Molecular Surveillance of Antimalarial Resistance *Pfcr*, *Pfmdr1* and *Pfk13* Polymorphisms in African *Plasmodium Falciparum* Imported Parasites to Wuhan, China

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Research

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Abstract

Background: The development of drug resistance in *Plasmodium falciparum* becomes a severe problem for malaria control globally. Before finding a practical solution, monitoring the susceptibility of *P. falciparum* resistance-related genes is crucial. It will offer valuable information on the drug resistance in malaria-endemic areas and guides the rational clinical use of antimalarial drugs.

Methods: Filter paper blood was taken from patients with positive *P. falciparum* during 2017-2019 in Wuhan, China. The target fragments from *pfcr*, *pfmdr1*, and *k13 propeller (pfk13)* genes of *P. falciparum* were amplified and sequenced. Subsequently, the polymorphisms of *pfcr*, *pfmdr1*, and *pfk13* and the haplotypes of Pfcrt and Pfmdr1 were analyzed.

Results: Totally, 106 samples were collected. Subsequently, 98.11% (104/106), 100% (106/106), and 86.79% (92/106) of these samples were successfully amplified and sequenced for the *pfcr*, *pfmdr1*, and *pfk13* genes, respectively. The prevalence of Pfcrt K76T, Pfmdr1 N86Y, and Pfmdr1 Y184F mutation were 9.62%, 4.72%, and 47.17%, respectively. At codons 72-76 of *pfcr* gene locus were showed three haplotypes, including CVMNK (wild-type), CVIET (mutation type), CV M/I N/E K/T (mixed type), with 87.50%, 9.62%, and 2.88% prevalence, respectively. For the *pfmdr1* gene, including NY (wild type), NF and YF (mutant type), N Y/F, Y Y/F, and N/Y Y/F (mixed type), accounted for 34.91%, 43.40%, 3.77%, 15.09%, 0.94%, and 1.89%, respectively. A total of eleven Pfcrt/Pfmdr1 combined haplotypes, including six types of combined haplotypes, and five combined haplotypes with mixed-type, For *pfk13*, no mutation was detected.

Conclusions: The wild-type SNPs and haplotypes for the *pfcr*, and *pfmdr1* genes become predominant in the current study. It indicates these isolates entirely or partly recovery their susceptibility to antimalarial drugs, including chloroquine, amodiaquine, and mefloquine. Moreover, it demonstrates these drugs can continue to be effective drugs for *P. falciparum* malaria cases treatment in Africa. Although no mutation is detected in *pfk13*, continuous molecular surveillance is still urgently necessary.

1. Introduction

Malaria is a mosquito-borne infectious disease that seriously endangers human health, among which falciparum malaria caused by *Plasmodium falciparum* is the most serious, mainly in tropical and subtropical regions, especially in sub-Saharan Africa and Southeast Asia (SEA) [1]. According to the latest WHO estimates in 2019, during 2018, there were 228 million new malaria cases worldwide and about 405,000 deaths from malaria [1]. Research demonstrates that since 2017, China has achieved zero reporting of indigenous malaria cases for three consecutive years [2]. In recent years, with the process of globalization, the number of migrant workers, tourists, and businessmen in China is increasing gradually, especially those returning from Africa and SEA [3], which has brought serious pressure for malaria control in China. It has brought severe challenges to the imported malaria control work of our country and even the whole world.

At present, drug therapy was the primary means for prevention and treatment of malaria [4]. However, with the use of increasingly antimalarial and anti-mosquito drugs, *P. falciparum* and its vector *Anopheles* mosquitoes gradually develop drug resistance and spread rapidly [5]. Chloroquine (CQ) is a safe, cheap, and effective antimalarial drug for malaria treatment. However, in the 1940s, *P. falciparum* parasites developed resistant to CQ. Since then, CQ-resistant (CQR) strains began to spread rapidly around the world [6]. After the discovery of artemisinin (ART) in the 1970s, malaria control was temporarily eased. Unfortunately, ART-resistant strains have been reported in SEA, which has raised global awareness of malaria treatment and management [7]. In our previous study, an R561H mutation of *pfk13* was found in a sample from Rwanda, Africa, in 2016 [8]. A recent study found the mutation was independent occurred in Africa [9], confirming our previous findings. In order to achieve better therapeutic results, artemisinin-based combination therapy (ACTs) was recommended by WHO as the most extensive and useful program for malaria treatment. As the core component of ACTs or other adjuvant combination drugs produces widespread drug resistance, the incidence of malaria treatment failure will be high. Therefore, in the absence of more choices, it is urgent to monitor drug resistance genes related to major antimalarial drugs.

At present, studies have confirmed that gene mutations such as *pfk13*, *pfcr1*, and *pfmdr1* have been used as the primary molecular markers of drug resistance in malaria parasites [7, 10–12]. The *pfk13* polymorphism has been considered to be related to ART resistance. However, studies have shown that the distribution of alleles for *pfk13* varies according to the mutation [13]. In SEA, the alleles of C580Y mutation was dominant [13]. In Africa, the mutation rate of the *pfk13* remains relatively low level. Recently, the newly discovered local drug resistance mutation site in Africa-R561H [8, 9, 14]. The 72–76 amino acid mutation in *Pfcr1*, especially the K76T mutation, was the primary marker of CQR [10, 15]. Several mutations in *pfmdr1* will cause the resistance of *P. falciparum* to CQ, amodiaquine (AQ) and mefloquine (MQ), and other drugs [11, 16].

In the current study, *pfcr1*, *pfmdr1*, and *pfk13* polymorphisms of *P. falciparum* isolates imported from Africa in Wuhan City, Hubei Province, China was surveyed. It will provide the gene mutation and the related data of *P. falciparum* resistance detection, and offer significant clues to prevent the spread of drug-resistant *P. falciparum* in Africa, and China.

2. Materials And Methods

2.1 Collection of samples

From May 2017 to December 2019, 3 ml of peripheral blood of returned migrant workers from Africa were collected in Wuhan, Hubei Province. These samples were examined by immune-gold assay kit (ICT Diagnostics) for *Plasmodium spp.* antigen (HRPII). Then, blood smears with patient samples were prepared and checked under microscopic. Finally, the species were identified by qPCR. Approximately 400 µl of blood was spotted on 3 MM Whatman filter paper and air-dried (identified and provided by the Center for Disease Control and Prevention of Wuhan City, Hubei Province). Then, these filter papers were

numbered and stored at -20 °C with a polyethylene bag. The consent of the owner and his legal guardian has been obtained before sampling.

2.2 Determination of *P. falciparum* gene mutations

Genomic DNA (gDNA) was extracted from blood-spots by TIANamp Blood Spots DNA Kit, to yield approximately 50 µl of supernatant containing DNA and stored – 20 °C until further use. Using the extracted gDNA as a template, the target fragments of *Pfcr*t gene (Gene ID: PF3D7_0709000), *pfmdr*1 gene (Gene ID: PF3D7_0523000), and *pfk*13 gene (Gene ID: PF3D7_1343700). Following the previously published primer information and following the previous procedure [8, 17, 18] to modify slightly and establish PCR reaction, the *pfcr*t, *pfmdr*1, and *pfk*13 genes were successfully amplified. The reaction system and reaction procedure for PCR were shown in Table 1. After all the reactions were finished, the 5 µl second-round PCR products were analyzed by 1.0% agarose gel electrophoresis. Then the remaining products were purified for sequenced in Genewiz, Soochow, China. The sequencing data were analyzed by Dnastar (DNASTAR Inc., Madison, WI, USA), and compared with *Pfcr*t, *Pfmdr*1, and *pfk*13 of *P. falciparum* 3D7 strain as a standard sequence. The new mutation was verified by a new PCR product and bi-directional DNA sequencing.

2.3 Data analysis

Analyze the data using SPSS 25 (SPSS Inc., Chicago, IL, USA). The allele frequencies and 95% confidence intervals of wild-type and mutant types for these genes were calculated [8].

3. Results

3.1 General information

A total of 151 individuals were diagnosed with malaria from 2017 to 2019. Of these, 106 were infected with *P. falciparum*, 17 with *Plasmodium vivax*, 20 with *Plasmodium ovale*, and 8 with *Plasmodium vivax*. Among 106 cases of *P. falciparum* infection, all from African (Table S1), including 48 cases in West Africa, 33 cases in Central Africa, 15 cases in South Africa, 9 cases in East Africa, and 1 case in North Africa. These cases were mainly from 21 African countries, dominating distributed in Congo (21.70%, 23/106), followed by Nigeria (16.98%, 18/106), Ivory Coast (9.43%, 10/106), Mozambique (6.60%, 7/106). No cases have been found in SEA.

3.2 Mutation prevalence of *Pfcr*t and *Pfmdr*1

The gDNA was extracted from 106 samples of *P. falciparum* patients. Then, PCR amplification was performed, and all positive PCR products were obtained. For *pfcr*t, a total of 104 samples were successfully sequenced (98.11%, 104/106). The prevalence of *Pfcr*t V73V, M74I, N75E, K76T allele mutation was 9.62% (10/104) in Africa (Table 2). These mutations were mainly concentrated in West Africa, followed by Central Africa. And the results exhibited that the *pfcr*t genotype had polymorphisms at codons 72–76, including CVMNK (wild-type), CVIET (mutation type), and CV M/I N/E K/T (mixed type).

Most isolates harboured parasites with the CVIET (87.5%, 91/104). The proportion of CVIET among these isolates was 9.62% (10/104). The last case was CV M/I N/E K/T, accounting for 2.88% (3/104), no SVMNT haplotype was found. (Table S2).

For *pfmdr1* gene, 100% samples were obtained the PCR products, and 100% PCR products were sequenced successfully (Table 2). Sequencing results showed the main epidemic mutation sites of *pfmdr1* were concentrated on N86Y and Y184F, with 4.72% and 47.17% mutation, respectively (Table 2). These mutations were also mainly focused in West Africa, followed by Central Africa and South Africa. In addition, six haplotypes encoding N86Y and Y184F were detected (Table S2), including NY (wild type), NF and YF (mutant type), N Y/F, Y Y/F, and N/Y Y/F (mixed type), accounted for 34.91%, 43.40%, 3.77%, 15.09%, 0.94%, and 1.89%, respectively. Besides, one non-synonymous mutation at position 199 and several synonymous mutations at position 87 and 102 were detected in these samples (Table 3 and Fig. 1).

With these polymorphisms in *pfprt* and *pfmdr1* genes, a total of eleven Pfcrt/Pfmdr1 combined haplotypes were assessed. There were six types of combined haplotypes, namely NF-CVIET, NF-CVMNK, NY-CVIET, NY-CVMNK, YF-CVIET, YF-CVMNK. They were mainly concentrated in West Africa and Central Africa. Among them, Congo and Nigeria accounted for 16.98% (18/106) and 13.21% (14/106) respectively. The other five combined haplotypes with mixed-type, including N Y/F-CV M/I N/E K/T, N Y/F-CVMNK, N/Y Y/F-CVMNK, NF-CV M/I N/E K/T, Y Y/F-CVMNK. Also concentrated in West Africa and Central Africa, mainly distributed in Ivory Coast 5.66% (6/106) and Congo 4.72% (5/106).

3.3 Analysis of mutation in *Pfk13* gene

For the *pfk13*, 93.40% (99/106) samples were amplified, and 92.93% (92/106) cases were sequenced successfully. Sequencing analysis displayed that these cases were all wild-type, and no mutation was detected at positions 474, 476, 493, 508, 527, 533, 537, 539, 543, 553, 568, 574, 578, and 580 of the Pfk13 gene. The previously detected mutations at positions 550, 561, 575, and 589 in Africa were also not detected.

4. Discussion

For decades, the rapid emergence of drug resistance of *P. falciparum* parasites has become one of the leading causes of the global burden for malaria [19]. The continuous influx of imported malaria infections in China increases the possibility of malaria re-spreading to all acceptable areas in China [20]. Therefore, we investigated the *pfk13*, *pfprt*, and *pfmdr1* polymorphisms of imported *P. falciparum* resistance genes from Africa in Wuhan City, Hubei Province, China. According to the results, at codons 72–76 of *pfprt* gene locus were showed three haplotypes, including CVMNK, CVIET, and CV M/I N/E K/T, which were 87.50%, 9.62%, and 2.88%, respectively. For *Pfmdr1* gene, the NY (34.91%) and NF (43.40%) were the most popular haplotypes. For the *pfk13*, both the mutations detected in SEA [21] and novel mutations detected in Africa [9] were not found in the current study. These results deliver information on

the mutations of *Pfcr*t, *Pfmdr*1, and *pfk*13 genes of imported *P. falciparum* isolate in Wuhan and thus provide valuable information for the status of drug-resistant *P. falciparum* in China.

Africa and SEA were the primary sources of imported malaria in Wuhan [8]. According to this survey, among the three haplotypes of the *Pfcr*t gene, CVMNK, CVIET and CV M/I N/E K/T demonstrates that the pressure on CQ drugs decreased significantly after most countries in sub-Saharan Africa stopped using CQ in the 1990s [22–25]. Compared with the previous investigation of four haplotypes of *Pfcr*t [8], the prevalence rates of CVMNK, SVMNT and CVIET, and CV M/I N/E K/T were 50.57%, 1.14%, 25.00%, and 23.30%, respectively. The relative increase in CVMNK genotypes of *Pfcr*t was also reported in Senegal and southeastern Cameroon [26, 27], which confirmed that the proportion of CQ-susceptibility parasites in Africa is increasing after drug withdrawal, which was remarkable phenomenon, but it is still not recommended to quote CQ at this time. Current national use and regulation of antimalarial drugs should continue to be implemented to prevent the recurrence of CQR development.

Previously reported N86Y, Y184F allele mutations in *Pfmdr*1 are most prevalent in Asia and Africa [28]. In this study, we monitored the situation of N86Y and Y184F in Africa, accounting for 4.72% and 47.17%. Compared with the previous survey [8], the prevalence rates of N86Y (22.28%) and Y184F (60.01%), Y184F allele mutation still dominates. It has been confirmed that N86Y mutation was near related to CQR and AQ resistance [29, 30]. The prevalence rates of N86Y dropped significantly from 22.28% in the previous survey [8] in 2011–2016 to 4.72% in this survey from 2017 to 2019, indicating that susceptibility to CQ and AQ drugs was recovering. In recent years, several studies demonstrate the N86Y, Y184F, and D1246Y were closely related to artemether-lumefantrine (AL) resistance [31, 32]. The prevalence rates of Y184F increased from 22.28% in the previous survey in 2011–2016 to 47.17% in this survey from 2017 to 2019, which remained at a high level. Also, we found a non-synonymous mutation at position 199 and synonymous mutations, including 87 and 102.

For the *pfk*13, no mutation was found in this survey, which may be a worthy symbol that there was still a high level of sensitivity to ART in Africa, and drugs can continue to be used. In our previous study, a mutation of R561H was reported in Rwanda [8]. It has also been reported in another study [14]. Recently, it was identified the mutation has ART resistance [9]. All these pieces of evidence indicate that ART resistance of *P. falciparum* strain was independent produced in Africa and not spread from SEA. The phenomenon needs to pay more attention to China and Africa. If there are no effective measures to curb this situation, then the general resistance of CQ is likely to be the end of ART.

However, there are also several limitations to the present study. First, because of the COVID-19 outbreak in 2019, especially in these low-income tropical countries [33, 34], these may hinder malaria control efforts, leading to a shortage of data. Second, some of the data were missing because of the failure of amplification or sequencing.

5. Conclusions

All in all, the wild-type SNPs and haplotypes for the *pfcr*t and *pfmdr*1 genes of *P. falciparum* become predominant in the current study. It indicates that these isolates entirely or partly recovery their susceptibility to antimalarial drugs, including CQ, AQ, and MQ. Moreover, it demonstrates these drugs can continue to be effective drugs for *P. falciparum* malaria cases treatment in Africa. Although no mutation is detected in *pfk*13, cautions should be made to ART therapy for *P. falciparum* in Africa, continuous molecular surveillance is still urgently necessary.

Declarations

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

WJC and JL conceived and designed the study. KW coordinated the field collections of patient isolates. KW carried out a microscopic examination. WJC and XNS performed the experiments. WJC, HBT, and JL analyzed the data. WJC and JL wrote the paper. All the authors read and approved the final manuscript.

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Availability of data and material

The datasets analyzed in this study are available from the corresponding author on reasonable request.

Consent for publication

Not applicable

Ethics approval and consent to participate

The current study was approved by the ethics committees of the Hubei University of Medicine and Wuhan City Center for Disease Prevention and Control Ethics Committee. Informed consent was obtained from all participated individuals.

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Tables

Due to technical limitations, table 1,2,3 is only available as a download in the Supplemental Files section.

Figures

Figure 1

Sequence profile of PCR products of the new mutation site of *pfmdr1* genes.

Supplementary Files

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