RESEARCH ARTICLE

Molecular surveillance of *Plasmodium falciparum* resistance to artemisinin-based combination therapies in the Democratic Republic of Congo

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Abstract

Malaria is a major public health problem in the Democratic Republic of Congo. Despite progress achieved over the past decade in the fight against malaria, further efforts have to be done such as in the surveillance and the containment of *Plasmodium falciparum* resistant strains. We investigated resistance to artemisinin-based combination therapies currently in use in Democratic Republic of Congo by surveying molecular polymorphisms in three genes: pfcrt, pfmdr1 and pfk13 to explore possible emergence of amodiaquine, lumefantrine or artemisinin resistance in Democratic Republic of Congo. This study essentially revealed that resistance to chloroquine is still decreasing while polymorphism related to amodiaquine resistance seems to be not present in Democratic Republic of Congo, that three samples, located in the east of the country, harbor Pfmdr1 amplification and that none of the mutations found in South-East Asia correlated with artemisinine resistance have been found in Democratic Republic of Congo. But new mutations have been identified, especially the M476K, occurred in the same position that the M476I previously identified in the F32-ART strain, strongly resistant to artemisinine. Antimalarial first-line treatments currently in use in Democratic Republic of Congo are not associated with emergence of molecular markers of resistance.

Introduction

In the Democratic Republic of Congo (DRC) malaria is still a major public health problem. Interventions conducted the last ten years led to reduce malaria related morbidity-mortality

[1], but further efforts have to be done to reach the aim fixed by the World Health Organization (WHO) to reduce malaria mortality and incidence by 90% in 2030 [2].

Efforts must be strengthened in many topics, such as surveillance and containment of *Plasmodium falciparum* resistant strains. Resistance, that could be defined as the ability of a parasite strain to survive or multiply in the presence of drug concentrations that normally kill parasites of the same species or prevent their multiplication [3], is a phenomenon that has always existed since drugs started to exert pressure phenomenon. All drugs submitted to the malaria parasite became, some years after their introduction, ineffective [4]. In response to it, WHO experts have recommended to use combination therapies including an artemisinin component instead of using monotherapies [5]. Currently, all endemic countries have officially adopted artemisinin-based combination therapies (ACT) as first-line treatment for non-severe malaria.

In DRC, two ACTs have been accepted as first-line treatment by the National Malaria Control Program (NMCP): Artesunate-Amodiaquine in 2005 followed by Artemether-Lumefantrine in 2010. Both combinations are simultaneously in use in the country.

Resistance to previous antimalarials has led to an increase of malaria mortality from 1980 to 2004 [6], an increase of the global cost for disease control [7, 8], and an increase of transmission [9].It is clear that the spread of artemisinin resistance from Asia to Africa would seriously threaten malaria control [10]. This resistance has been clearly established in South-East Asia but currently, no report of artemisinin resistance has been described in Africa [11].

Monitoring parasite resistance to antimalarial drugs is mandatory in malaria control strategies. Molecular markers are a good alternative to *in vivo* treatment trials or *in vitro* drug susceptibility testing to detect drug-resistant strains as they allow analyzing large sample and assessing resistance to many antimalarial drugs simultaneously [12]. Unfortunately, molecular studies require expensive infrastructures and reagents and also qualified persons that are not always available in sub-Saharan Africa. This can explain the small number of studies conducted in DRC [13].

Some molecular markers associated with malaria resistance have been clearly described. The K76T mutation occurring on the *Plasmodium falciparum* chloroquine-resistance transporter (*pfcrt*) gene is the key element of chloroquine resistance [14] and the SVMNT haplotype, defined by specific mutations at amino acid positions 72–76, found in the same gene has been clearly linked to amodiaquine resistance [15]. Moreover, Ariey et al. provided the link between mutations in the propeller region of the *Kelch* 13 gene (K13, PF3D7_1343700) and artemisinin resistance [16]. Mutations on the *Plasmodium falciparum* multidrug resistance 1 (*pfmdr1*) gene and increase of its copy number have been related to resistance to many monotherapies [17–20] but also linked to ACT introduction [21, 22].

In previous studies, we have detected a high prevalence of *pfcrt* mutants in Kinshasa [23] so in the present work we have extended the analysis to five other geographic areas in DRC. We investigated resistance to ACT currently in use in DRC by surveying molecular polymorphisms in three genes: *pfcrt*, *pfmdr1* and *pfk13* to explore possible emergence of amodiaquine, lumefantrine or artemisinin resistance in DRC.

Materials and methods

Ethical considerations

The protocol and the informed consent received the ethical approbation from the Ministry of Public Health of the DRC and from the Institutional Committee of the Faculty of Medicine, University of Kinshasa. All the participants involved in the study (or the parents/guardians of children) provided a written consent.

Study sites and participants

We conducted this study in six areas with different dynamics of transmission: Bolenge, Luzizila and Mweka in the equatorial facies; Punia and Kapolowe in the tropical facies and Butembo in the mountain facies. Malaria transmission is perennial in the equatorial and tropical facies but seasonal in the mountain one. In each site, one hundred individual has been randomly selected in a household survey (except for Punia where only eighty individuals could be selected). The survey was conducted between March and November 2014.

Blood collection and parasite identification

For each individual, blood samples were collected from finger prick and DNA was extracted by using the QiaAmp® DNA mini kit (Qiagen, Hilden, Germany) as previously described [23].

One real-time PCR consisting in two duplex reactions (identifying *P. falciparum* + *P. ovale* and *P. malariae* + *P. vivax*) was run to detect *Plasmodium* species [24] on a Lightcycler 480 instruments (Roche®) in the Clinical microbiology Unit of the University Hospital of Liege, Belgium. *P. falciparum* positive samples were stored at −20˚C for further molecular analysis.

Assessing *Pfmdr1* copy number

A relative quantification multiplex real-time PCR was run by using a couple of primers plus a FAM-labelled probe for *pfmdr1* detection and another couple of primers with a VIC-labeled probe for β-tubullin detection [25]. Assays were run on a Lightcycler 480 instrument (Roche®) in the presence of one single copy control reference strain 3D7. The results were analyzed by the comparative ΔΔCt method. Parasites were considered to have an amplified *pfmdr1* gene if copy number was > 1.5 [26].

Assessing *Pfcrt* 72–76 haplotypes

A conventional PCR was run to amplify an approximately 152 pb fragment on the *pfcrt* gene containing the region of interest followed by sequencing of amplicons, as described in a previous work [23]. Both 3D7 and K1 reference strains, respectively sensitive and resistant to chloroquine, were used as control during the assays.

Assessing polymorphisms on the K13 propeller gene

Another conventional PCR was run by using primers recently described by Ariey et al. [16] to amplify a fragment including positions where mutations related to artemisinin resistance were found. All PCR assays were run in the presence of the F32-ART reference strain provided by Centre National de la Recherche Scientifique—CNRS, France. All amplicons were purified on a Sciclone G3 Automated Liquid Handling Workstation (Perkin Elmer, USA) using AgencourtCleanSEQ® kit (Agencourt Bioscience, USA) and then sequenced jointly in the GIGA centre of University of Liege, Belgium and in the Molecular biology platform of the University Hospital of Liege, Belgium using a 3130xl DNA sequencer (Applied Biosystems, USA).

The K13-propeller SNPs were analyzed by comparing with the reference 3D7 strain (PF3D7_1343700) using Sequencher® Software Ver. 5.4.5 (Gene Codes corporation, Michigan, USA) and the online BLASTx tool (National Center for Biotechnology Information, USA).

Table 1. Analysis of molecular markers related to Pf resistance in DRC.

Mol. marker	Status	Bolenge N	Bolenge N (%)	Mweka N	Mweka N (%)	Kapolowe N	Kapolowe N (%)	Luzizila N	Luzizila N (%)	Butembo N	Butembo N (%)	Punia N	Punia N (%)
pfcrt		51		31		63		62		22		51	
	W		15 (29.4)		10 (32.3)		22 (34.9)		24 (38.7)		6 (27.3)		24 (47.1)
	M		36 (70.6)		21 (67.7)		41 (65.1)		38 (61.3)		16 (72.7)		27 (52.9)
*pfmdr1**		51		31		63		62		22		51	
	W		51 (100.0)		31 (100.0)		63 (100.0)		62 (100.0)		19 (86.4)		51 (100.0)
	M		0 (0.0)		0 (0.0)		0 (0.0)		0 (0.0)		3 (13.6)		0 (0.0)
K13		51		31		63		62		22		51	
	W		50 (98.1)		29 (93.5)		61 (93.7)		59 (95.2)		21 (95.4)		51 (100.0)
	M		1 (1.9)		2 (6.5)		2 (3.2)		3 (4.8)		1 (4.5)		0 (0.0)

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Results

Out of the 580 samples collected over the six geographic sites, 280 (48.2%) were PCR-positive to *P. falciparum*, among which 6 (2.14%) were mixed infections (combined only with *P. malariae*). Distribution of these prevalences by area and by age group has been described in a previous published study [27]. All results obtained for these molecular markers are presented in Table 1.

Pfmdr1 copy number

Pfmdr1 analysis was successful for all *P. falciparum* positive samples. Only three samples (1.07%), all found in Butembo (Nord-Kivu province) have a copy number amplification beyond 1.5.

Pfcrt haplotype

One hundred and seventy-nine samples (63.9%) harbored the 76T mutation among which two had the relatively rare CVMNT haplotype (in Mweka and Kapolowe) and the rest got the CVIET one.

Pfk13 propeller polymorphism

On the 280 samples analyzed, sequencing was correctly done for 261 samples. We identified 9 samples (3.4%) with mutations in the propeller domain of the K13 among which 3 mutations previously described (F495L, S522C and V520A) and 3 new mutations (M476K, N523T and E509D)(Fig 1).

Discussion

Since the Congolese NMCP introduced ACT as first-line treatment in 2005, whatever some local studies have assessed ACT efficacy, only one national survey, conducted by the NMCP, has been performed to assess malaria first-line treatment efficacy. But results of this study have not yet been published.

In this study, we reported only 1% (3/280) of the samples that have increased *pfmdr1* copy number and all of these samples are from Butembo, in the east of the country. This is the first time that amplification of *pfmdr1* gene copy number is assessed in DRC. Our results are similar to those reported from African samples by Uhlemann et al. in Gabon [28], Ngalah et al. in Kenya [22] or Gadalla et al. [29] in Sudan where low prevalence (< 10%) of *pfmdr1 gene* copy
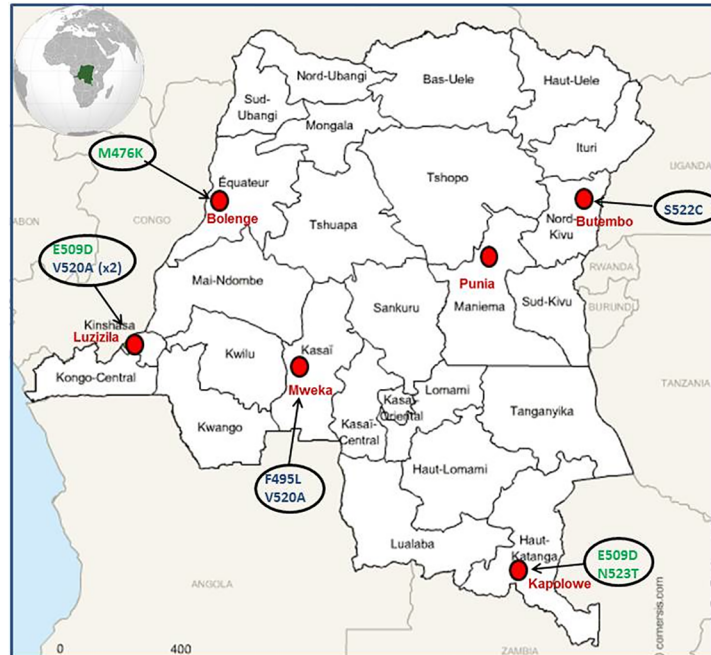
Fig 1. Distribution of mutations found in the k13 gene across DRC. New mutations are in green and already described mutations are presented in blue.

number was detected. That seems to be different than in South-east Asia where amplification of *Pfmdr1* copy number is frequently high [21, 26, 30]. Duah et al. have incriminated the use of ACT in the occurrence of *P. falciparum* strains with *pfmdr1* amplification in African samples [31]. One other reason to explain this difference could be that mefloquine, a drug that selects lines with increased *pfmdr1* copy number [9], was widely used in Asia both in monotherapy and as partner drug in ACT, comparatively than in Africa.

Increased *pfmdr1* copy number has been linked with treatment failure (or reduced sensitivity) to some ACT [18, 21, 22, 29, 32]. The evidence for the first time of this increased *pfmdr1* copy number in the East of the DRC emphasizes the importance of maintaining regular monitoring.

As previously observed for Sulfadoxine-Pyrimethamine or chloroquine, *pfmdr1* resistance-based is maybe emerging in the East of the country before to expand to the rest of the country. Before the present work, only one study assessed *pfmdr1* polymorphism in DRC but by exploring the N86Y mutation [33].

Despite the fact that prevalence of the K76T mutation (related to chloroquine-resistance) is still in a high level in our results (65.7%) and that a cross resistance has been described between chloroquine and amodiaquine [34], the SVMNT haplotype linked to *in vivo* and *in vitro* amodiaquine resistance [35], has not been found in our samples. Currently, this haplotype has been reported to be present in only five African countries, including two bordering the DRC (Angola and Tanzania) [36–40]. It seems that this haplotype is quite rare in Africa compared to Asia. But continuous use of the artesunate-amodiaquine combination in DRC as first-line treatment could make selection for this resistant strain in the future.

After analysis of the *pfk13* gene, six different mutations in the propeller domain were detected into nine samples (3.2%). Two of these mutations have been previously described in other African countries. The S522C mutation was isolated in the present study in a sample

from Butembo and has been previously reported in Uganda [41], which is relatively close to Butembo (~80 Km). Due to the important trade and the frequent movement of population between both countries around this geographic area, we can hypothesize that this mutant has disseminated, but it's not excluded that this mutant has independently emerged in DRC.

On the other hand, we found the V520A mutation in two sites relatively distant (Luzizila and Mweka). Our results are similar to those reported by Taylor et al. who found this mutation in DRC too, in the same geographic area than us. In the results provided by Taylor et al. in 2015, this mutation appears to be the most common in African parasites as it has been reported in several African countries (Gambia, Mali, Ghana, Burkina-Faso, Kenya, Tanzania, Malawi and DRC) [41]. One must ask if this mutation has spread out from one point or if it has appeared spontaneously in many areas. Further phylogenetic analysis could put light on that. However, a recent published multi-countries study has not found this mutation but has reported the A578S mutation as the most prevalent in Africa [42]. We have not either found this mutation in our samples.

Surprisingly, the F495L mutation that we found in Mweka was described for the first time in samples from the China Myanmar border [43] then in Mayotte [44].

We also found three undescribed mutations on the *pfk13* gene (M476K, E509D and N523T). The M476K mutation could be of a particular interest because one mutation on this position, the M476I, that appeared *in vitro* after artemisinin pressure in an African line from Tanzania (F32-ART) [16, 45]. This M476I mutation was also largely found in isolates from Myanmar [46]. Unfortunately, we cannot presently define what are the clinical implications of this new M476K mutation.

The newly reported E509D and N523T mutations are not related or close to resistant polymorphisms described in Asia.

None of the mutations clearly correlated to increased parasite clearance time in Asian samples have been found in Africa yet.

Conclusion

The data reported in this study reports the occurrence of some polymorphisms onto *P. falciparum* genes related to drug resistance. None of the mutations clearly associated to ACT resistance have been found in this study.

As in the rest of Africa, resistance to artemisinin seems not to be yet present there. DRC is one of the rare countries that have officially adopted multiple first-line treatment in its policy. This could provide a protective effect to the emergence of resistant strains but discovery of new mutations on the *pfk13* gene highlights the importance of a continuous monitoring. We unfortunately have not assessed in vitro susceptibility of the isolates with these new mutations.

Supporting information

S1 Table. Basic data related to individuals with *P. falciparum* positive samples.
(DOCX)

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Author Contributions

Conceptualization: MPH DMM.

Funding acquisition: PDM GLM.

Investigation: DMM TLB.

Methodology: JMNK HNTS.

Resources: PM GLM.

Validation: MPH FBV.

Writing – original draft: DMM.

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