Different patterns of *pfcrt* and *pfmdr1* polymorphism in *Plasmodium falciparum* isolates from Tehama region, Yemen

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ABSTRACT

Introduction. Despite the efforts of the malaria control programme, malaria morbidity is still a common health problem in Yemen, with 60% of the population at risk. *Plasmodium falciparum* is responsible for 99% of malaria cases. The emergence in Yemen of parasite resistance to chloroquine (CQ) prompted the adoption of artemisinin combination therapy (ACT) in 2009, which involves the use of artesunate plus sulphadoxine-pyrimethamine (AS + SP). However, CQ was retained as the drug of choice for vivax malaria. To assess the impact of the change in the malaria treatment policy five years after its introduction, the present study investigated the mutations in the CQ resistance transporter (*pfcrt*) and multidrug resistance 1 (*pfmdr1*) genes.

Method. A molecular investigation of 10 codons of *pfcrt* (72–76, 220, 271, 326, 356, and 371) and five codons of *pfmdr1* (86, 184, 1034, 1042, and 1246) was conducted on *P. falciparum* isolates from districts with the highest malaria endemicity in the Hodeidah and Al-Mahwit governorates in Tehama region, Yemen. A total of 86 positive cases of falciparum monoinfection were investigated for the presence of mutations related to CQ and other antimalarials using a PCR-RFLP assay.

Results. There was a wide prevalence of *pfcrt* gene mutations with the *pfcrt* 76T CQ resistance marker being predominant (97.7%). The prevalence of other *pfcrt* mutations varied from high (75E: 88%) to moderate (74I: 79.1%, 220S: 69.8%, 271E and 371I: 53.5%) or low (326S: 36%, 72S: 10.5%). Mutated *pfcrt* 72–76 amino acids haplotypes were highly prevalent (98.8%). Among these, the CVIET classic, old-world African/Southeast Asian haplotype was the most predominant, and was mostly found in the isolates from the Khamis Bani Saad district of Al-Mahwit (93.1%) and the AdDahi district of Hodeidah (88.9%). However, it was only found in 26.3% of the isolates from the Bajil district of Hodeidah. Surprisingly, the SVMNT new-world South American haplotype was exclusively detected in 9.3% of the isolates from the Bajil district of

Hodeidah. Mutations at Y184F of *pfmdr1* were found in all isolates (100%) from all districts. The mutation for codons 1034C and 86Y were found only in the isolates from the AdDahi and Khamis Bani Saad districts. Overall, the AdDahi and Khamis Bani Saad districts were similar in terms of carrying most of the mutations in the *pfcrt* and *pfmdr1* genes, while there was a lower prevalence of mutation in the isolates from the Bajil district.

Conclusion. The high prevalence of mutations in *pfcrt* 5 years after the official cessation of CQ use against *P. falciparum* suggests that there is sustained CQ pressure on *P. falciparum* isolates in the study area. Moreover, the low prevalence of mutations in the *pfmdr1* gene could be a good indicator of the high susceptibility of *P. falciparum* isolates to antimalarials other than CQ. A new strategy to ensure the complete nationwide withdrawal of CQ from the private drug market is recommended.

INTRODUCTION

Chloroquine (CQ), a safe and cheap antimalarial, has been the drug of choice for treating uncomplicated falciparum malaria over the past few decades. However, its effectiveness has been greatly hampered by the emergence of resistant *Plasmodium falciparum* strains that emerged along the Thai–Cambodian border early in 1957 and then spread to other foci in Asia, and also to South America, Papua New Guinea, and Africa (*Moore & Lanier*, *1961*; *Harinasuta, Suntharasamai & Viravan*, *1965*; *Grimmond, Donovan & Riley*, *1976*; *Campbell et al.*, *1979*; *Fogh, Jepsen & Effersoe*, *1979*). The emergence and spread of *P. falciparum* strains that are resistant to CQ and other antimalarials has necessitated a switch to new drugs to ensure maximum effectiveness and prevent the development of ongoing parasite resistance. It has been suggested that a combination therapy rather than monotherapies is highly efficient for falciparum malaria case management and malaria control.

Currently, artemisinin combination therapy (ACT) is the cornerstone of malaria treatment policies and control worldwide (*Nosten & White*, *2007*; *White*, *2008*; *World Health Organization*, *2015*). Interestingly, the replacement of CQ with an ACT in areas where falciparum isolates were CQ-resistant resulted in the re-emergence of CQ-sensitive strains a few years after the cessation of CQ use (*Liu et al.*, *1995*; *Kublin et al.*, *2002*; *Kublin et al.*, *2003*; *Mita et al.*, *2004*).

Chloroquine resistance is determined by the major point mutation at codon 76 of the *P. falciparum* CQ resistance transporter (*pfcrt*) gene, through the substitution of lysine amino acid (K) by threonine (T) (*Fidock et al.*, *2000*), which is highly correlated with increased clinical CQ tolerance and treatment failure (*Djimde et al.*, *2001*; *Wellems & Plowe*, *2001*; *Wongsrichanalai et al.*, *2002*; *White*, *2004*). However, the accumulation of mutations along the *pfcrt* gene, especially at codons A220S, Q271E, N326S, I356T, and R371, in addition to the leader *pfcrt* K76T point mutation was found significantly associated with CQ resistance (*Djimde et al.*, *2001*). Also, point mutations in the *P. falciparum* multidrug

resistance 1 (*pfmdr1*) gene have been reported to modulate sensitivity and resistance to multiple antimalarials (*Basco et al.*, *1995*; *Reed et al.*, *2000*; *Sisowath et al.*, *2005*).

In Yemen, malaria is still a major public health problem that threatens more than 60% of the population, with *P. falciparum* causing 99% of the malaria cases (*World Health Organization*, *2015*). The emergence in Yemen of parasite resistance to CQ as well as to other antimalarials prompted the adoption of ACT in 2009, which includes artesunate plus sulphadoxine-pyrimethamine (AS + SP) and artemether plus lumefantrine (AL) as the first line and second line treatment, respectively, for uncomplicated falciparum malaria infections. However, CQ was retained as the drug of choice for vivax malaria.

Some *in vivo* and *in vitro* studies have been conducted in different malaria-endemic districts of Yemen in order to evaluate the efficacy of CQ against *P. falciparum* isolates. These studies found that CQ resistance varied from 16.1% in the Taiz governorate (*Alkadi, Al-Maktari & Nooman*, *2006*) to more than 40% in the Lahj (*Mubjer et al.*, *2011*) and Hodeidah governorates (*Al-Maktari et al.*, *2003*; *Al-Shamahy et al.*, *2007*). However, despite these reports and the introduction of a new malaria treatment policy, CQ continues to be available in drug markets and private drug stores in Yemen, is still prescribed for falciparum malaria in some private health facilities, and is also used by individuals for self-administration, especially in remote malarious areas (*Bashrahil, Bingouth & Baruzaig*, *2010*; *Ghouth*, *2013*).

Some molecular studies have reported a high prevalence of *pfcrt* 76T mutations (ranging from 88% to 100%) in different districts of Yemen (*Al-Mekhlafi et al.*, *2011*; *Mubjer et al.*, *2011*; *Abdul-Ghani, Farag & Allam*, *2013*; *Al-Hamidhi et al.*, *2013*). Nevertheless, there is still a paucity of information about the full genetic profile of *pfcrt* and *pfmdr1* in the country. Yet, such genetic markers are useful tools for monitoring parasite susceptibility to various antimalarials. In addition, they help researchers to monitor changes in parasite genotypes after a change in treatment policy. Therefore, the present study aimed to investigate the susceptibility of *P. falciparum* to CQ (and some other antimalarials) five years after the adoption of an ACT by conducting a molecular analysis of the related mutations in the *pfcrt* and *pfmdr1* genes in isolates from the Tehama region in northwestern Yemen. Furthermore, because there is a diverse market for antimalarials in this region, improving our knowledge about drug pressure would be useful in targeting interventions aimed at reducing inappropriate drug use in the region.

MATERIALS AND METHODS

Study area and subjects

An active case survey that targeted individuals with fever (as a group suspected to have malaria) was conducted from March to May 2014 in two malaria-endemic districts of the Hodeidah governorate and one in the Al-Mahwit governorate in the Tehama region in northwestern Yemen (Fig. 1). The Hodeidah governorate (14.79°N, 42.97°E) is located about 226 km from Sana'a, the capital of Yemen. Hodeidah is a coastal area along the Red Sea with a total area of 117,145 km^2 and a total population of 2.16 million (*National Information Centre, Yemen*, *2014*). The Al-Mahwit governorate (16.25°N, 44.717°E) is
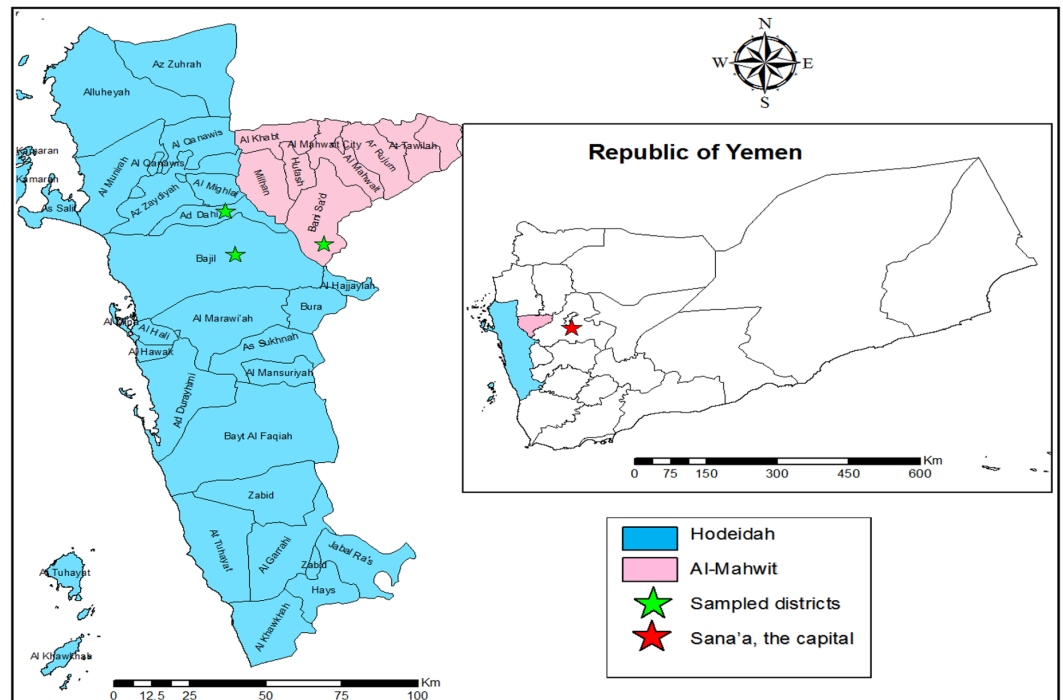
Figure 1 A geographic map showing study area (Hodeidah and Al-Mahwit governorates).

located between Hodeidah and Sana'a (about 111 km west of Sana'a), covers a total area of 2,858 km^2, and has a total population of 597,000 (*National Information Centre, Yemen, 2014*). The climate in the Tehama region is a combination of tropical monsoons with occasional rain in summer and dry weather in winter with a mean rainfall of 200 mm/year. The mean temperature is 37.5 °C in summer and 24 °C in winter with humidity ranging between 70% and 90%. Malaria transmission is perennial, but there is a high malaria transmission peak from January to March. Transmission is known to be more prevalent in Hodeidah.

Villages in the districts with the highest malaria endemicity in the region were chosen based on the national malaria records for the period 2010–2013 that were provided by the National Malaria Control Programme (NMCP), Yemen. Based on these records, samples were collected from 10 villages in the AdDahi and Bajil districts in Hodeidah and the Khamis Bani Saad district in Al-Mahwit. The villages in question were Halalah, Al-Huamarah, Al-Meshaahra, Al-Rakib, Al-Rufae, Al-Sharjah, Deer Shareef, Kidf Zumailah, Shat Hajal, and Siraj. Samples were also collected from the city of Bajil.

The present study employed an *in vivo* efficacy trial that was designed to investigate the effectiveness of the AS + SP therapy in treating uncomplicated malaria and that used the World Health Organization (WHO) 28-day follow-up protocol. Initially, 622 individuals with fever in the 11 mentioned sites were examined. From this number, 86 individuals with falciparum malaria monoinfection, who voluntarily agreed to take part in the *in vivo* study, were recruited based on the inclusion criteria in the WHO guidelines (*WHO, 2009*). All 86

were considered for the investigation of different molecular markers related to resistance to antimalarial drugs, including the *pfcrt* and *pfmdr1* genes.

Plasmodium falciparum isolates

A finger-prick blood sample was collected from each participant for use in the RDT test (*CareStart*[TM] Malaria HRP2-RDT) and for preparing thick and thin blood film and a filter paper blood spot. Blood films were stained with 5% of buffer-diluted Giemsa stain for 30 min and examined microscopically for malaria parasites. Filter papers were left to dry in air away from dust and then kept in an aluminium pouch together with desiccated silicon bags until used.

Parasite species and stages were recorded and parasitaemia (parasite density) was determined by counting only the asexual stages against 300 white blood cells (WBCs) and then multiplying by 25, assuming the average of the total WBC count of the individuals was equal to 7,500 cells per µl of blood (*Billo et al.*, *2013*). The level of parasitaemia was graded as low (<1,000 parasites/µl of blood), moderate (1,000–9,999 parasites/µl of blood), or severe (≥10,000 parasites/µl of blood).

DNA extraction

Two to three discs (6 mm diameter) of 3MM Whatman's filter paper blood spots (cut by a flamed-sterile puncher) were used for DNA extraction, which was performed using a Qiagen blood and tissue kit (Cat. no. 69506; QIAGEN, DNeasy® Blood & Tissue Kit, Germany) according to the manufacturer's instructions. The DNA was eluted using 100 µl of AE (10 mM Tris-Cl; 0.5 mM EDTA; pH 9.0) elution buffer (included in the kit) and kept at –20 °C until used.

Detection of gene mutations in *pfcrt* and *pfmdr1*

All extracted DNA samples of *P. falciparum* were subjected to mutation analysis using polymerase chain reaction (PCR) amplification followed by restricted fragment length polymorphism (RFLP) to investigate the mutations in CQ resistance transporter (*pfcrt*) and multidrug resistance1 (*pfmdr1*) genes.

Amplification of *pfcrt* was performed using the protocol designed by *Djimde et al.* (*2001*) with the aim of detecting mutations at codons 72–76, 220, 271, 326, 356, and 371 of the *pfcrt* gene and at codons 86 and 1246 of the *pfmdr1* gene. The other codon mutations of *pfmdr1* (184, 1034, and 1042) were analysed using the PCR-RFLP protocol in *Duraisingh et al.* (*2000*). However, the PCR amplification and restriction enzyme (RE) digestion in the protocol was modified for codon 184 by designing a forward primer MDR184-F (5′-ATAATAATCCTGGATCTAAATTAAGA-3′) to replace A4 to amplify an amplicon of 155 bp instead of 560 bp and by using Swa1 endonuclease which cuts once into 123 bp and 32 bp for the mutated allele but not the wild.

Restriction enzyme digestion was done in 20 µl of reaction mixture that consisted of 6–8 µl of PCR product, 1X of the specific buffer (included in the RE kit), and 1 unit of the specific RE (New England Biolabs Inc., Hitchin, UK), then was incubated for 15–60 min (based on the RE) according to the manufacturer's instructions, and then visualised in 2.5%–4.0% of TAE buffered agarose gel (based on the size of the cleaves) stained with

Sybr® safe DNA gel stain (Invitrogen, USA) using a Bio-Rad Molecular Imager Gel Doc XR System (Bio-Rad, Hercules, CA, USA).

Genomic DNA of *P. falciparum* strains 3D7 (MRA-102G) and HB3 (MRA-155G) were used as positive controls for the wild types of *pfcrt* and *pfmdr1*, while the Dd2 strain (MRA-150G) was used as positive control for the mutated types. All the strains were provided by the Malaria Research and Reference Reagents Resources Center (MR4, ATCCW, Manassas VA, USA).

The study protocol was approved by the Medical Ethics Committee of the University of Malaya Medical Centre, Kuala Lumpur (Ref. 974.19), and by the Ministry of Health and Population, in conjunction with the National Malaria Control Programme in Yemen.

RESULTS

A total of 86 malaria-positive individuals participated in the present study; 40 (46.5%) were male and 46 (53.5%) were female. The ages of participants ranged from 8 months to 65 years, with a mean age of 12.4 years. The age group of 5–15 years represented 58.1% of the study participants. Asexual parasite density (parasitaemia) varied from 561 to 55,555 parasites/μl, with a mean parasitaemia of 8,199 parasites/μl.

The *pfcrt* gene mutations were screened as molecular markers of CQ, and possibly of other antimalarials such as amodiaquine and lumefantrine. All isolates were successfully amplified (100%). Mutation at *pfcrt* 76T was found at a virtual fixation level in almost all districts (97.7%), followed by 88.4% for position 75E. The prevalence of mutations at other codons of *pfcrt* was found to vary from moderate as in codons 74I (79.1%), 220S (69.8%), and 271E and 371I (53.5%) to low as in 326S (36%) and 72S (10.5%). None of the isolates considered in the present study carried the mutated type at codon 356 of *pfcrt* (see Table 1).

Eight haplotypes of *pfcrt* 72–76 amino acids were found to be present in the study area. The CVIET classical, old-world African/Southeast Asian haplotype was the most prevalent among all *pfcrt* haplotypes (77.9%), followed by SVMNT (9.3%) and CVMET (7%). Only one isolate (1.2%) was found to carry each of the other mutated haplotypes, namely CVMEK, CVINT, SVMET and CVMNT, as well as the CVMNK wild haplotype.

Among all 10 types of codon mutation in *pfcrt*, sextuple mutations (i.e., mutations at six codons) had the highest presence among the isolates (33.7%), followed by quadruple mutations (four codons) with 23.3%, quintuple (five codons) with 14.4%, septuple (seven codons) with 12.2%, and triple (three codons) with 8.1%. The frequency of double and single mutations was very low; 3.5% and 1.2%, respectively.

As regards the results of the mutation analysis of the *pfmdr1* gene, *P. falciparum* field isolates from the Tehama region showed at least a single point mutation in the *pfmdr1* gene (see Table 2). Mutation at position 184 of *pfmdr1* was found at the fixation level (100%), together with a low prevalence of mutation for codons 1034 and 86 of *pfmdr1* of 20.9% and 16.3%, respectively. No mutation was detected for codons 1042 and 1246.

The NFSND single-mutated haplotype of phenyl alanine amino acid at position 184 was predominant (64%), followed by NFCND (19.8%), and YFSND (15.1%), with an

Table 1 Frequency distribution of *pfcrt* mutations and haplotypes for *P. falciparum* isolates from different districts of Tehama, Yemen.

Marker		AdDahi		Bajil		Khamis Bani Saad		Total	
		n	%	*n*	%	*n*	%	*n*	%
Crt	wild	9	100	10	52.6	58	100	77	89.5
72	mutated	0	0	9	47.4	0	0	9	10.5
Crt	wild	1	11.1	14	73.7	3	5.2	18	20.9
74	mutated	8	88.9	5	26.3	55	94.8	68	79.1
Crt	wild	0	0	8	42.1	2	3.4	10	11.6
75	mutated	9	100	11	57.9	56	96.6	76	88.4
Crt	wild	0	0	0	0	2	3.4	2	2.3
76	mutated	9	100	19	100	56	96.6	84	97.7
Crt	wild	4	44.4	4	21.1	18	31	26	30.2
220	mutated	5	55.6	15	78.9	40	69	60	69.8
Crt	wild	0	0	18	94.7	8	13.8	26	30.2
271	mutated	9	100	1	5.3	50	86.2	60	69.8
Crt	wild	5	55.6	2	10.5	48	82.8	55	64
326	mutated	4	44.4	17	89.5	10	17.2	31	36
Crt	wild	3	33.3	18	94.7	19	32.8	40	46.5
371	mutated	6	66.7	1	5.3	39	67.2	46	53.5
	CV**IET**	8	88.9	5	26.3	54	93.1	67	77.9
	SVMN**T**	0	0	8	42.1	0	0	8	9.3
	CVM**ET**	1	11.1	5	26.3	0	0	6	7
	CVIN**T**	0	0	0	0	1	1.2	1	1.2
Haplotypes	CVME**K**	0	0	0	0	1	1.2	1	1.2
	CVMN**K**	0	0	0	0	1	1.2	1	1.2
	CVMN**T**	0	0	0	0	1	1.2	1	1.2
	SVM**ET**	0	0	1	1.2	0	0	1	1.2

Table 2 Frequency distribution of *pfmdr1* mutations and haplotypes for *P. falciparum* isolates from different districts of Tehama, Yemen.

Marker		AdDahi		Bajil		Khamis Bani Saad		Total	
		n	%	*n*	%	*n*	%	*n*	%
Mdr1	wild	5	55.6	19	100	48	82.8	72	83.7
86	mutated	4	44.4	0	0	10	17.2	14	16.3
Mdr1	wild	8	88.9	19	100	41	70.7	68	79.1
1034	mutated	1	11.1	0	0	17	29.3	18	20.9
	N**F**SND	4	44.4	19	100	32	55.2	55	64
Haplotypes	N**FC**ND	1	11.1	0	0	16	27.6	17	19.8
	YFSND	4	44.4	0	0	9	15.5	13	15.1
	YF**C**ND	0	0	0	0	1	1.7	1	1.2

overall presence of 34.9% for all double-mutated haplotypes. The YFCND triple-mutated haplotype was found in only one isolate (1.2%) carrying the mutated amino acids tyrosine (codon 86), phenyl alanine (184), and cysteine (1034).

Interestingly, falciparum malaria in the study area, which consisted of districts known to have the highest malaria endemicity in the country, showed unexpected variation in *pfcrt* and *pfmdr1* gene mutations. Isolates from the AdDahi district in Hodeidah and the Khamis Bani Saad district in Al-Mahwit were almost similar in terms of carrying the majority of mutated alleles for most of the codons, and consequently carrying the mutated haplotypes. In contrast, the malaria isolates from the Bajil district in Hodeidah were found to be mostly of the wild type and consequently carried less mutated haplotypes.

Isolates from the study area showed a high prevalence of CQ resistant haplotypes, with a predominance of CVIET classical old-world African/Southeast Asian haplotypes in the Khamis Bani Saad district of Al-Mahwit (93.1%) and the AdDahi district in Hodeidah (88.9%) compared to only 26.3% for isolates from the Bajil district of Hodeidah. In the same context, mutation at the *pfcrt* 72 codon was only reported in isolates from the Bajil district (47.4%), whereas it was totally absent from the other two districts. Accordingly, the SVMNT haplotype was found exclusively in 42.1% of isolates from Bajil. (Refer to Table 1.)

Similarly, mutations at *pfmdr1* showed a geographic variation in terms of *pfmdr1* allele and haplotype frequencies. That is to say, in addition to the fixation level of mutation at *pfmdr1* 184, mutations at 86 and 1034 of *pfmdr1* were found only in the AdDahi and Khamis Bani Saad districts, whereas they were not present in isolates from the Bajil district. The triple-mutated haplotype of 86, 1084 and 1034 (YFCND) was found in one isolate from Khamis Bani Saad. (See Table 2.)

DISCUSSION

The present study provides information on the *pfcrt* and *pfmdr1* genetic profile of *P. falciparum* isolates from the districts with the highest malaria endemicity in the Tehama region of Yemen. The isolates were found to mostly carry mutated alleles, especially for *pfcrt* codons 74I, 75E, 76T, 220S, 271E, and 371I, while no mutations were detected at codon 356 of *pfcrt*. This is consistent with a previous study conducted in Yemen that reported a high prevalence of mutated alleles for codons 74I and 75E (89%) and 76T (93%) (*Al-Hamidhi et al., 2013*). The present study found that the overall prevalence of *pfcrt* 76T was 74%–100%, which is similar to the figure previously reported for isolates from other parts of Yemen (*Al-Mekhlafi et al., 2011*; *Mubjer et al., 2011*; *Bamaga, Mahdy & Lim, 2015*).

The high level of mutation at position 76 of *pfcrt* (96.6%–100%) appears to indicate that, 5 years on from changing the malaria treatment policy to one that is ACT based and the official cessation of CQ use for *P. falciparum*, the re-emergence of a CQ-sensitive strain of *P. falciparum* has not yet occurred in the study area. One possible explanation for the virtual fixation of the *pfcrt* 76 mutation in the study area could be the continued availability of CQ; it is still being prescribed to falciparum malaria patients in Yemen particularly in private health facilities and is also being used as a self-administered treatment (*Bashrahil, Bingouth & Baruzaig, 2010*; *Ghouth, 2013*; *Bamaga et al., 2014*). Moreover, CQ has not yet

been totally withdrawn from governmental drug stores as it is still the drug of choice for treating vivax malaria infections.

The findings of the present study support those of previous research conducted elsewhere in Yemen that have reported 79%–100% mutated alleles for *pfcrt* 76T (*Al-Mekhlafi et al.*, *2011*; *Mubjer et al.*, *2011*; *Abdul-Ghani, Farag & Allam*, *2013*; *Al-Hamidhi et al.*, *2013*). The finding in previous studies such as these that there was a decrease in the prevalence of the *pfcrt* 76T mutated allele and a re-emergence of CQ-sensitive strains some years after abandoning CQ was controversial, particularly as many studies in Africa documented an increase in the re-emergence of the wild type of *pfcrt* 76 after complete CQ withdrawal (*Laufer et al.*, *2006*; *Mohammed et al.*, *2013*; *Mbogo et al.*, *2014*; *Mekonnen et al.*, *2014*). In contrast, South American isolates of *P. falciparum* have been reported to retain their mutated types despite the cessation of CQ use (*Vieira et al.*, *2004*; *Griffing et al.*, *2010*; *Adhin, Labadie-Bracho & Bretas*, *2013*). Similarly, studies conducted in Ethiopia have found a fixation of *pfcrt* 76T mutations after cessation of CQ use for more than a decade (*Mula et al.*, *2011*; *Golassa et al.*, *2014*).

The results of the present study also revealed that the CVIET classical old-world, African/Southeast Asian mutated haplotype was predominant (77.9%) among all *pfcrt* haplotypes in the study area and that seven other haplotypes were also present, including 9.3% (eight cases) of the SVMNT new-world South American mutated haplotype (only in the Bajil district) and 7.0% (six cases) of CVMET. This finding is in agreement with the only two other available studies based in Yemen that attempted to examine the mutations along the *pfcrt* gene (*Al-Hamidhi et al.*, *2013*; *Mubjer et al.*, *2011*). For instance, CVIET was reported for the majority of the isolates from different parts of Yemen (Dhamar 100%, Taiz 88%, and Hodeidah 70.6%), with a presence of only 4% of the SVMNT haplotype in isolates from the Hodeidah governorate (*Al-Hamidhi et al.*, *2013*). Similarly, CVIET was the only haplotype reported for isolates from the Al-Musaimeer malaria-endemic district in Lahj Province (*Mubjer et al.*, *2011*).

Interestingly, the present study found the SVMNT haplotype exclusively in 42.1% of isolates from the Bajil district in Hodeidah. This finding might be due to a 'parasite response' to a particular drug pressure other than CQ. However, data on the use of a unique antimalarial in this particular area were not available, so it is not possible to relate the occurrence of the SVMNT haplotype to the increased use of any of the antimalarials. A previous study conducted in Tanzania reported the unusual existence of the SVMNT haplotype in Africa and attributed the finding, without supporting evidence, to the increased drug pressure of amodiaquine (AQ) use in the area under study (*Alifrangis et al.*, *2006*).

Moreover, malaria transmission in the Bajil district, an area with the SVMNT haplotype, was found to be lower than that in the AdDahi and Khamis Bani Saad districts. This is in line with the study on Tanzania, which reported a higher existence of the SVMNT mutated haplotype in areas with low malaria transmission than in areas with high transmission (*Alifrangis et al.*, *2006*). As mentioned above, it has been suggested that the SVMNT haplotype occurs as a response to the intense pressure of AQ, and *P. falciparum* isolates with SVMNT are supposedly less susceptible to AQ monotherapy and combination therapy. Interestingly, the NMCP in Yemen conducted six *in vivo* clinical trials for CQ and AQ drug

efficacy from 2002 to 2004; four studies looked at CQ efficacy and found that there was 30%–57% treatment failure, while two studies monitored the efficacy of AQ monotherapy and artesunate-amodiaquine (AST-AQ) combination therapy and found 44.3% and 18.5% treatment failure rate, respectively (National Malaria Control Programme (NMCP), 2013, unpublished data).

The existence of SVMNT in Bajil rather than in the other two districts raises an interesting question about the source of the evolution of this CQ resistant haplotype in the country. Typically, SVMNT is associated with new-world South American or Papua New Guinea isolates, and importation of this haplotype from South America or Papua New Guinea, or even from the Philippines or India in Asia, was logically unaccepted based on the population characteristics of the study area. Hence, the apparent independent evolution of SVMNT in Bajil isolates will need further study to confirm the finding presented herein. However, the results of a study conducted in Papua New Guinea suggest that there is a recombination of de novo point mutation that transforms the old-world classical African CVIET haplotype into a new-world South American SVMNT one (*Mehlotra et al.*, *2001*).

As regards the *pfmdr1* gene, the present study found that mutations varied among the isolates from the three districts, from fixation (100%) for 184F to 20.9% for 1034C and 16.3% for 86Y, while no mutation was detected for the 1042 and 1246 codons. These results are consistent with those of a previous study in Hodeidah that reported a high prevalence (99%) of 184F and a relatively low existence (20%) of the 86Y mutated allele (*Al-Hamidhi et al.*, *2013*). Likewise, a recent study conducted in Hadramout in the southeastern part of Yemen, which has low malaria transmission, reported a low frequency (16.7%) of *pfmdr1* 86 mutated allele (*Bamaga, Mahdy & Lim*, *2015*). However, a high prevalence (70%) for mutations of 1034C and 1042D has been reported for the Taiz, Dhamar, and Hodeidah governorates (*Al-Hamidhi et al.*, *2013*).

It has been found that polymorphism in the *pfmdr1* gene is related to an increase in parasite tolerance/resistance to some antimalarials (*Gamo*, *2014*). Nevertheless, the findings regarding the association of mutations in the *pfmdr1* codons with antimalarial drug resistance are mixed. For instance, 86Y has been linked to CQ resistance, while mutations at positions 184, 1034, 1042, and 1246 were found to be mostly related to resistance to AQ, mefloquine, halofantrine, and quinine (*Reed et al.*, *2000*; *Danquah et al.*, *2010*; *Gamo*, *2014*). Moreover, the occurrence of *pfmdr1* N86 and 184F has been found to increase with the use of lumefantrine (*Sisowath et al.*, *2007*; *Nwakanma et al.*, *2008*; *Malmberg et al.*, *2013*). In the same vein, another study reported an increase of *pfmdr1* N86 and 184F, together with the wild alleles of D1246, and concluded that this was linked to the extensive and prolonged use of AQ in combination with artesunate (*Fröberg et al.*, *2012*). In other studies, *P. falciparum* isolates carrying *pfmdr1* haplotypes of mutated 86Y and Y184 wild alleles were found to be associated with the usage of CQ or AQ, which continued to change over time with changes in the antimalarials used. For instance, mutated 86Y and Y184 wild alleles changed to a haplotype carrying wild N86 and 184F mutated alleles years after the implementation of ACT, and the selection of parasites to one of artemisinin's derivatives (*Humphreys et al.*, *2007*; *Dlamini, Beshir & Sutherl*, *2010*; *Mungthin et al.*, *2010*; *Thomsen et al.*, *2011*). Overall, the findings of the present study provide a broad view on

the polymorphism of *pfcrt* and *pfmdr1* genes in areas with the highest level of malarial transmission in Yemen, 5 years after official cessation of CQ.

CONCLUSION

The present study aimed to provide a genetic profile of *pfcrt* and *pfmdr1* as molecular markers of the *P. falciparum* parasite's resistance to antimalarials, especially CQ. The high prevalence of *pfcrt* mutations and mutated haplotypes suggests a high CQ resistance in *P. falciparum* and a continuation of CQ pressure in the study area. It is therefore recommended that the availability of CQ in private hospitals, clinics, and drugstores should be controlled to ensure its complete withdrawal. Moreover, treatment of vivax malaria using CQ should only be available in governmental health facilities to prevent the random, uncontrolled use of CQ.

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Competing Interests
The authors declare there are no competing interests.

Author Contributions
- Wahib M. Atroosh conceived and designed the experiments, performed the experiments, analyzed the data, wrote the paper, prepared figures and/or tables.

- Hesham M. Al-Mekhlafi conceived and designed the experiments, analyzed the data, wrote the paper, prepared figures and/or tables.
- Adel Al-Jasari provided logistic support for data collection and supervises fieldwork.
- Hany Sady, Salwa S. Dawaki, Fatin N. Elyana, Mona A. Al-Areeqi, Nabil A. Nasr, Awatif M. Abdulsalam and Lahvanya R. Subramaniam performed the experiments.
- Meram Azzani analyzed the data.
- Init Ithoi and Yee Ling Lau reviewed drafts of the paper.
- Johari Surin conceived and designed the experiments.

Human Ethics

The following information was supplied relating to ethical approvals (i.e., approving body and any reference numbers):

The study protocol was approved by the Medical Ethics Committee of the University of Malaya Medical Centre, Kuala Lumpur (Ref. 974.19), and by the Ministry of Health and Population, in conjunction with the National Malaria Control Programme in Yemen.

Data Availability

All data related to this work are included in the manuscript.

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