

Assessment of Molecular Markers of Anti-malarial Drug Resistance in Pfk13-propeller, Pfprt and Pfmdr1 Genes in Plasmodium Falciparum Isolated From Patients Returning for Malaria Retreatment in Democratic Republic of Congo

Doudou Malekita Yobi (✉ doudou.yobi@unikin.ac.cd)

Université de Kinshasa, Faculté de Médecine, Département de Sciences de Base, Service de Biologie Moléculaire
<https://orcid.org/0000-0002-8703-1269>

Nadine K. Kayiba

School of Public Health, Faculty of Medecine, University of Kinshasa, Democratic Republic of Congo; School of Public Health and Research Institute of Health and Society, Catholic University of Louvain, 1200 Brussels, Belgium

Dieudonné M. Mvumbi

University of Kinshasa: Universite de Kinshasa

Raphael Boreux

Laboratory of Clinical Microbiology, University of Liege, 4000 Liege, Belgium

Pius Z. Kabututu

Department of Basic Sciences, Faculty of Medicine, University of Kinshasa, Kinshasa, Democratic Republic of Congo

Hippolyte NT. Situakibanza

Department of internal Medicine, Faculty of Medicine, University of Kinshasa, Kinshasa, Democratic Republic of Congo

Solange E. Umesumbu

National Malaria Control Programme, Kinshasa, Democratic Republic of Congo

Patrick De Mol

Laboratory of Clinical Microbiology, University of Liege, 4000 Liege, Belgium

Niko Speybroeck

School of Public Health and Research Institute of Health and Society, Catholic University of Louvain, 1200 Brussels, Belgium

Georges L. Mvumbi

Department of Basic Sciences, Faculty of Medicine, University of Kinshasa, Kinshasa, Democratic Republic of Congo

Marie-Pierre Hayette

Laboratory of Clinical Microbiology, University of Liege, 4000 Liege, Belgium

Research

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Abstract

Background

The Democratic Republic of Congo (DRC) malaria treatment policy recommends two first-line artemisinin-based combination therapies (ACTs) for the treatment of uncomplicated malaria: Artesunate-amodiaquine (ASAQ) and Artemether-lumefantrine (AL). This study investigated resistance to the ACTs currently in use in DRC through molecular markers in *pfk13*, *pfcr1* and *pfmdr1* genes in *Plasmodium falciparum* isolated from patients returning for malaria retreatment.

Methods

From November 2018 to November 2019, dried blood spots were taken from patients returning to health structures for fever within 28 days after an initial malaria treatment in 6 sentinel sites of National Malaria Control Programme (NMCP) across DRC. The new episode of malaria was first detected by a rapid diagnostic test (RDT) and then confirmed by a real-time PCR assay. Fragments of interest in *pfk13* and *pfcr1* genes were amplified by conventional PCR before sequencing and *Pfmdr1* gene copy number was determined by a TaqMan real-time PCR assay.

Results

Out of 474 enrolled patients, 364 (76.8%) were confirmed positive by PCR for *P. falciparum*. Of the 325 *P. falciparum* isolates successfully sequenced in *pfk13*-propeller gene, 7 (2.2%) carried non-synonymous (NS) mutations among which 3 previously reported (N498I, N554K and A557S) and 4 not yet reported (F506L, E507V, D516E and G538S). Of the 335 isolates successfully sequenced in *pfcr1* gene, 139 (41.5%) harbored the K76T mutation known to be associated with CQ resistance. The SVMNT haplotype associated with resistance to AQ has not been found. None of the isolates carried increased copy number of *pfmdr1* gene among the 322 *P. falciparum* isolates successfully analyzed.

Conclusion

No molecular marker known to date as associated with resistance to first-line ACTs in use was detected in *P. falciparum* isolated in patients returning for retreatment. Regular monitoring through *in vivo* drug efficacy and molecular studies must continue to ensure the effectiveness of the treatment of malaria in DRC.

Introduction

Plasmodium falciparum (*P. falciparum*) is the most widespread Plasmodium species and is responsible for most severe forms and deaths related to malaria in sub-Saharan Africa. *P. falciparum* has succeeded in developing resistance mechanisms against almost all existing antimalarial drugs, which is a major threat to malaria control worldwide. The high level of *P. falciparum* resistance to chloroquine (CQ) and then to sulfadoxine-pyrimethamine (SP) has led the Democratic Republic of Congo (DRC), like all endemic countries, to change its anti-malarial drug policy for the treatment of uncomplicated malaria. The DRC national policy currently supports two first-line artemisinin-based combinations therapies (ACT) for the treatment of uncomplicated *P. falciparum* malaria: artemether–lumefantrine (AL) and artesunate–amodiaquine (ASAQ). In case of confirmed treatment failure by microscopy to both first-line ACTs, the patient should be given dual therapy of quinine plus clindamycin or plus doxycycline [1]. The World Health Organization (WHO) recommends regular surveillance of ACT efficacy to provide an early warning against the emergence and spread of resistance [2]. Thanks to the discovery of several *P. falciparum* genes involved in anti-malarial drug resistance, molecular markers have become a precious tool in resistance surveillance. Numerous polymorphisms in the *P. falciparum* genome have been suggested to provide resistance to ACTs, both to the artemisinin and the associated drugs [3, 4]. Mutations in the propeller domain of *P. falciparum* Kelch 13 gene (*pfk13*) have been identified as associated with *in vivo* delayed parasite clearance

and *in vitro* artemisinin (ART) resistance in ring stage survival assay (RSA) [5, 6]. These mutations spread into the Greater Mekong Sub-region (GMS) of Southeast Asia and have been classified as validated markers and candidate/associated markers of ART resistance [7]. Some of these mutations are increasingly detected in some sub-Saharan African countries providing evidence for *de novo* emergence of resistance to artemisinin in Africa [8–10]. Mutations in the chloroquine resistance transporter gene (*pfcr1*) originally identified as a marker of CQ resistance, have also been associated with resistance to amodiaquine (AQ) [11]. The *pfcr1* gene is highly polymorphic in codon position 72–76 determining different haplotypes that include the key mutation K76T associated with CQ resistance while the SVMNT haplotype has been found associated with AQ resistance [12]. Additionally, increased copy number of the gene encoding the multidrug resistance 1 transporter (*pfmdr1*) has been postulated to confer resistance to lumefantrine (LU) [13].

Since it is not possible to distinguish routinely between recrudescence and reinfection in endemic areas, the absence of resolution of fever and parasitaemia or their recurrence within 28 days of treatment is considered treatment failure with currently recommended ACTs, while all recurrence of fever and parasitaemia after 28 days of an initial treatment should, from an operational standpoint, be considered as re-infections and treated with first-line ACT [14]. The distinction between recrudescence and re-infection may be confirmed only by genotyping. This concern is addressed by therapeutic efficacy studies according to WHO protocol [15]. Many factors can contribute to treatment failure, including resistance and inadequate exposure to drug due to sub-optimal dosing, poor patient compliance, poor drug quality, vomiting, poor drug absorption and drug interactions. The present study investigated the resistance to each associated drug in the two first-line ACTs currently in use in the DRC through molecular markers in *pfk13*, *pfcr1* and *pfmdr1* genes in case of malaria retreatment.

Methods

Study area

Six sentinel sites of National Malaria Control Programme (NMCP) were selected among 26 provinces of the DRC. The sites included 2 of the 3 largest cities of country and 4 other sites selected based on their epidemiological facies and their accessibility. Thus, the following sentinel sites were selected: Kingasani in Kinshasa and Kabondo in Kisangani for the largest cities; Bolenge in Equateur province and Vanga in Kwilu province for the equatorial facies; Fungurume in Lualaba province for the tropical facies and Katana in Sud-Kivu province for the mountainous facies. In each study site, 1 to 3 health structures were selected based on the accessibility and the attendance for the realization of the study.

Study participants

From November 2018 to November 2019, patients of all ages who returned to health structures for fever within 28 days from an ACT-based treatment for an initial episode of malaria that had to be documented in the patient's medical record and who had, during investigation, a positive rapid diagnostic test (RDT) for malaria were enrolled after that informed consent was given. The ACT which was used to treat the initial episode depended on the ACT available on each study site during the investigation.

Blood sample collection

Screening tests were performed on blood samples taken by finger prick. Malaria was detected using RDTs available on site: SD Bioline Malaria Ag Pf (Standard Diagnostics) or CareStart Malaria Pf (Access Bio). After enrollment, a blood sample was taken by finger prick and three spots were deposited on Whatman Grade GB003 filter paper (Whatman, GE Healthcare). Dried blood spots (DBS) were placed in an individual ziploc plastic bag containing silica gel desiccant and were then stored at – 20° before molecular analysis.

DNA Extraction

DNA was extracted from blood spots using the QIAamp DNA Mini Kit (Qiagen, Germany) following the manufacturer's recommended protocol for DBS. The extracted DNA was stored at - 20 °C before PCR testing.

Plasmodium falciparum **real-time PCR**

A real-time PCR for the detection of *P. falciparum* was performed according to a previously described procedure [16]. Assays were run on an ABI 7500 Fast real-time thermocycler (Applied Biosystems) at the Laboratory of Molecular biology of school of Medicine, University of Kinshasa, DRC.

pfk13 **PCR**

The target sequence was a 506-nucleotide fragment of the pfk13-propeller gene containing codons 427–595, as previously described [17]. The interest segment included recently described mutations associated with ART resistance [7]. PCR was run using a conventional thermal cycler Hybaid HBPXE 0.2 (Thermo Scientific) at Laboratory of Molecular biology of school of Medicine, University of Kinshasa, DRC.

pfcr1 **PCR**

The fragment of interest (containing codons 72–76) on the *pfcr1* gene was amplified following a previously described procedure [18]. The PCR was run on the conventional Thermal Cycler cited above.

Estimation of pfmdr1 copy number by real-time PCR

Pfmdr1 copy number was assessed by a relative quantification real-time PCR using ABI 7500 thermocycler (Applied Biosystems, Warrington, UK) as previously described [19]. In each run of real-time PCR, 3D7 and Dd2 clones were used as calibration controls with single and multiple copies of *pfmdr1* gene, respectively 1 copy and 4 copies, and negative control containing no template DNA was also included. Test samples were assayed in triplicate, the copy number of *pfmdr1* was determined using the comparative $\Delta\Delta C_t$ method and calculated as $2^{-\Delta\Delta C_t}$. Samples with a spread of $\Delta\Delta C_t$ among the three triplicates of more than 1.5 or with a $C_t > 35$ were repeated and the second result used. Analyses were performed at Laboratory of Clinical Microbiology of University of Liege, Belgium.

Pfcr1 and pfk13 **genotyping**

After purification using AMPure XP magnetic beads (Beckman Coulter, CA, US), the PCR products were added to a mix of Big Dye Terminator V3.1 for the sequencing reaction. The resulting nucleotide sequences were analyzed on an ABI 3730 DNA Analyzer automated sequencer (Applied Biosystems) using the Sanger method at GIGA (University of Liège's Interdisciplinary Research Institute in the Biomedical Sciences). Sequences (forward and reverse) were aligned using Vector NTI (Thermo Fisher Scientific, US) and compared to the reference sequence PF3D7_0709000 (https://www.ncbi.nlm.nih.gov/gene/?term=PF3D7_0709000 accessed on March 2020) for *pfcr1* and PF3D7_1343700 (<http://www.plasmodb.org>, accessed on March 2020) for *pfk13* using the online Basic Local Alignment Search Tool (BLAST) (National Center for Biotechnology Information-NCBI) for identifying mutations.

Statistical analysis

Data were entered in a 2010 Excel sheet by an independent data clerk. Statistical analysis was performed using SPSS V. 20.0 (IBM corp, Armonk, NY). Samples for which genotype profile could not be determined were excluded from the analysis. Categorical variables were expressed as frequencies with 95% confidence intervals (95% CI) while quantitative variables as median with interquartile range (IQR). The mutant and wild-type alleles identified in the sequenced isolates were used to generate the prevalence of the alleles.

Results

In total, 474 patients were enrolled in the study, their age ranged from 0 to 76 years with a median age of 10 years (IQR: 4–21 years). The male to female sex ratio was 0.74.

Real-time PCR analysis of DNA extracted from DBS samples confirmed the initial diagnosis of *P. falciparum* infection for 364 (76.8%; 95% CI: 72.7% – 80.5%) patients.

Pfcr1 72–76 haplotype

Of the 364 *P. falciparum* isolates detected by real-time PCR, 335 were successfully sequenced among which 139 (41.5%; 95% CI: 36.2% – 47.0%) harbored the K76T mutation known to confer resistance to CQ. The CVIET haplotype was found in 98 (70.5%) isolates harboring K76T mutation followed by CVIKT (20.1%), CVINT (5.8%) and CVMNT (3.6%). The SVMNT haplotype associated with resistance to AQ has not been detected.

Pfk13 -propeller polymorphism

Of the 325 successfully sequenced *P. falciparum* isolates, 318 (97.8%; 95% CI: 95.6% – 99.1%) were wild-type and 7 (2.2%; 95% CI: 0.9% – 4.4%) and carried non-synonymous (NS) mutations in *pfk13*-propeller among which 3 previously described (N498I, N554K and A557S) and 4 not yet report described (F506L, E507V, D516E and G538S). None of validated and candidate mutations associated with ART resistance was found by this study. The N498I, D516E, G538S mutations were found each in isolates harboring also the K76T mutation. Table 1 shows the distribution of polymorphisms in *pfk13*-propeller gene and the key mutation K76T in *pfcr1* gene among enrolled patients per site.

Table 1
Distribution of *pfk13*-polymorphisms and *pfprt*-K76T mutation per site.

| Site | Enrolled patient N | Positive PfPCR N (%) | <i>Pfprt</i> gene | | <i>pfK13</i> gene | | | | NS mutation |
|-----------|-----------------------|-------------------------|--------------------------------|-----------|-----------------------|--------------------------------|----------------|----------------------------|--------------|
| | | | Pf successfully sequenced N | K76T N | K76T % (95% CI) | Pf successfully sequenced N | Mutant Pf N | mutant Pf % (95% CI) | |
| Bolenge | 88 | 70 (79.5) | 62 | 21 | 33.9 (22.3–47.0) | 60 | 2 | 3.3 (0.4–11.5) | F506L, N554K |
| Fungurume | 83 | 63 (75.9) | 60 | 6 | 10.0 (3.8–20.5) | 58 | 1 | 1.7 (0.0–9.2) | A557S |
| Kabondo | 20 | 17 (85.0) | 16 | 3 | 18.8 (4.0–45.6) | 17 | 0 | 0.0 (0.0–16.2) | - |
| Katana | 143 | 95 (66.4) | 91 | 70 | 76.9 (66.9–85.1) | 86 | 2 | 2.3 (0.3–8.1) | N498I, D516E |
| Kingasani | 113 | 92 (81.4) | 81 | 27 | 33.3 (23.2–44.7) | 80 | 2 | 2.5 (0.3–8.7) | E507V, G538S |
| Vanga | 27 | 27 (100.0) | 25 | 12 | 48.0 (27.8–68.7) | 24 | 0 | 0.0 (0.0–11.7) | - |
| Total | 474 | 364 (76.8) | 335 | 139 | 41.5 (36.2–47.0) | 325 | 7 | 2.2 (0.9–4.4) | |

N = number; PfPCR = *Plasmodium falciparum* Polymerase chain reaction; NS: non-synonymous.

The treatment of initial episode of malaria was AL, ASAQ and Dihydroartemisinin-piperaquine (DP) used respectively in 273 (57.6%), 196 (41.3%) and 5 (1.1%) patients, as shown in Table 2. Globally, among samples successfully sequenced, 65/133 (48.9%) of the K76T mutations were found in patients treated with ASAQ while 74/198 (37.4%) in patients treated with AL (p-value = 0.038).

Table 2
 Repartition of patients per ACT treatment of the initial episode of malaria per site

| Site | Total enrolled patients N | ALU N (%) | ASAQ N (%) | DP N (%) |
|-----------|------------------------------|--------------|---------------|-------------|
| Bolenge | 88 | 88 (100) | 0 (0.0) | 0 (0.0) |
| Fungurume | 83 | 40 (48.2) | 43 (51.8) | 0 (0.0) |
| Kabondo | 20 | 16 (80.0) | 4 (20.0) | 0 (0.0) |
| Katana | 143 | 30 (21.0) | 113 (79.0) | 0 (0.0) |
| Kingasani | 113 | 84 (74.3) | 24 (21.2) | 5 (4.4) |
| Vanga | 27 | 15 (55.6) | 12 (12) | 0 (0.0) |
| Total | 474 | 273 (57.6) | 196 (41.3) | 5 (1.1) |

ALU: Artemether-lumefantrine, ASAQ: Artesunate-amodiaquine, DP: Dihydroartemisinin piperaquine.

Pfmdr1 copy number

In total, 322 *P. falciparum* isolates were successfully analyzed for copy number variation in the *pfmdr1* gene. Using a copy number threshold of 1.5 to define multiple copies, all isolates harbored single copy of *pfmdr1* gene.

Discussion

The present study has not detected molecular markers associated with resistance to first-lines ACT in use in the DRC in patients returning to health facilities for malaria retreatment.

Although none of the mutations associated with ART resistance in Southeast Asia has been found by the study, 7 coding substitutions that are of unknown phenotype were observed, among which 3 previously reported notably N498I in Kenya [20], N554K in Comoros and A557S in Togo and DRC [21] and 4 others not yet reported (F506L, E507V, D516E, G538S). Numerous *pfk13*-propeller mutations of unknown function are commonly reported in the Sub-Saharan African countries like in the DRC [17, 21, 22]. There are criteria for prioritizing further laboratory studies notably the frequent observation of a new allele with a non-synonymous mutation, the evidence of dissemination and preliminary association with clinical data whenever possible [22]. Several independent single nucleotide polymorphisms (SNPs) could be responsible of the ART-R in Sub-Saharan Africa, the known mutations that confer drug resistance would differ from one location to another, depending on the parasite genetics. There is the possibility that *pfk13* mutations do not cause ART-R in isolation but would act in combination with other genetic or non-genetic factors that are different in African and Southeast Asian parasite populations [23, 24]. Since African *pfk13*-propeller mutations were shown to be different from those found in Southeast Asia, further molecular and biochemical studies should investigate whether other factors such as additional mutations could be associated to alter the functions of PFK13 protein, resulting in altered ART sensibility. However, some of the validated and candidate mutations associated with ART resistance in Southeast Asia have been detected in some neighbouring countries such as in Rwanda [8, 9] and in Uganda [10], providing evidence of de novo emergence of ART resistance in Sub-Saharan Africa. Thus, surveillance must be strengthened to avoid the worst.

The global prevalence of the K76T mutation known to be associated with CQ resistance was 41.5%, but it remained variable from one site to another, ranging from 10.0% in Fungurume to 76.9% in Katana. In 2017, a study conducted in 10 sites including 6 sites of the present study reported a global prevalence of K76T mutation of 28.5% but with a high between regions variability ranging from 1.5% in Fungurume to 89.5% in Katana [16]. In the present study, the prevalence of K76T in patients treated with ASAQ (48.9%) was higher than in those treated with AL (37.4%) with low statistical

difference (p-value = 0.038). The possibility that AQ would continue to contribute to the selection for the K76T mutant even after discontinuance of CQ usage has been previously raised [25]. The simultaneous presence of very low and high prevalence of CQ resistance could be related to between-regions difference of CQ pressure and also to effect of selection for CQ resistance depending on the genetic structure of parasite populations which have been shown to vary significantly across the country [26]. Data concerning current CQ use in the country are not available, further studies at the community level and parasite genetic studies should be conducted to explain the persistence of high CQ resistance rate in some provinces despite the withdrawal of this molecule from the national policy of malaria treatment. The SVMNT haplotype associated with AQ resistance was not detected in the present study, which is encouraging for the DRC national policy for the continued use of AQ in ACT. This haplotype has not yet been reported in the DRC [16, 18, 27, 28] whereas it was found in neighbouring countries such as Tanzania and Angola [29, 30].

None of the *P. falciparum* isolates had multiple copies number of *pfmdr1* gene in this study, consistent with the general absence [31, 32] or low frequencies [28, 33, 34] of *pfmdr1* copy number variation in *P. falciparum* from sub-Saharan Africa. Multiple copy number of *pfmdr1* gene has been postulated to confer resistance to LU [13], which is the associated drug in AL, one of the ACTs used in the DRC.

The ACT used to treat the initial episode was that available on each study site during investigation and best tolerated by the patient. In practice, AL tends to be primarily used in urban areas because patients have more options to obtain it from private pharmacies, while ASAQ is used in rural areas [1]. The study assessed molecular markers of anti-malarial resistance in patients who returned to health structures for fever within 28 days of the ACT treatment, which is considered as treatment failure [14]. Treatment failure is the inability to clear parasites from a patient's blood or to prevent their recrudescence after the administration of an anti-malarial drug. When possible, treatment failure must be confirmed by microscopy, as histidin rich protein-2 (HRP-2)-based RDT may remain positive for weeks after successful treatment due to persistent antigenemia, even without recrudescence [35, 36]. In this study, *P. falciparum* real-time PCR assay was used afterwards to confirm *P. falciparum* malaria in patients returning to health structures for fever within 28 days of an initial malaria treatment.

The study contributes to the ongoing surveillance of the resistance to anti-malarial drugs in use in the DRC as recommended to track the emergence and spread of *P. falciparum* resistance to different molecules used in malaria management. However, although the study was carried out in six provinces of the DRC with varied geography and malaria endemicity, the results were not representative of either any one province or the entire country.

Conclusion

No molecular marker known to date as associated with resistance to first-line ACTs components in use in the DRC was detected in *P. falciparum* isolated from patients returning for malaria retreatment. The findings are encouraging for the current DRC malaria treatment policy, however, the appearance of coding substitutions that are of unknown functions calls for further investigations and other factors, apart from the resistance, that contribute to treatment failure should be assessed in order to ensure the effectiveness of the treatment of malaria in the DRC.

Abbreviations

WHO

World Health Organization; DRC:Democratic Republic of Congo; *pfcr1*:*Plasmodium falciparum* chloroquine resistance transporter; CQ:Chloroquine; AQ:Amodiaquine; LU:Lumefantrine; ACT:Artemisinin-based combination therapy; DNA:Deoxyribonucleic acid; *pfcr1*:*Plasmodium falciparum* chloroquine resistance transporter; *pfmdr1*:*Plasmodium falciparum* multidrug resistance 1; *pfk13*:*Plasmodium falciparum* kelch 13; PCR:Polymerase chain reaction; RDT:Rapid

diagnostic test; SP:sulfadoxine-pyrimethamine; SNP:single nucleotide polymorphisms; DP:Dihydroartemisinin piperaquine; ASAQ:Artesunate-Amodiaquine; AL:Arthemether-Lumefantrine.

Declarations

Ethics approval and consent to participate

The protocol and the informed consent form were approved by the Ethics Committee of the Faculty of Medicine, University of Kinshasa (Approval N°: ESP/CE/111/2018). All participants involved in the study signed an informed consent form. Where participants were young (children), the consent form was approved and signed by their parents or guardians.

Consent for publication

Not applicable.

Availability of data and materials

All data generated and analysed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

DMY, NKK, DMM, PDM, GLM and MPH conceived and wrote the study protocol. DMY and NKK collected the samples and data. DMY and RB carried out the molecular analyses. DMY, DMM, RB and MPH analysed molecular data. DMY wrote the first draft of the manuscript and all the authors were involved in editing and approval of the final manuscript.

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