Kelch 13 propeller gene polymorphism among *Plasmodium falciparum* isolates in Lagos, Nigeria: Molecular Epidemiologic Study

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Abstract

OBJECTIVE To assess polymorphism in Kelch 13 gene of *Plasmodium falciparum* isolates in Lagos, Nigeria.

METHODS 195 *Plasmodium falciparum*-positive dried blood spots collected from individuals that accessed diagnostic care at some health facilities and during community surveys across several Local Government Areas of Lagos State, Nigeria, were investigated for the presence of mutations in the K13 gene by nested polymerase chain reaction (PCR) using haplotype-specific probes and sequencing.

RESULTS Three mutant genotypes of K13 gene were observed: A578S in 0.5%, D464N in 0.5% and Q613H in 1.5%. The frequency of K13 polymorphism was 3.1%, while the remaining parasite population had the wild K13 propeller genes.

CONCLUSION No validated Kelch 13 polymorphism associated with artemisinin resistance was seen among *P. falciparum* isolates from Lagos, Nigeria. As no clinical study was done, this could not be correlated with artemisinin sensitivity.

keywords artemisinin, *Plasmodium falciparum*, resistance, *Kelch* 13 gene

Introduction

Malaria case management currently relies on the use of artemisinin combination therapy (ACT). However, emergence of artemisinin-resistant *Plasmodium falciparum* (*Pf*) isolates in Greater Mekong Sub-region [1,2] which clinically manifested as delayed clearance of parasiteamia after treatment with artemisinin derivatives now poses a threat to the global malaria elimination goal. This makes it imperative for the continual monitoring of antimalarial resistant genes in order to promptly respond to emerging resistance. Several validated polymorphisms of Kelch 13 genes are associated with artemisinin resistance, although reports of African parasite populations that possess these resistance genotypes are scarce.

Nigeria has the highest malaria burden on the African continent, as about 29% of global estimated malaria cases in 2015 occurred there [3]. As Nigeria has many travellers especially to Asian countries, there is a threat of possible dissemination of artemisinin-resistant parasites from Western Cambodia to the Greater Mekong Sub-region and to Africa, or of independent emergence of artemisinin-resistant strains in Africa, as happened previously with chloroquine and sulphadoxine/pyrimethamine-resistant parasites. Thus, any potential impact of artemisinin resistance on the ongoing control programme that relies on artemisinin-based combination therapies would have catastrophic consequences on the global malaria elimination target. Surveillance of resistance marker genes is very important so as to promptly detect/identify and respond to emerging resistance.

ACT was introduced in 2005 in Nigeria with artemether–lumefantrine (AL) as first-line treatment for uncomplicated malaria and Artesunate + Amodiaquine as the alternative [4]. Artemisinin derivatives (artesunate, artemether and dihydroartemisinin (DHA)) are fast-acting, well-tolerated drugs that are often paired with longer-acting partner drugs ACTs. The short half-life (typically <1 h) of ART derivatives in plasma necessitates the use of longer-lasting partner drugs that can eliminate residual parasites once the ART component has dropped to subtherapeutic concentrations [5].

The Kelch 13 (K13) gene belongs to the kelch superfamily of proteins that harbours multiple protein–protein

interaction sites and mediates different cellular functions, including ubiquitin-regulated protein degradation and oxidative stress responses [6]. This gene resides on chromosome 13 of the *P. falciparum* genome, near regions earlier associated with slow parasite clearance rates [7–10]. It is usually produced by all stages of *P. falciparum* [asexual (trophozoites and schizonts) and sexual blood stages (gametocytes)]. Mutation at certain codons on K13 gene is associated with *in vitro* artemisinin resistance and *in vivo* slow clearance of parasiteamia and therefore used as a marker of artemisinin resistance [11].

Mutation alters the amino acid sequence of the transporter genes and results in changing their physiochemical properties and functional character. Each amino acid has certain distinguishing properties that determine its impact on its mechanism of action, and these changes are associated with antimalarial drug resistance.

Genomic analysis of *Plasmodium falciparum* isolates from Cambodia identified four K13-gene mutations (Y493H, R539T, I543T and C580Y) that were associated with elevated ring stage assay (RSA) 0–3h survival rates *in vitro* and delayed clearance of parasiteamia (>5 h) in patients [11–13]. These K13 gene polymorphisms are used as predictors of artemisinin resistance.

WHO classified artemisinin resistance into (i) suspected artemisinin resistance, defined as high prevalence of the delayed parasite clearance phenotype or high prevalence of K13 mutants; and (ii) confirmed artemisinin resistance, defined as the combination of delayed parasite clearance and presence of K13 gene resistance-validated mutations in the same patient [14]. K13 gene mutations that have been validated to be associated with artemisinin resistance are N458Y, Y493H, R539T, I543T, R561H and C580Y [14]. Effects of partner drugs, immunity, insufficient levels of drug in the blood and non-validated K13 mutations could be confounding factors in these definitions.

Polymorphisms in the K13 gene have been identified from different countries [11,15–18], but in Nigeria information on either polymorphism in the K13 gene or resistance of *P. falciparum* to artemisinin is scarce. Previous studies showed that chloroquine and sulphadoxine/pyrimethamine-resistant parasites emerged from the Thai-Cambodia border and consequently spread to sub-Saharan Africa across Asia [19,20]. Surveillance of the resistance marker genes to artemisinin is imperative especially in a megacity such as Lagos, Nigeria with migrants from parts of the world where artemisinin resistance has been reported. Surveillance on artemisinin resistance in Nigeria is an important global health issue that could prompt actions to deter resistance and will assist in promptly informing treatment policy. WHO recommends monitoring the efficacy of first-line and second-line ACTs every 2 years in all falciparum-endemic countries. The aim of this study was to determine K13 polymorphism in *Plasmodium falciparum* isolates in Lagos, Nigeria.

Materials and methods

Study design and sites

Mutations in K13 were investigated in 195 of 404 dried blood spots from malaria-positive individuals from health facilities across different Local Government Areas (LGA) of Lagos State, Nigeria. Lagos state is situated in the south-west region of Nigeria, shares boundaries with Ogun State both in the North and East and is bordered in the West by the Republic of Benin. In the South, it stretches for 180 km along the coast of the Atlantic Ocean. Transmission of malaria is moderate and stable in Lagos but peaks during the wet season corresponding to the increase in the population of mosquitoes. Lagos has been described as mesoendemic during the dry season [21].

Lagos is a low-lying coastal state and is Nigeria's centre of commerce. It has a diverse and fast-growing population, resulting from heavy and ongoing migration to its cities from all parts of Nigeria as well as neighbouring and foreign countries. Lagos was the capital city of the country before the capital was moved to Abuja on 12th December 1991. The land surface in the state generally slopes gently downwards from north to south and consists of depositional landforms which include wetlands, barrier islands, beaches, low-lying tidal flats and estuaries.

This health institution-based study was conducted on self-presenting individuals at the health facilities with a history of fever and other clinical symptoms of malaria between January 2013 and February 2014. Research subjects for community study were asymptomatic and symptomatic individuals recruited on a voluntarily bases during the community survey conducted from October 2010 to September 2011. The locations of health centres used for the study in Lagos South-West zone, Nigeria, are as shown in Figure 1 and include St Mathew (Catholic) Primary Health Center, Amukoko, Ajeromi Ifelodu LGA. Lagos Central zone: Shomolu General Hospital in Shomolu LGA; Lagos East zone: St. Kizito Primary Health Centre, Lekki in Ibeju Lekki LGA and four (4) health centres situated in Ikorodu LGA: Ijede General hospital, three primary health centres at Imota, Bayioku, Agura and St. Kizito Health Post Oreta. The communities used for the study as shown in Figure 1 are as follows: in Lagos East senatorial zone, Ipakodo in Ikorodu LGA, Agbowa, Ajagunmagbe, Noforija in Epe LGA, Lekki in Ibeju Lekki LGA, Badagry town, Apa in Badagry LGA;

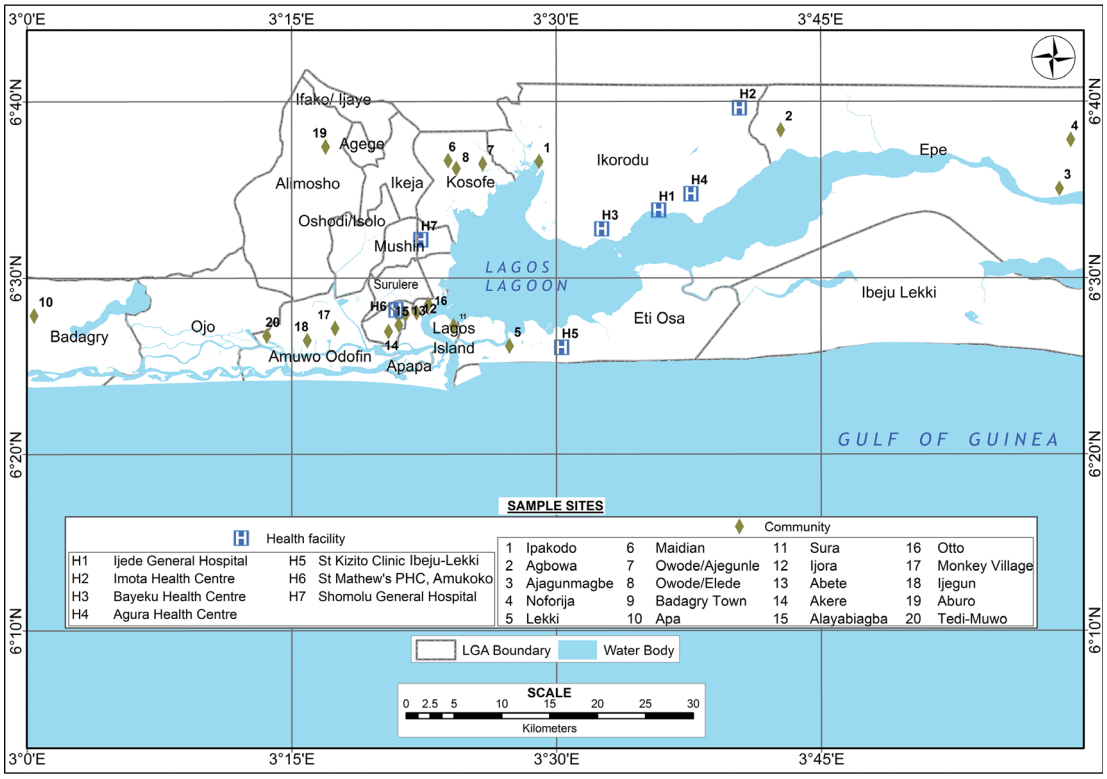

Figure 1 Locations of health facilities and communities from where patients and individuals were enrolled for the study in Lagos State, Nigeria

Tedi-Muwo in Ojo LGA, Aburo in Alimosho LGA. Communities in Lagos Central senatorial zone include Maidian Community, Owode/Ajegunle, Owode/Elede in Kosofe LGA, Sura in Lagos Island LGA, Ijora, Abete, Akere, Alayabiagba in Apapa LGA, Otto in Lagos Mainland LGA. Communities in the Lagos West senatorial zone include: Monkey Village and Ijegun in Amuwo Odofin LGA.

Collection of samples and DNA extraction

Blood samples collected from the study participants were spotted on Whatman® #3 filter paper (Whatman International Ltd., Maidstone, England) and also used to prepare thick and thin films on clean, grease-free frosted end slides and screened for malaria by microscopy using Giemsa stained thick and thin blood smears. Blood spots were air-dried, individually inserted in a zip-lock bag and kept at 2–8 °C for molecular analysis.

We used 195 dried blood spots (DBS) from the samples positive for *P. falciparum* for this study. 3 mm diameter punches were made from the dried blood spot with a single-hole paper puncher. These were placed into a 1.5 ml microcentrifuge tube; the genomic DNA from all the DBSs was extracted using the QIAamp® DNA Mini kit according to the manufacturer's recommendations (Qiagen, Germany).

Nested PCR was done using oligonucleotide primers and cycling conditions listed in Table 1. Total volume of amplification reaction mixture was 25 μl, composed of 2 μl of template genomic DNA prepared from the dried blood spots, 1 μl of primer F, 1 μl of primer R, 12.5 μl of 2*Taq DNA master mix and 8.5 μl of double-distilled water (ddH_2O).

The products of nested PCR were visualised using 1% of agarose gel on a Bio-Rad molecular imager$^{(R)}$ Gel Doc^{TM} and then sequenced on an ABI 3130xl Genetic analyzer (Applied Biosystems, Foster City, CA, USA). Sequence alignment and analysis was performed using BLAST search (www.ncbi.nlm.nih.gov) using the reference *P. falciparum* K13 gene sequence (PF3D7_134700).

Ethical considerations

All research participants and caregivers (in the case of children) gave written informed consent to participate

Table 1 Primer sequences and cycling conditions used for nested Polymerase chain reaction of *Kelch* 13 genes of *Plasmodium falciparum* isolates

Genes	Primer sequence 5′→3′	PCR cycling conditions
K13 (P) F:R:	5′-GGGAATCTG GTGGTAACAGC-3′5′-CGG AGTGACCAAATCTG GGA-3′	95 °C 15 min; {95 °C 30 s, 58 °C 2 min, 72 °C 2 min} 30 cycles; 72 °C 10 min
Nested PCR: K13 (S) F:R:	5′-GCCTTGTTGAAA GAAGCAGA-3′5′-GC CAAGCTGCCATTC ATTTG-3′	95 °C 15 min; {95 °C 30 min, 60 °C 1 min, 72 °C 1 min} 40 cycles; 72 °C 10 min

Note: P: Primary PCR reaction, S: Secondary PCR reaction, F: Forward primer, R: Reverse primer.

using the template approved by the Ethics Committee before the study. The aim and procedures of the research were explained to the research participants, with provision of willingness to participate or withdraw at any point of the study without affecting the standard of care they should receive in the health facilities. All consent documents were stored separately from other study tools in an access-controlled cabinet. Approval to conduct this study was obtained from The Research Grants and Experimentation Ethics Committee, College of Medicine, University of Lagos, Lagos, Nigeria. Patients who declined to participate in the study were not denied access to the available routine care. Generally, the study was conducted in line with the principles of Good Clinical Laboratory Practice.

Results

Kelch 13 genes were amplified and sequenced from 195 of 404 microscopically confirmed *Plasmodium falciparum*-positive dried blood spots (DBS). The prevalence of parasites with mutations on the chromosome 13 in the 'propeller region' of the K13 protein was 3.1%; thus, 96.9% of the K13 propeller genes studied were of the wild type (Figure 2). Mutations at codon 464 resulting in the amino acid change from aspartic acid to asparagine (N) (D464N) and 613 glycine (Q) to histamine (H) (Q613H) were found in four (4) samples from Amukoko in Ajeromi Ifelodu LGA, Shomolu in Shomolu LGA, Ijede and Ipakodo in Ikorodu LGA. One (1) nonsynonymous polymorphism, 578 alanine (A) to serine (S) (A578S), was found in Agbowa in Epe LGA in Lagos state.

Ikorodu LGA in the Lagos East senatorial zone had the largest number of samples analysed as well as the highest

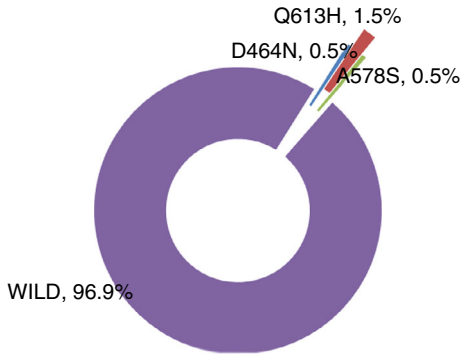

Figure 2 Genotypes of K13 propeller gene in Lagos State, Nigeria

number of *P. falciparum* isolates with Kelch 13 polymorphism (Table 2). Two mutant genotypes, D464N and Q613H, were seen in Ikorodu LGA, while A578S mutant genotype was seen in Epe LGA all from Lagos East senatorial zone. The Q613H mutation was seen in 2 *P. falciparum* isolates, in Lagos central and Lagos west zones (Table 2).

Reports of K13 polymorphism were further segregated into health facilities and communities as shown in Table 3. A578S and D464N mutations were present in parasites from asymptomatic individual in the communities in Epe and Ikorodu LGA situated in the Eastern senatorial zone of Lagos state, respectively (Table 3), while the Q613H mutant genotype was reported each from health facilities located in Amukoko in Ajeromi Ifelodu LGA, Shomolu in Shomolu LGA and Ijede in Ikorodu LGA of Lagos State. One mutant genotype (Q613H) was present in parasites from individuals that accessed diagnostic services in 3 health facilities located in 3 LGAs, namely Ikorodu (Lagos East) Ajeromi Ifelodu LGA (Lagos west) and Shomolu LGAs (Lagos Central) (Table 3).

Discussion

This study demonstrates a low prevalence of single nucleotide polymorphisms (SNPs) in the Kelch 13 gene among *P. falciparum* parasite isolates in Lagos, Nigeria. One (A578S) of the three reported mutations (A578S, D464N and Q613H) was among the eighteen SNPs described in South-East Asia and also reported in Kenya, DRC, Gabon, Ghana and Mali [11,15]. Other SNPs reported in this study are new mutations and have not been reported elsewhere. This study did not detect any of the validated mutations associated with artemisinin resistance (N458Y, Y493H, R539T, I543T, R561H and C580Y) [14].

Table 2 Distribution of *Plasmodium falciparum* kelch 13 gene polymorphism by Local Government Areas and Senatorial zones in Lagos, Nigeria

Senatorial Zone	Name of local govt area	Total no. of DBS collected	K13 nested PCR positive and sequencing *N* (%)	A578S	D464N	Q613H	Total mutation	Wild
Lagos West	Ajeromi Ifelodu	48	38 (79%)			1	1	37
	Alimosho	3	0					
	Amuwo Odofin	4	1 (25%)					1
	Badagry	6	0					
	Ojo	7	0					
Lagos East	Epe	23	3 (13%)	1			1	1
	Ibeju Lekki	12	7 (58%)					7
	Ikorodu	248	127 (52%)		1	1	2	125
	Kosofe	7	3 (43%)					3
Lagos Central	Shomolu	38	15 (39%)			1	1	14
	Lagos Island	2	0					
	Lagos Mainland	1	0					
	Apapa	5	1 (20%)					1
	Grand total	404	195	1	1	3	5	189

Table 3 Distribution of *Plasmodium falciparum* kelch 13 gene polymorphism in Health Facilitates and Communities in Lagos State, Nigeria

Health facility study				Mutation (frequency)		
S/N	Senatorial Zone	Local Government Area (LGA)	Town	A578S	Q613H	D464N
1	Lagos West	Ajeromi Ifelodu	Amukoko	–	1 (0.5%)	–
2	Lagos Central	Shomolu	Shomolu	–	1 (0.5%)	–
3	Lagos East	Ikorodu	Ijede	–	1 (0.5%)	–
Total mutations				–	3 (1.6%)	–

Community study				Mutation (frequency)		
S/N	Senatorial Zone	Local Government Area (LGA)	Town	A578S	Q613H	D464N
1	Lagos East	Ikorodu LGA:	Ipakodo			1 (0.5%)
2		Epe LGA	Agbowa	1 (0.5%)		
Total mutations				1 (0.5%)	–	1 (0.5%)

K13 gene single nucleotide polymorphisms (SNPs) have been searched in many locations to assess the presence of resistant parasites and halt them before clinical resistance spreads. To date, more than 200 mutations in K13 gene have been described in Africa and South-East Asia [3,11,15,22–25], However, no resistance-associated mutations have been found outside the Greater Mekong subregion [14].

The efficacy of ACTs is being monitored in most malaria-endemic countries. There have been some reports of delayed parasite clearance during routine therapeutic efficacy studies (TES) of ACTs conducted in Africa. However, these reports have not been consistent over time. The most frequent allele observed in Africa is A578S [22,23]. This allele was not previously associated with clinical or *in vitro* resistance to artemisinin but a study [26] associated it with prolonged parasite clearance in Ugandan children who had severe malaria and were treated with intravenous artesunate.

WHO defined 'Suspected endemic artemisinin resistance as prevalence of ≥5% of patients carrying K13 resistance-confirmed mutations' [13]. Our study did not detect any of the WHO-validated K13 mutation associated with artemisinin resistance and importantly, no clinical correlative study was conducted to show either tolerance or resistance to artemisinin or its derivatives – a critical requirement in real-time resistance assessment.

Conclusion

There was no validated mutation associated with artemisinin resistance in this study, as there were highly artemisinin-sensitive K13 genotypes among the *Plasmodium falciparum* isolates studied. Nevertheless, sensitivity of *P. falciparum* to artemisinin can only be established by a corresponding *in vivo* clinical therapeutic efficacy testing protocol.

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