmodium falciparum* malaria after cessation of chloroquine use in Malawi. J Infect Dis 187:1870–1875. https://doi.org/10.1086/375419.
33. Lashin A, Al Arifi N (2012) The geothermal potential of Jizan area, southwestern parts of Saudi Arabia. Int J Phys Sci 7:664–675.
34. Laufer MK, Thesing PC, Eddington ND, Masonga R, Dzinjalamala FK, Takala SL, Taylor TE, Plowe CV (2006) Return of chloroquine antimalarial efficacy in Malawi. N Engl J Med 355:1959–1966. https://doi.org/10.1056/NEJMoa062032.
35. Lwanga SK, Lemeshow S, World Health Organization. Sample size determination in health studies: a practical manual. Geneva: World Health Organization; 1991. https://apps.who.int/iris/handle/10665/40062 Accessed 20 July 2021.
36. Madkhali AM, Al-Mekhlafi HM, Atroosh WM, Ghzwani AH, Zain KA, Abdulhaq AA, Ghailan KY, Anwar AA, Eisa ZM (2020) Increased prevalence of pfdhfr and pfdhps mutations associated with sulfadoxine-pyrimethamine resistance in *Plasmodium falciparum* isolates from Jazan Region, Southwestern Saudi Arabia: important implications for malaria treatment policy. Malar J 19:446. https://doi.org/10.1186/s12936-020-03524-x.
37. Malik GM, Khan AM, Abdalla SEA (1997) No response to chloroquine therapy in a case of a Saudi falciparum malaria. Saud Med J 18:99–100.
38. Malik GM, Seidi O, El-Taher A, Mohammed AS (1998) Clinical aspects of malaria in the Asir region, Saudi Arabia. Ann Saudi Med 18:15–17.
39. May J, Meyer CG (2003) Association of *Plasmodium falciparum* chloroquine resistance transporter variant T76 with age-related plasma chloroquine levels. Am J Trop Med Hyg 68:143–146.
40. Mayengue P, Kalmbach Y, Issifou S, Kremsner P, Ntoumi F (2007) No variation in the prevalence of point mutations in the *pfcrt* and *pfmdr1* genes in isolates from Gabonese patients with uncomplicated or severe *Plasmodium falciparum* malaria. Parasitol Res 100:487–493. https://doi.org/10.1007/s00436-006-0287-8
41. MEWA. Annual Statistical Book 2019. Riyadh: Ministry of Environment, Water, and Agriculture, 2019. Available at: https://mewa.gov.sa/ar/Pages/default.aspx Accessed 30 June 2021.
42. Ministry of Health. National Malaria Drug Policy. Riyadh: Ministry of Health, 2018. Available at: https://www.moh.gov.sa/Ministry/About/Health%20Policies/029.pdf Accessed 10 July 2021.
43. Mwai L, Ochong E, Abdirahman A, Kiara SM, Ward S, Kokwaro G, Sasi P, Marsh K, Borrmann S, Mackinnon M, Nzila A (2009) Chloroquine resistance before and after its withdrawal in Kenya. Malar J 8:106. https://doi.org/10.1186/1475-2875-8-106
44. Payne D (1987) Spread of chloroquine resistance in *Plasmodium falciparum*. Parasitol Today 3:241–246.
45. Roux AT, Maharaj L, Oyegoke O, Akoniyon OP, Adeleke MA, Maharaj R, Okpeku M (2021) Chloroquine and Sulfadoxine-Pyrimethamine Resistance in Sub-Saharan Africa-A Review. Front Genet 12:668574. https://doi.org/10.3389/fgene.2021.668574.
46. Sahar S, Tanveer A, Ali A, Bilal H, Muhammad Saleem R (2015) *Pfcrt* Gene in *Plasmodium falciparum* field isolates from Muzaffargarh, Pakistan. J Arthropod Borne Dis 9:204–214.
47. Shah M, Omosun Y, Lal A, Odero C, Gatei W, Otieno K, Gimnig JE, ter Kuile F, Hawley WA, Nahlen B, Kariuki S, Walker E, Slutsker L, Hamel M, Shi YP (2015) Assessment of molecular markers for anti-malarial drug resistance after the introduction and scale-up of malaria control interventions in western Kenya. Malar J 14:75. https://doi.org/10.1186/s12936-015-0588-4.
48. Snow RW, Amratia P, Zamani G, Mundia CW, Noor AM, Memish ZA, Al Zahrani MH, Al Jasari A, Fikri M, Atta H (2013) The malaria transition on the Arabian Peninsula: progress toward a malaria-free region between 1960–2010. Adv Parasitol 82:205–251. https://doi.org/10.1016/B978-0-12-407706-5.00003-4.
49. Soliman RH, Garcia-Aranda P, Elzagawy SM, Hussein BE, Mayah WW, Martin Ramirez A, Ta-Tang TH, Rubio JM (2018) Imported and autochthonous malaria in West Saudi Arabia: results from a reference hospital. Malar J 17:286. https://doi.org/10.1186/s12936-018-2438-7.
50. Ursing J, Zakeri S, Gil JP, Björkman A (2006) Quinoline resistance associated polymorphisms in the pfcrt, pfmdr1 and pfmrp genes of *Plasmodium falciparum* in Iran. Acta Trop 97:352–356. https://doi.org/10.1016/j.actatropica.2006.01.002.
51. US-Saudi Arabian Business Council. Working in Saudi Arabia: a labor market update. Vienna, 2017. http://www.ussaudi.org/wp-content/uploads/2018/02/Labor-Industry-Brief-2017.pdf. Accessed 13 March 2021.
52. Wélé M, Djimdé AA, Guindo A, Beavogui AH, Traoré IZ, Sadou A, Blaise D, Diallo DA, Wellems TE, Doumbo OK (2011) High frequency of PfCRT 76T in two Malian villages and its prevalence in severe relative to non-severe malaria. Acta Trop 119:11–13. https://doi.org/10.1016/j.actatropica.2011.01.002.
53. Wellems TE, Plowe CV (2001) Chloroquine-resistant malaria. J Infect Dis 184:770–776. https://doi.org/10.1086/322858.
54. WHO. Microscopy for the detection, identification and quantification of malaria parasites on stained thick and thin blood films in research settings. Geneva: World Health Organization, 2015. Available at: https://apps.who.int/iris/handle/10665/163782 Accessed 01 December 2020.

55. WHO. World malaria report 2020. Geneva: World Health Organization; 2020. https://www.who.int/publications/i/item/9789240015791. Accessed 15 July 2021.
56. Wicht KJ, Mok S, Fidock DA (2020) Molecular Mechanisms of Drug Resistance in *Plasmodium falciparum* Malaria. Annu Rev Microbiol 74:431–454. https://doi.org/10.1146/annurev-micro-020518-115546.

Supplementary Material

Table S1 Distribution of *pfcrt* mutant alleles and haplotypes for *P. falciparum* isolates from Jazan region according to governorates involved in the study (n = 235).

Marker	Baysh	Abu Arish	Alharth	Aldair	Samtah	Jazan	Sabya	Aldarb	AlAridah	Eidabi	Dhamad	Ahad Almsariha	*P*
Pfcrt 72**S**	0 (0.0)	2 (9.1)	0 (0.0)	0 (0.0)	0 (0.0)	1 (2.6)	1 (7.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0.295
Pfcrt 74**I**	26 (27.8)	9 (40.9)	16 (59.3)	21 (91.3)	6 (31.6)	18 (47.4)	9 (69.2)	6 (50.0)	6 (66.7)	6 (46.2)	7 (70.0)	2 (50.0)	0.021*
Pfcrt 75**E**	26 (27.8)	9 (40.9)	16 (59.3)	21 (91.3)	6 (31.6)	18 (47.4)	9 (69.2)	6 (50.0)	6 (66.7)	6 (46.2)	7 (70.0)	2 (50.0)	0.021*
Pfcrt 76**T**	35 (77.8)	12 (54.5)	16 (59.3)	22 (95.7)	7 (36.8)	23 (60.5)	10 (76.9)	7 (58.3)	6 (66.7)	9 (69.2)	7 (70.0)	2 (50.0)	0.017*
CVMNK	10 (22.2)	10 (45.5)	11 (40.7)	1 (4.3)	12 (63.2)	15 (39.5)	3 (23.1)	5 (41.7)	3 (33.3)	4 (30.8)	3 (30.0)	2 (50.0)	0.017*
CVMN**T**	9 (20.0)	1 (4.5)	0 (0.0)	1 (4.3)	1 (5.3)	4 (10.5)	0 (0.0)	1 (8.3)	0 (0.0)	3 (23.1)	0 (0.0)	0 (0.0)	0.072
SVMN**T**	0 (0.0)	2 (9.1)	0 (0.0)	0 (0.0)	0 (0.0)	1 (2.6)	1 (7.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0.295
CV**IET**	26 (27.8)	9 (40.9)	16 (59.3)	21 (91.3)	6 (31.6)	18 (47.4)	9 (69.2)	6 (50.0)	6 (66.7)	6 (46.2)	7 (70.0)	2 (50.0)	0.021*

All values are number (%).

Pfcrt 73 was of wild type (V73) and thus, not included in the analysis.

Mutant alleles are bold and underlined.

*Significant association ($P < 0.05$)

Figures

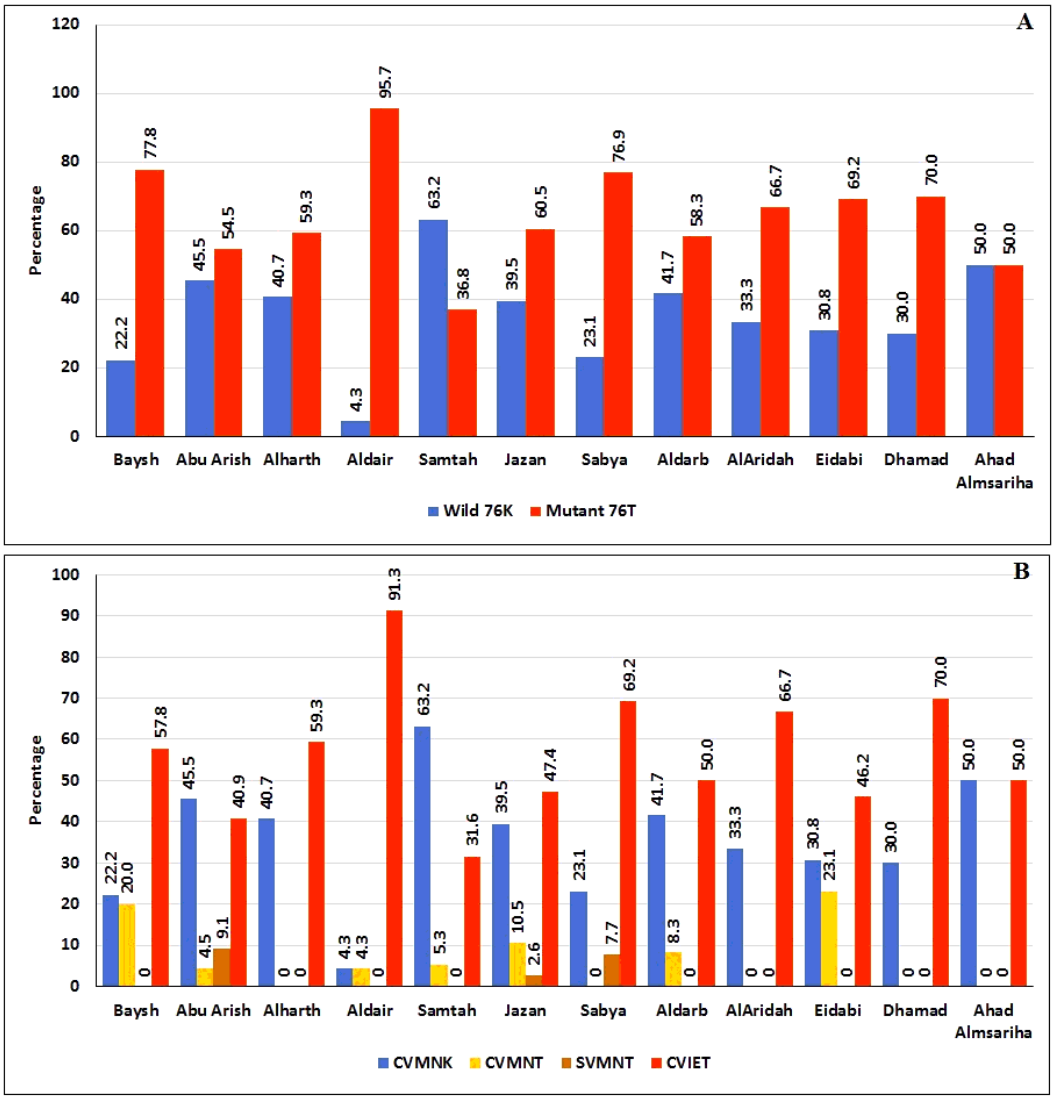

Figure 1

Distribution of pfcrt 76T point mutation and haplotypes for P. falciparum isolates from Jazan region according to governorates involved in the study (n = 235). (A) pfcrt 76T mutation and (B) pfcrt haplotypes