Influx of diverse, drug resistant and transmissible *Plasmodium falciparum* into a malaria-free setting in Gulf Cooperation (GCC) countries.

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Abstract

Background Successful malaria control programs have interrupted local malaria transmission in almost all the Gulf Cooperation Council (GCC) countries. However, a massive influx of imported malaria via migrant workers from endemic areas sustains a threat for the re-introduction of local transmission. Here we examined the origin of imported malaria into one of the GCC countries (Qatar) and assessed the extent of genetic diversity, and carriage of drug resistance genes of imported Plasmodium falciparum and it’s potential to re-introduce the disease.

Methods We examined imported malaria reported in Qatar, between 2013 and 2016. We focused on P. falciparum infections and estimated total parasite and gametocyte density using qPCR and qRT-PCR, respectively. In addition, we examined ten neutral microsatellites and four drug resistance genes, Pfmrp1, Pfcrt, Pfmdr1 and Pfkelch13 , to assess the extent of diversity of imported P. falciparum and its potential carriage of drug resistance genotypes respectively.

Results The majority of imported malaria comprised P. vivax , while P. falciparum and mixed species infections (P. falciparum /P. vivax) were less frequent. The main origin of P. vivax was the Indian subcontinent, while P. falciparum was most apparent among expatriates from Africa. Imported P. falciparum was highly diverse carrying multiple genotypes as well as early and late gametocytes. We observed a high prevalence of SNPs implicated in drug resistance among imported P. falciparum , with some novel SNPs in Pfkelch13 .

Conclusions The high influx of genetically diverse P. falciparum, with multiple drug resistance marker gene mutations and high capacity of producing gametocytes, sustains threat for re-introduction of drug resistant malaria into GCC countries. This scenario highlights the impact of current globalisation of movement of humans in reintroducing malaria infections to areas targeted for elimination.

Background

The Gulf Cooperation Council (GCC) countries have achieved great success in malaria control. The increased investment in control efforts beginning in the 1950s is responsible for the interruption of local transmission, and led to malaria-free status in all of the GCC countries, with the exceptions of Saudi Arabia, where limited foci of indigenous malaria exist [1, 2] and Oman, where sporadic

outbreaks still occur [3]. Such success has encouraged health ministries in GCC countries to shift policy towards a malaria-free Arabian Peninsula [4] and a focus on preventing re-introduction via sustainable vector control policy, improved surveillance, and prompt case management [5].

Qatar in particular has been free from local malaria transmission since the 1970s [6], with no reports of indigenous malaria occurring since [7]. Despite this, the influx of migrant workers from malaria-endemic countries in the Indian subcontinent and Africa has sustained a high number of imported cases, representing a major threat for the re-introduction of local transmission. Émigrés constitute the majority of residents in GCC countries, reaching > 80% in Qatar [8]. In the last two decades there has been an increase in the flow of migrant workers to Qatar, associated with a positive trend in reported cases of imported malaria [7, 9, 10]. In addition to the increase in imported cases, the receptivity and risk of malaria reintroduction is evident by the presence of the mosquito vectors, *Anopheles stephensi* and *An. multicolor* [11]. Similar risk factors for reintroduction of local malaria prevail in other GCC countries, with relatively high percentages of expatriates from malarious areas, hindering current efforts to curb local transmission. Some of the immigrant freelance construction workers from the Indian subcontinent are subjected to poor environmental conditions, work for several months at building construction sites and in agriculture, live in close proximity to water tanks, and are thus subject to mosquito bites [3] . For example, repeated outbreaks of locally acquired cases, linked to imported malaria, were seen in Oman [3]. The most recent Ministry of Health's annual report (2018), shows a persistent and constant number of imported malaria cases, which presumably, exist as asymptomatic infection prior to the onset of the disease signs. Consequently, there has been regular local malaria outbreaks, presumably seeded from imported cases that are not identified and treated in time to prevent transmission to the indigenous *Anopheles* population [3].

The chronic nature of asymptomatic malaria infection, most common in adults, in conjunction with population mobility, creates a threat to effective, long-term malaria elimination [12]. Imported asymptomatic infection often carries drug resistant strains of the *Plasmodium* parasite, and the transmissible stages [9, 13], posing an imminent threat to receptive areas where transmission has been interrupted or targeted for elimination [14]. An improved understanding of transmission rates,

Plasmodium species involved, extent of genetic diversity, and the possession of drug resistant strains of imported malaria, would permit the deployment of effective cross-border measures to limit impacts on regions targeted for elimination.

The present study examines the source of imported malaria to the transmission-free country of Qatar, and assesses the genetic diversity, prevalence of drug resistance mutations, and ability of *P. falciparum* to produce gametocytes and thus be transmitted to mosquito vectors. Such knowledge would allow control programs to develop targeted policies to reduce circulating parasites, define the source of outbreaks and limit the risk of reintroduction of malaria.

Methods

Subjects

A total of 583 patients, reporting to Hamad General Hospital and Al-Khor Hospital, Doha, two main centers of Hamad Medical Corporation (HMC) for malaria treatment, were tested for malaria between January 2013 and October, 2016. All cases were diagnosed using microscopic examination of Giemsa-stained thick (100 fields) and thin blood (1000 RBCs) films. A total of 448 (76.8%) of patients tested positive for malaria parasites, and based on disease history, the origin of infection in all patients was traced to an endemic region outside of Qatar [15]. Genomic DNA from capillary blood was isolated and purified using a QIAamp DNA blood mini kit as per the manufacturer's instructions (Qiagen, CA, USA). Species identification was confirmed using species-specific PCR as described by Snounou [16]. Quantification of *P. falciparum* was carried out using qPCR of *18s rRNA* [17]. Demographic information on the patient was collected using a structured questionnaire, including age, sex, nationality, travel history, and any previous malaria treatment. Patients were provided treatment as per the current guidelines for malaria treatment at HMC [11].

Microsatellite genotyping and multiplicity of infection (MOI)

A panel of ten unlinked polymorphic microsatellites of *P. falciparum* were genotyped as described by Anderson *et al*. and Anderson *et al*. [18, 19]. Labelled PCR products were mixed with Gene-Scan™ 500 ROX internal size standard (Applied Biosystems, UK) for capillary electrophoresis on an ABI 3130XL Genetic Analyzer (Applied Biosystems, UK). GeneMapper software version 4 (Applied Biosystems, UK)

was used for scoring allele sizes and quantifying electropherogram peak heights for samples containing multiple alleles per locus. Multiple alleles per locus were scored if electrophoretic peaks corresponding to minor alleles were >32% of the height of the predominant allele [19].

Detection and quantification of early and late *P. falciparum* gametocytes

Quantitative RT-PCR (qRT-PCR) was used to detect and quantify mRNA from the early gametocyte-specific gene, *Pfpeg4* [20], and the late gametocyte-specific gene, *pfs25* [21].

Total RNA was first isolated from 100 µL of capillary blood (collected by finger stick) using the SV Total RNA Isolation System (Promega, U.K.). Quantitative reverse transcription and subsequent amplification (qRT-PCR) of cDNA was carried out using the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher, U.K.). The RT-PCR conditions and primers used were previously described by Hemson *et al*. and Schneider *et al*. [17, 22].

Amplicon sequencing for characterisation of *P. falciparum* drug resistance loci

SNPs in four *P. falciparum* genes, *Pfmrp1* (PF3D7_0112200)*, Pfcrt* (PF3D7_0709000)*, Pfmdr1* (PF3D7_0523000)*,* and *PFK13* (PF3D7_1343700), implicated in resistance to several different antimalarial drugs, were typed by amplicon sequence according to the prescribed methodology of Rao *et al*. [23]. The analysis combined multiplex PCR and custom-designed sequence analysis using the Illumina® Miseq sequencing (for the high throughput SNP-profiling of drug resistance genes) protocol [23]. Seventy *P. falciparum* isolates were examined together with two controls, laboratory *P. falciparum* clones 3D7 and Dd2, with known alleles of the examined genes and phenotypic responses to some antimalarial drugs [23]. The *PfK13 and Pfcrt,* genes were each amplified as one fragment, while the longer *Pfmdr1* and *Pfmrp1* genes were each amplified as two fragments. PCR was carried out in a volume of 25 µl, containing 1 µl (10 pmol) of primers, 0.4 µl of dNTPs (200 µlmol/L), 4 µl of Phusion HF buffer (5x) and 1 U of Phusion high-fidelity polymerase enzyme. The cycling profile for all loci was: 98 °C / 30 s, followed by 30 cycles of (98 °C / 10 s, 64 °C /4 min), and a final extension at 64 °C / 5 min.

The PCR amplicons of all genes, for each isolate were pooled and purified using Agencourt AMPure XP purification system and quantified using a Qubit double-stranded DNA (dsDNA) HS assay kit (Thermo

Fisher Scientific). Sequencing was conducted on Illumina® MiSeq systems. Initially, libraries were prepared using Nextera XT kit according to the manufacturer's protocol (Illumina Nextera® XT DNA Sample Preparation Guide, 2012). Following PCR cleanup, the libraries were quantified using a Qubit dsDNA BR kit and evaluated for fragment size using an Agilent High Sensitivity DNA Kit, designed for the 2100 Bioanalyzer Instrument (Agilent Technologies, Santa Clara, CA, USA). Each library was normalized for sequencing to 10 pM according to the manufacturer's protocol and following Illumina's (Illumina®, San Diego, CA, USA) directions for cluster optimization (Illumina Nextera® Library Validation and Cluster Density Optimization, 2013). Sequencing reactions were carried out using a MiSeq Reagent Kit V2, for 50 cycles (MiSeq, Illumina). SNPs in all genes were called using the reference sequence of the *P. falciparum* 3D7 clone, version 3 (PlasmoDB, PF3D7 v3).

Data Analysis

All samples that contained gametocyte transcripts, as detected by qRT-PCR, were analysed for association between gametocyte carriage and total parasitemia. A Mann-Whitney U test was used to examine the difference in density between late and early gametocytes. Spearman's rank correlation was used to test for association between total parasite density (18S rRNA copy number) and the density of either late gametocytes (*Pfs25* copy number) or early gametocytes (*Pfpeg4* copy number). Microsatellite allele data were filtered to retain only minor alleles having a peak height of > 33% of the corresponding predominant alleles if more than one allele was present at any given locus. Genetic diversity metrics were calculated for the entire dataset using GenAlEx v6.5 [24]. Expected heterozygosity was calculated using the formula for ‘unbiased heterozygosity’ also termed haploid genetic diversity, $H_e = [n/(n\text{-}1)][1\text{-}\sum p^2]$ where n is the number of isolates and p the frequency of each different allele at a given locus [25]. Population differentiation was assessed by estimating Wright’s F_{ST} index using the *F*stat computer package version 2.9.3.2. Two estimators of F_{ST} (G'_{ST} and θ) [26, 27] were used to estimate genetic differentiation between imported parasites from the Indian subcontinent and Africa.

Multiplicity of infection (MOI), defined as the presence of multiple genotypes per infection, was

determined by the detection of more than one allele at a given locus. To avoid over-estimation of low-abundance alleles, only minor alleles having a peak height of > 33% of the corresponding predominant alleles were accepted. The proportion of samples with more than one allele across ten loci was used to represent MOI. The maximum number of alleles across the ten loci was used as an index for minimum number of clones per infection (MNC). The overall mean of the index value for each sample was then calculated.

Results

Demographic characteristics of imported malaria cases in Qatar

Among the 583 patients (all expatriates) tested for malaria between January, 2013 and October, 2016 in Hamad General Hospital and Al-Khor Hospital, Qatar, 448 (76.8%) tested positive: 318 for *P. vivax* (*70.9%),* 118 for *P. falciparum* (*26.3%*) and 12 (2.7%) for *P. vivax / P. falciparum co*infection (*Table 1*). The majority of patients that tested positive for malaria were men, *P. vivax (94.3%)*, and *P. falciparum* (84.0%), with a mean age of 32 yrs and 33 yrs, respectively, reflecting the fact that the predominate attending patients were young men (Supplementary Table 1).

The primary origin of those presenting with *P. vivax* was the Indian Subcontinent: India (46.0%, n = 146), Pakistan (32.1%, n = 102) and Nepal (3.8%, n = 12). A smaller proportion of *P. vivax* cases were from Africa (16%, n = 53) as a whole (*Table 1*). Unlike *P. vivax*, the primary origin of *P. falciparum* infection was Africa: East Africa (76.1%, n = 67) West and Central Africa (23.9%, n = 21), followed by the Indian Subcontinent (20.3%, n = 24) and other countries (5.1%, n = 6) (*Table 1*).

Parasitaemia and gametocytaemia among imported malaria cases

Ninety of the 118 discovered *P. falciparum* infections were further examined for total parasite and gametocyte density (using qPCR and qRT-PCR, respectively) as well as the diversity of 10 microsatellites and the alleles of four genes linked to drug resistance. The total *P. falciparum* density among imported cases varied widely, ranging between 32 and 9,218,498 parasites/ml blood with a median of 82,783 parasites/ml. The median parasite density among imported cases from the Indian Subcontinent (99,572 parasites/ml) was not statistically greater than that of Africa (88,504 parasites/ml) (P = 0.394).

Seventy-three *P. falciparum* isolates were successfully examined by qRT-PCR to detect and quantify transcripts of genes expressed in early (*Pfpeg4*) and late gametocytes (*Pfs25*). The prevalence of all gametocytes was 74% (n =54), with 9.6% (n =7) of patients carrying only early stages, 37% (n =27) only late stages, and 27.4% (n =20) a mixture of both stages. Early gametocytes were found at a relatively lower density, ranging between 14 and 3,781/ml blood, with a median of 1,011 early gametocytes/ml blood, compared to late gametocytes, which possessed a higher density overall (range 16 – 15,289 gametocytes/ml blood, median 136 gametocytes/ml blood) (Mann-Whitney U test, P = 0.003). No correlation was found between total parasitaemia (18S rRNA copy numbers) and either late gametocytes (*Pfs25* copy number) (r_S = 0.008, P = 0.946) or early gametocytes (*Pfpeg4* copy number) (r_S = 0.031, P = 0.835) (*Figure 1*) and early gametocyte density (P = 0.857).

Genetic diversity and structure of imported *P. falciparum* parasites

Microsatellite polymorphisms

All of the examined microsatellites were highly polymorphic among *P. falciparum* isolates originating from Africa and the Indian Subcontinent (*Table 2*). The number of alleles per locus was higher among African isolates, ranging from 5 for *pfg377,* to 18 for *polyα*, compared to Indian Subcontinent isolates, which ranged from 3 for *2490* to 7 for *TA1* and *PfPK2* (Table 2; Supplementary Table 2). Nevertheless, allelic diversity (summarized as mean expected heterozygosity (*H*e) across 10 microsatellite loci) was not significantly different among parasites in the Indian Subcontinent (mean *H*e = 0.78) compared to those in Africa (mean *H*e = 0.76).

Multi-locus haplotypes were constructed using predominant alleles at all examined loci. All 90 isolates differed from each other in at least one of the examined loci, with the exception of two isolates from Sudan, Africa, that shared an identical haplotype. Thus, almost every isolate in each of the examined sites carried a unique genotype.

Multiplicity of infection (MOI)

Seventy-six (84.4%) out of the 90 imported *P. falciparum* isolates (with complete data) harbored multiple genotypes. A similar mean of multiple genotype infections was observed among parasites of

the Indian Subcontinent (84.6%). The minimum number of genotypes per infected person (the mean maximum number of alleles observed at all loci) was slightly lower in Africa (2.16 genotypes) than in the Indian Subcontinent (2.38 genotypes), but this trend was not calculated to be statistically significant ($a < 0.05$).

Genetic differentiation

Alleles of most microsatellites were distributed widely across *P. falciparum* among imported malaria cases from both Africa (n = 77) and the Indian Subcontinent (n = 13). A large number of private alleles (detected only in one region) were seen in Africa (n = 50) compared to the Indian Subcontinent (n = 5), which may reflect the smaller sample size. Nonetheless, no evidence of genetic differentiation was observed between imported *P. falciparum* from Africa and those from the Indian Subcontinent ($F_{ST} = 0.055$). The genetic relatedness between *P. falciparum* populations was further illustrated using PCoA analysis (*Figure 2*). Analysis of molecular variance (AMOVA), comparing between *P. falciparum* isolates imported from the Indian Subcontinent, revealed that the majority of differences were due to variation between individuals within the same group (95%), while only 5% could be attributed to differences between populations ($P < 0.001$).

Distribution of drug resistance genes among imported cases

Seventy imported *P. falciparum* isolates were examined using amplicon sequencing for four putative drug resistance genes, *PfK13, Pfmdr1, Pfcrt and Pfmrp1* (Table 3). With the exception of *PfK13*, there was no difference in the prevalence of wild type genes among parasites originating from Africa versus the Indian subcontinent. There was, however, a significantly higher prevalence of mutant *PfK13* haplotypes among parasites from Africa than the Indian subcontinent. One nonsynonymous mutation in *PfK13* (K189T) was observed at a high prevalence (36%) among parasites originating from Sudan, analogous to reported findings from other African countries [28]. Furthermore, ten additional nonsynonymous SNPs: K108E (2%), L119L (1%), H136N (1%), T149S (2%), K189N (2%), N217H (1%), R255K (3%), I354V (1%), E433D (1%), G453A (1%), all existed at very low prevalence ranging from 1-3% (Table 3; Supplementary Table 3).

PfK13 mutations including the amino acid substitutions of C580Y, Y493H, R539T and M579I associated

with slow artemisinin clearance of *P. falciparum* [29, 30] were not detected among the *P. falciparum* isolates imported into Qatar. However, *Pfmdr1* alleles encoding the polymorphisms N86**Y** (33%) and Y184**F** (77%) were prevalent among imported *P. falciparum* isolates. In addition, six rare nonsynonymous SNPs were detected (Table 3). The N_{86}**F**$_{184}D_{1246}$ and **Y**$_{86}$**F**$_{184}D_{1246}$ haplotype associated with Artemether-Lumefantrine (AL) and Chloroquine/Amodiaquine (CQ/AQ) treatment failure were prevalent among imported *P. falciparum* cases, at 43% and 33%, respectively. Notably, while the *Pfcrt* K76**T** substitution associated with CQR was found at low frequency (n = 70, 6%), other SNPs implicated in CQR were observed at high prevalence, e.g. A220S (53%), Q271E (49%), N326D/S (36%), I356L (6%) and R371I (47%). Overall, the CQ sensitive haplotype $C_{72}V_{73}M_{74}N_{75}K_{76}$ was common (94%), while the CQ resistant haplotypes, **S**$_{72}V_{73}M_{74}N_{75}$**T**$_{76}$ and $C_{72}V_{73}M_{74}N_{75}$**T**$_{76}$, were detected in only one and three isolates, respectively.

Regarding *Pfmrp1*, eight mutations were observed among imported *P. falciparum* in Qatar, ranging from high I876V (46%) to low D1533V (3%) (Table 3). The most prevalent six mutations (20% to 46%) detected among imported cases in Qatar have been previously reported in the Indian subcontinent and Africa [31, 32]. *Pfmrp1* polymorphisms that have previously been associated with decreased *in vitro* susceptibility to SP, artemisinin, mefloquine, and lumefantrine were common among imported malaria in Qatar. For example, the most prevalent SNP, I876V (46%), was found to be under significant selection pressure after AL treatment [31].

Discussion

Sustainable interventions, driven by global support and WHO efforts, have resulted in significant malaria contraction in the past three decades (WHO 2019). For example, in 2016, 21 countries have been identified by WHO to have potential to eliminate malaria by the year 2020 on the basis of ongoing intervention activities in these countries [33]. However, drug and insecticide resistance, social and demographic factors could drift back the progress attained so far. Expanding business and demographic ties can play a major role in re-introduction of malaria to transmission-free areas, driven by travelers from endemic countries where a large proportion of the semi-immune inhabitants sustain asymptomatic low levels of parasitemia. Asymptomatic infection can often develop into clinical

malaria after as long as 8 years of settlement in a malaria-free country [34]. Unlike *P. falciparum*, *P. vivax* includes persistent liver stages (hypnozoites) that permit dormant infection, and can lead to a relapse within a 9-year period [35]. Therefore, asymptomatic migrants with malaria parasites can maintain a long-lasting reservoir for secondary local transmission in receptive malaria-free areas, where elimination has been accomplished [3, 36]. This potential is evident by the high prevalence of gametocyte carriage seen among imported *P. falciparum* malaria in Qatar. Fifty-four (74%) of 73 imported *P. falciparum* isolates, successfully examined by qRT-PCR [42], carried gametocyte stages, with a large proportion (37%) harbouring both early and mature gametocytes. This is indicative of ongoing gametocytogenesis from the asexual population present in the patient. Low-density gametocytes can readily infect *Anopheles*, even at sub-microscopical levels [37, 38]. Secondary transmission, originating from imported malaria, is often reported in GCC countries in areas where the *Anopheles* vector is present and a favorable ecological habitat prevails [3, 39]. The surge in mosquito abundance in highly seasonal transmission settings has been linked to an upsurge in gametocyte numbers in asymptomatic carriers [21], in line with enhanced malaria parasite infectivity in response to increased exposure to uninfected mosquitoes at the start of the transmission season [21, 40]. Although the resumption of endemic transmission in GCC countries is unlikely, the high rate of imported malaria can readily seed outbreaks in receptive areas, if vector control wanes [3].

The high level of diversity among imported *P. falciparum* in Qatar from Africa (*He* = 0.76) and the Indian subcontinent (*He* = 0.78), as well as genotype multiplicity, are similar to that reported in local parasites in both sites [41, 42], and sites where local transmission occurs in the region, in southwest of Saudi Arabia and Yemen [43].This highlights the role of imported malaria to enrich genetic diversity in areas moving towards elimination, were local transmission occurs. The introduction of novel malaria lineages via migrants into the region, in areas heading towards elimination, such as Saudi Arabia [44] and Oman [3], with limited parasite reservoir can enhance the parasite diversity and effective population size (*Ne*) as there is a direct relationship between expected level of diversity and *Ne* [45]. In addition, the combination of high genotype multiplicity and gametocyte carriage, as seen

in the present study, increases the likelihood that imported malaria infections will generate novel genotypes, should transmission occur [46]. Thus, the imported malaria cases represent not only a risk for initiating local transmission, but also a risk of disseminating novel strains that can escape the effect of current drug regimens.

Efforts to prevent the reintroduction of malaria in transmission-free areas of the GCC rely on effective case management using artemisinin-combination therapy. The present study revealed high prevalence of SNPs in 4 unlinked genes implicated in drug resistance among imported malaria cases in Qatar, including, *Pfcrt, pfmdr1, P fmrp1 and K13*.

With exception, of K13, there is no differences in the distribution of alleles of drug resistance genes between imported parasite in the Indian subcontinent compared to Africa. There was higher prevalence of wild type allele *PfK13,* nonetheless, known mutations C580Y, Y493H, R539T and M579I associated with slow artemisinin clearance [28, 30], were not detected in *P. falciparum* imported from both regions. Numerous low-frequency SNPs (ranging between 1 to 3%) were observed (K108E, L119L, H136N, T149S, K189N, N217H, R255K, I354V, E433D, G453A), while one, K189T, was observed at a high prevalence (36%) among parasites originating from Sudan [28]. Nevertheless, parasites carrying mutation K189T were found to have a similar response to that of wild type parasites [28] and may not impact the current ACT regimen in Qatar artesunate/doxycycline for uncomplicated and complicated *P. falciparum* malaria [11].

However, the presence of SNPs/haplotypes linked to tolerance/resistance to artemisinin derivatives and common partners can impede this strategy, and results in persistence and increased parasite reservoir and vulnerability. There is a high prevalence of the *Pfmdr1*-$N_{86}F_{184}D_{1246}$ haplotype (43%) associated with artemether-lumefantrine (AL) treatment failure [47]. Similarly, the common variants of *Pfmrp1*, F1390 (79%) and I876V (46%), have been linked to decreased susceptibility to artemisinin mefloquine, and lumefantrine [31, 48]. Together, these findings suggest that regular monitoring of

the above SNPs coupled with clinical response should be considered to combat the spread of *P. falciparum* parasites with potential AL resistance/tolerance. Previous surveys in Saudi Arabia and Yemen revealed a high prevalence of drug resistance genotypes among locally acquired *P. falciparum* infection and linked the source of some of them to Africa and Indian Subcontinent [31, 48].

An interesting observation in the present work was the divergence in the frequencies of *pfcrt* mutation K76T compared to other mutations (A220S, Q271E, N326D/S, I356L and R371I). Similar to K76T, the latter mutations have been associated with reduction in CQ transport activity and CQR [49]. Nonetheless, the low frequency of K76T mutation may reflect the fitness cost due to reduced exposure to CQ [50], while the other mutations are probably less susceptible to fitness cost. Alternatively, these other *pfcrt* mutations are under selection pressures from both CQ and another antimalarial drugs**.**

Conclusions

The present study underscores the threat of imported plasmodium parasites to the reintroduction of malaria in receptive, malaria-free areas, such as Qatar. The high levels of genetic diversity and the capacity of imported *P. falciparum* to produce gametocytes highlight the threat of drug resistance genotypes, should local transmission commence. There is an urgent need for molecular tools that can be used for the surveillance of imported malaria cases in Qatar and the GCC, to limit the risk of reintroduction of malaria.

Abbreviations

GCC: Gulf Cooperation Council

HMC: Hamad Medical Corporation

MOI: Multiplicity of infection

qRT-PCR: Quantitative Reverse Transcriptase-Polymerase Chain Reaction

MNC: Minimum number of clones per infection

H_e: Expected heterozygosity

F_{ST}: Fixation index

PCoA: Principle coordinates analysis

AMOVA: Analysis of molecular variance

ACT: Artemisinin-based combination therapy

AL: artemether-lumefantrine

Declarations

Ethics approval and consent to participate: Ethical clearance for the study was obtained from the Institutional Review Board of HMC and Weill Cornell Medicine-Qatar (Protocol no. 14-00097). A written consent was obtained from each participant before any interview or clinical examination was conducted.

Consent for publication: Not applicable.

Availability of data and materials: The datasets used in the current study are available from the corresponding author on reasonable request.

Competing interests: The authors declare that they have no competing interests.

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Authors' contributions: HAB, AAS, LRC and AP conceived the study and designed the experiments. AR, ZH, SA, AG and RN contributed to data collection and data analysis.

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Tables

Table 1: Origin of imported malaria cases in Qatar between 2013-2016. The percentage values in brackets represent the proportion of one species originating from the country listed. The information on the originating country of the expatriates was obtained in response to the questionnaire, and may not reflect all countries through which the individual travelled prior to arrival in Qatar.

Country	Origin	*P. vivax (%)*	*P. falciparum (%)*	*P. vivax + falciparum*
The Indian Subcontinent	India	148 (46.5%)	18 (15.3%)	2
	Pakistan	104 (32.7%)	4 (3.4%)	0
	Sri Lanka	0	1 (0.8%)	0
	Nepal	12 (3.8%)	1 (0,8%)	0
Africa	Mauritania	1 (0.3%)	0	0
	Sudan	34 (10.7%)	36 (30.5%)	4
	Kenya	3 (0.9%)	16 (13.6%)	3
	Nigeria	3 (0.9%)	11 (9.3%)	2
	Eritrea	5 (1.6%)	10 (8.5%)	1
	Ethiopia	5 (1.6%)	3 (2.5%)	0
	Ghana	1 (0.3%)	3 (2.5%)	0
	Rwanda	0	2 (1.7%)	0
	Cameroon	0	2 (1.7%)	0
	Tanzania	1 (0.3%)	1 (0.8%)	0
	Djibouti	0	1 (0.8%)	0
	Democratic Republic of Congo	0	1 (0.8%)	0
	Republic of Ivory Coast	0	1 (0.8%)	0
	Chad	0	1 (0.8%)	0
Others*	Romania	0	1 (0.8%)	0
	USA	0	1 (0.8%)	0
	Syria	0	1 (0.8%)	0
	Qatar	0	1 (0.8%)	0
	Saudi Arabia	0	1 (0.8%)	0
	Spain	0	1(0.8%)	0
	Canada	1(0.3%)	0	0
Total		318	118	12

Others*: Reported by patients who have been on visit to malaria endemic countries.

Table 2: Number of alleles and expected heterozygosity (*He*) at ten microsatellite loci among imported *Plasmodium falciparum* from the Indian Subcontinent and Africa.

Origin of isolates		*2490*	*Pfg377*	*polyα*	*TA109*	*TA81*	*ARA2*	*PfPK2*	*TA1*	*TA60*	*TA87*
The Indian Subcontinent (n = 13)	Alleles	3	4	6	5	5	5	7	7	6	6
	He	0.67	0.68	0.87	0.81	0.74	0.71	0.91	0.87	0.72	0.86
Africa (n =77)	Alleles	7	5	18	10	10	11	9	13	6	10
	He	0.48	0.58	0.93	0.81	0.76	0.81	0.78	0.85	0..76	0.83

Table 3. Haplotypes of drug resistance genes, that exist at a prevalence of more than 5%, among imported *P. falciparum* to Qatar. Haplotypes are shown as amino acids (wild-type in normal case, substitutions in **bold underlined**).

Locus	Genotype	**Haplotype**	Prevalence	the Indian subcontinent (n=7)	A
Pfcrt	Wild type	C_{72} K_{76} A_{220} Q_{271} N_{326} I_{356} R_{371}	44%	1(14%)	3
	Mutant	C_{72} K_{76} **S_{220}** **E_{271}** N_{326} I_{356} R_{371}	13%	6(85%)	3
	Mutant	C/S_{72} **T_{76}** **S_{220}** **E_{271}** N_{326} I_{356} R_{371}	5%		
	Mutant	C_{72} K_{76} **S_{220}** **E_{271}** **S_{326}** I_{356} R_{371}	29%		
Pfmdr1	Wild type	N_{86} F_{184} F_{938} G_{968} D_{1246}	39%	4(57%)	2
	Mutant	N_{86} **Y_{184}** F_{938} G_{968} D_{1246}	10%	3(43%)	4
	Mutant	**Y_{86}** F_{184} F_{938} G_{968} D_{1246}	30%		
PfK13	Wild type	H_{136} T_{149} K_{189} N_{217} R_{255} E_{433} G_{453}	47%	7(100%)	2
	Mutant	H_{136} T_{149} **T_{189}** N_{217} R_{255} E_{433} G_{453}	34%	0(0%)	3
Pfmrp1	Wild type	H_{191} K_{202} S_{437} I_{876} L_{1342} F_{1390} K_{1466} D_{1533}	31%	1(14%)	2
	Mutant	H_{191} K_{202} S_{437} **V_{876}** L_{1342} F_{1390} K_{1466} D_{1533}	6%	6(86%)	4
	Mutant	H_{191} K_{202} S_{437} **V_{876}** L_{1342} F_{1390} **R_{1466}** D_{1533}	10%		
	Mutant	**Y_{191}** K_{202} **A_{437}** **V_{876}** L_{1342} **I_{1390}** K_{1466} D_{1533}	5%		

Figures

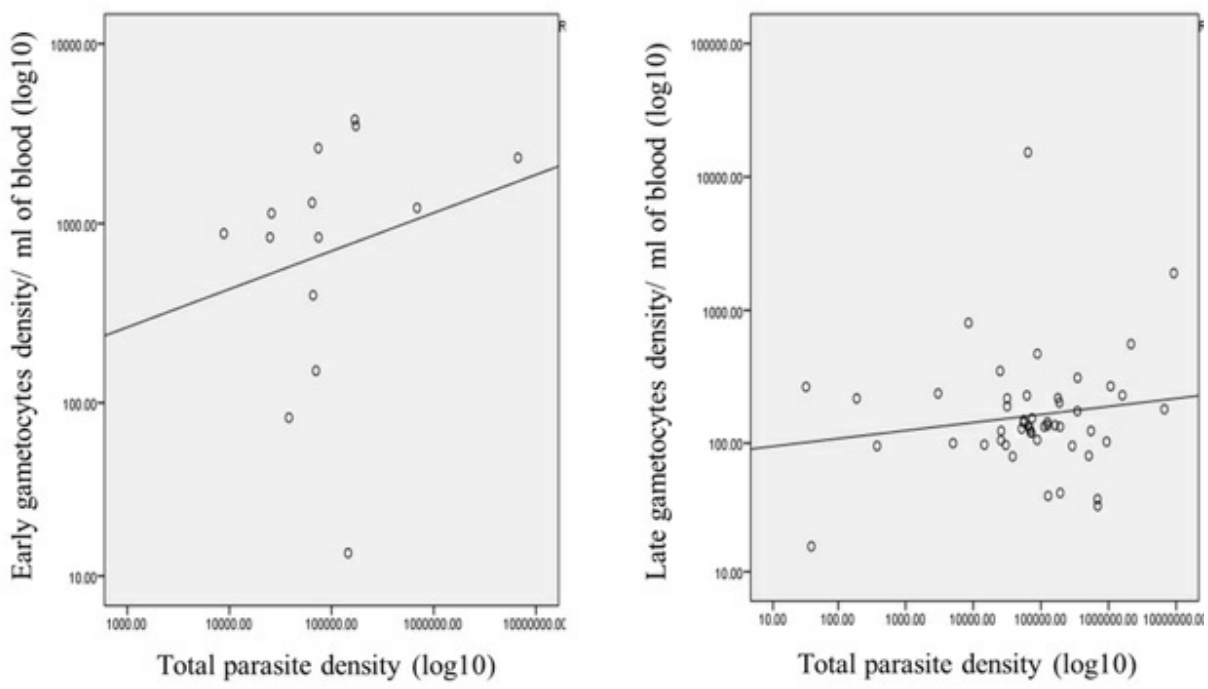

Figure 1

Correlation between total parasite density with both early gametocyte and late gametocyte density. (A) log total parasitaemia (X axis) and log early gametocyte density (Y axis), the fit line in scatter plot shows a weak/non-significant correlation coefficient (r =0.031, p = 0.835). (B) log total parasitaemia (X axis) and log late gametocyte density (Y axis), the fit line in scatter plot shows a weak/non-significant correlation coefficient (r= 0.008, p = 0.946).

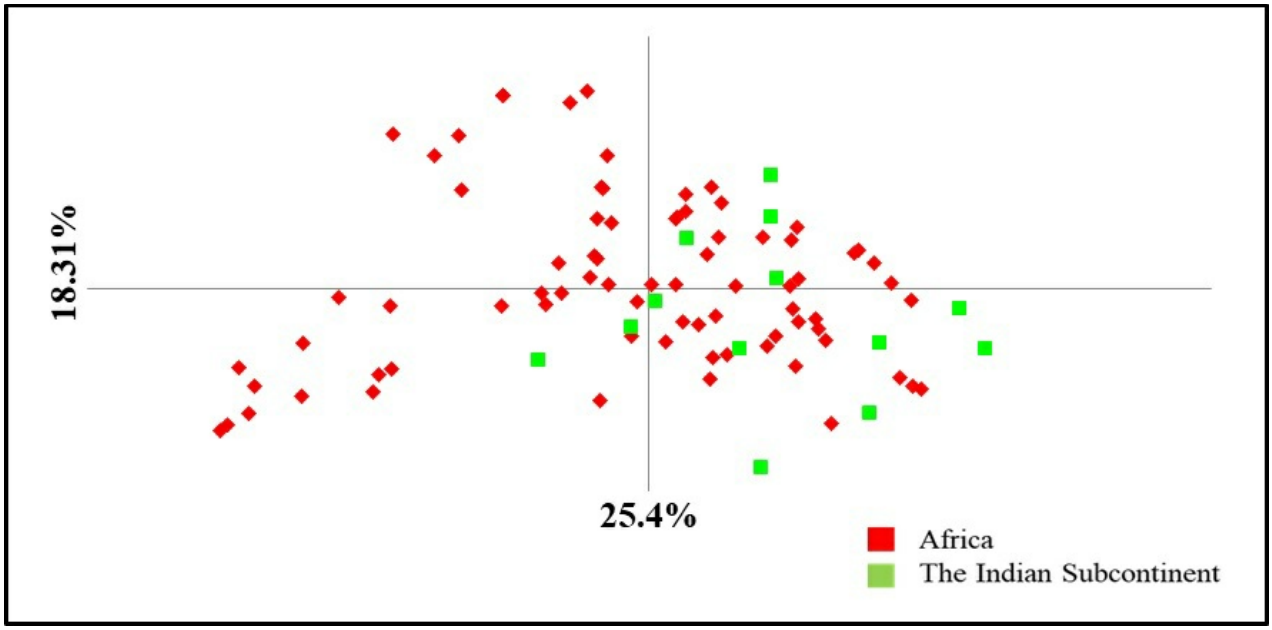

Figure 2

Principal Co-ordinates Analysis (PCoA) of P. falciparum populations in two regions (the Indian Subcontinent and Africa). Values within () after the coordinate number, are the percentage of variation explained by the coordinate.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

Supplementary Table 2.docx
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