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Original Article

Asymptomatic falciparum malaria and genetic polymorphisms of *Pfcrt* K76T and *Pfmdr1* N86Y among *almajirai* in northeast Nigeria

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Abstract

Introduction: Malaria remains a public health challenge, especially in sub-Saharan Africa where asymptomatic malaria is not uncommon. In the present study, the prevalence of asymptomatic falciparum malaria was investigated in *almajirai*, and the genetic polymorphisms of chloroquine (CQ) resistance biomarkers were assessed.

Methodology: A total of 440 apparently healthy *almajirai* between 3 and 12 years of age were randomly enrolled in Maiduguri, northeast Nigeria, between July and December 2010. Parasitemia and gametocytemia were assessed by light microscopy, and polymorphisms of *Pfcrt* K76T and *Pfmdr1* N86Y were detected by nested polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) techniques.

Results: The mean age of the subjects was 8.3 ± 4.5 years, with subjects ≤ 5 years accounting for 10.7% (47/440) of the population. Prevalence of asymptomatic falciparum parasitemia and gametocytemia were 12.7% (56/440) and 8.6% (38/440), respectively. Geometric mean parasite density (GMPD) was 240 (160–630) parasites/µL, while geometric mean gametocyte density (GMGD) was 53 (24–96) gametocytes/µL. The GMPD was higher among subjects ≤ 5 years of age (p = 0.027). *Pfcrt* 76T was detected in 5.4% (3/56) of the isolates, and no isolates harbored *Pfmdr1* 86Y mutant.

Conclusions: The study reveals asymptomatic falciparum malaria in *almajirai* and low levels of *Pfcrt* 76T and *Pfmdr1* 86Y alleles. These findings could hinder malaria control measures, and hence *almajirai* should be incorporated into malaria control programs.

Key words: asymptomatic malaria; *almajirai*; *Pfcrt*; malaria control; Nigeria.

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Introduction

Falciparum malaria remains a major public health challenge in sub-Saharan Africa, especially among pregnant women and children under five years of age [1]. It contributes the largest proportion of malaria morbidity and mortality and is responsible for most cases of complicated malaria [2]. In northeast Nigeria, it accounts for about 98% of malaria cases with a mean annual prevalence of 22.0%, lowest (7.5%) in April and highest (80.7%) in September [3]. Asymptomatic falciparum malaria (AFM), the presence of *Plasmodium falciparum* in peripheral blood without presenting clinical symptoms, is common among adult populations in malaria-endemic areas of Africa, Asia, and South America [4-9]. It is associated with factors such as low parasitemia, increasing age, repeated malaria episodes, and increasing gestational age [10,11]. In addition, AFM has also been reported in children [9,12,13], creating a major challenge to malaria diagnosis, treatment, and control.

Chloroquine (CQ) was widely used in Nigeria prior to the withdrawal in 2004 [14] owing to the widespread resistance to the drug [15,16]. CQ resistance is associated with specific point mutation at various codons in *P. falciparum* CQ resistance transporter (*Pfcrt*) [17,18] and modulates by mutations in the *P. falciparum* multidrug resistance locus 1 (*Pfmdr1*) [19]. The mutations at codons 76 (*Pfcrt* K76T) and 86

(*Pfmdr1* N86Y) are most important for *Pfcrt* and *Pfmdr1*, respectively [20,21]. However, attention is gradually being shifted towards CQ due to evidence of returned sensitivity [22,23].

Almajirai (*almajiri* – singular) are individuals usually under 15 years of age who attend informal Islamic schools and are allowed to wander in search of alms when there are no classes [24]. An estimated 10 million of these children are distributed in most cities, towns, and villages in the northern Nigeria, and are without adequate shelter. *Almajirai* usually sleep outside [25] and are thus exposed to mosquito bites; despite this, they are left out in most malaria control programs. Repeated malaria exposure could induce pre-immunity resulting in asymptomatic infection and increased gametocyte carriage among this cohort; therefore, there is a need for malaria assessment among the *almajirai*. In the present study, the prevalence of AFM and *Pfcrt* K76T and *Pfmdr1* N86Y mutations were determined in a cohort of Nigerian children, the *almajirai*.

Methodology

Study area

This study was conducted at the University of Maiduguri Teaching Hospital (UMTH), Maiduguri, northeast Nigeria with subjects (*almajirai*) recruited from Maiduguri, northeast Nigeria, between July and December 2010. Maiduguri, a malaria endemic area, is the capital of Borno State, with an estimated population of 1,860,000 *almajirai* [26]. The mean annual prevalence of malaria among the general population is 22.0% (78.9% among children ≤ 5 years of age); this is lowest in April (7.5%) and highest in September (80.7%) [3].

Study design and subject enrolment

This was a cross-sectional study aimed at determining the epidemiology of AFM among *almajirai* in Maiduguri. Ethical approval and research permission was obtained from the Borno State Ministry of Health and Ministry of Religious Affairs, respectively. Between July and December 2010, 440 *almajirai* between 3 and 12 years of age who met the inclusion criteria were enrolled after informed consent was obtained. A concise medical history of each subject was obtained using a standard case record form, and each subject underwent a comprehensive clinical examination by a physician.

Collection of samples

From finger-prick blood samples, Giemsa-stained thick and thin blood smears were prepared for malaria microscopy [27]. Capillary blood samples were collected for hematocrit estimation [28], and blood-spotted filter paper (Whatman 3 MM, Whatman, United Kingdom) samples were collected for molecular analyses [29]. The sampling was carried out using standard operating procedures, and all samples were stored appropriately.

Assessment of falciparum parasitemia and gametocytemia

The Giemsa-stained thick smear was used for quantification of parasitemia and gametocytemia, while thin smear was used for species identification by light microscopy. A slide was declared negative if no parasites seen after examination of 100 high power field. Parasitemia was estimated by counting asexual parasites against 200 leukocytes and gametocytemia by counting gametocytes against 1,000 leukocytes. Parasite densities (/µL blood) were calculated assuming a leukocyte count of 8,000 cells/µL blood using the formulae below [30]:

$$\text{Parasite density (parasitemia)} = \frac{\text{Number of asexual parasites counted}}{200 \text{ leukocytes}} \times 8{,}000$$

$$\text{Gametocyte density (gametocytemia)} = \frac{\text{Number of gametocytes counted}}{1{,}000 \text{ leukocytes}} \times 8{,}000$$

Determination of hematocrit

The blood-filled capillary samples were spun at 8,000rpm for 5 minutes using a microhematocrit centrifuge (Hawskey Ltd., High Wycombe, United Kingdom), and hematocrit was determined using a microhematocrit reader (Hawskey Ltd.). Values < 30% were adjudged to be anemic [31].

Extraction of genomic DNA

The genomic DNA (gDNA) of the 56 positive samples was extracted from the blood-spotted filter paper using a QIAamp DNA Mini Kit (QIAGEN, Valencia United States) according to the manufacturer's instructions. The gDNA were stored at -20°C until use [32].

Assessment of genetic polymorphisms of Pfcrt K76T and Pfmdr1 N86Y

The mutation at codon 76 of the *Pfcrt* gene was detected by nested polymerase chain reaction (PCR) (Table 1) followed by restriction fragment length polymorphism (RFLP) (Table 2), as previously described [21,33]. The restriction enzyme *Apo*I (New England Biolabs, Beverly, United States) digests the wild allele *Pfcrt* K76, giving two fragments of 100 bp and 34 bp, but not the mutant allele *Pfcrt* 76T, which remains at 134 bp. The mutation at codon 86 of *Pfmdr1* gene was detected by nested PCR (Table 3) followed by RFLP (Table 2), as previously described [21,34]. The restriction enzyme *AflIII* (New England Biolabs, Beverly, United States) digests the mutant allele *Pfmdr1* Y86, giving two fragments of 190 bp and 120 bp, but not the wild allele *Pfmdr1* N86, which remains at 310 bp. The digestion products of both genes were resolved on 1.5% agarose gel containing ethidium bromide (5 µL/100 mL) and visualized under ultraviolet light. Dd2 and 3D7 clones were used as positive controls for mutant and wild alleles, respectively [21,33,34].

Data analyses

The results were analyzed using SPSS version 15.0 software. Student's t test and analysis of variance (ANOVA) were used to compare mean values, and Chi squared (χ^2) was used to assess proportion. Statistical significance was inferred at $p \leq 0.05$.

Table 1. Conditions of nested polymerase chain reaction for amplification of *Pfcrt* K76T.

Components	Initial Amplification	Nested Amplification
Forward primer (FP)		
Name	pfct-pf (23 bp)	pfct-nf (21 bp)
Sequence	CGGTTAATAATAAATACAC GCAG	TGTGCTCATGTGTTTAAAC TT
Reverse primer (RP)	pfct-pr (25 bp)	pfct-nr (23 bp)
Name		
Sequence	CGGATGTTACAAAACTATAGTTACC	CAAAACTATAGTTACCAAT TTTG
Master mix (25 µl)		
10X PCR buffer	2.5 µl (1X)	2.5 µl (1X)
25 mM $Mgcl_2$	2.0 µl (1.5 mM)	2.0 µl (1.5 mM)
10 mM dNTP	0.5 µl (0.2 µM)	0.5 µl (0.2 µM)
10 µM FP	1.0 µl (0.2 µM)	1.0 µl (0.2 µM)
10 µM RP	1.0 µl (0.2 µM)	1.0 µl (0.2 µM)
5 U/µl Hot Taq	0.3 µl (1 U)	0.3 µl (1 U)
Sterile water	15.7 µl	15.7 µl
gDNA template	2.0 µl	-
**Initial PCR products*	-	2.0 µl
PCR cycling conditions		
Hot start	95 °C - 15 minutes	95 °C - 15 minutes
Denaturation	94 °C - 30 seconds**	94 °C - 30 seconds***
Annealing	45 °C - 45 seconds**	45 °C - 45 seconds***
Extension	72 °C - 1 minute**	72 °C - 1 minute***
Final extension	72 °C - 10 minutes	72 °C - 10 minutes
Hold	4 °C Hold	4 °C Hold

* Diluted into 1:20 of sterile distilled water; dNTP dinucleotides; gDNA Genomic deoxyribonucleic acid; PCR Polymerase chain reaction; [Adapted from 21, 34]; ** 40 cycles; *** 25 cycles.

Table 2. Master mix for restriction fragment length polymorphism.

Components	*Pfcrt* K76T		*Pfmdr1* N86Y	
	Final Concentration	Volume (µl)	Final Concentration	Volume (µl)
Water	-	6.8	-	7.4
NEB3 (10 X)	1 X	2.0	1 X	2.0
BSA (100 X)	1 X	0.2	1 X	0.2
*Apo*I (10 U/µl)	1 U	1.0	-	-
*Afl*III (5 U/µl)	-	-	2 U	0.4
Template	unknown	10.0	unknown	10.0
Final volume	**-**	**20.0**	**-**	**20.0**

Incubated at 56 °C for 3 hours; [Adapted from 34,35].

Table 3. Conditions of nested polymerase chain reaction for amplification of *Pfmdr1* N86Y.

Components	Initial PCR	Nested PCR
Forward primer (FP)		
Name	pfmd-pf (29 bp)	pfmd-nf (23 bp)
Sequence	GCGCGCGTTGAACAAAAAGAGTACCGCTG	TTTCCGTTTAAATGTTTACCTGC
Reverse primer (RP)		
Name	pfmd-pr (28 bp)	pfmd-nr (24 bp)
Sequence	GGGCCCTCGTACCAATTCCTGAACTCAC	CCATCTTGATAAAAAACACTTCTT
Master mix (25 µl)		
10X PCR buffer	2.5 µl (1X)	2.5 µl (1X)
25 mM $Mgcl_2$	2.0 µl (1.5 mM)	2.0 µl (1.5 mM)
10 mM dNTP	0.5 µl (0.2 µM)	0.5 µl (0.2 µM)
10 µM FP	1.0 µl (0.2 µM)	1.0 µl (0.2 µM)
10 µM RP	1.0 µl (0.2 µM)	1.0 µl (0.2 µM)
5 U/µl Taq	0.3 µl (1 U)	0.3 µl (1 U)
Sterile water	15.7 µl	34.3 µl
DNA template	2.0 µl	-
**Initial PCR products*	-	2.0 µl
PCR cycling conditions		
Hot start	94 °C - 2 minutes	94 °C - 2 minutes
Denaturation	94 °C - 1 minute **	94 °C - 1 minute ***
Annealing	45 °C - 1 minute **	52 °C - 1 minute ***
Extension	72 °C - 45 seconds **	72 °C - 45 seconds ***
Final extension	72 °C - 5 minutes	72 °C - 5 minutes
Hold	4 °C Hold	4 °C Hold

* Diluted into 1:50 of sterile distilled water; dNTP dinucleotides; gDNA Genomic deoxyribonucleic acid; PCR: Polymerase chain reaction; [Adapted from 21,35]; ** 40 cycles; *** 25 cycles.

Table 4. Demographic and clinical characteristics of the subjects.

Variables	Values
Number enrolled	440
Sex	
Female (%)	68 (15.5)
Male (%)	372 (84.5)
Age (years)	
Mean ± SD (range)	8.3 ± 4.5
Range	3.0 – 12.0
Number ≤ 5 (%)	47 (10.7)
Weight (kg)	
Mean ± SD	16. 7 ± 5.6
Range	7.0 – 35.0
Hematocrit (%)	
Mean ± SD	37.6 ± 4.8
Range	18.0 – 47.0
Number < 30	26 (5.9)
Use of ITN (%)	4 (0.9)
Previous medication	
Antimalarials (%)	0 (0.0)
Herbs (%)	48 (10.9)

SD: Standard deviation; ITN: Insecticide-treated net.

Results

Demographic and clinical characteristics of the subjects

The mean age of the 440 enrolled subjects was 8.3 ± 4.5 (range, 3–12) years; subjects ≤ 5 years accounted for 10.7% (47/440) of the population. The mean hematocrit value was 37.6% ± 4.8% (range, 18–47); the proportion of anemic subjects was 5.9% (26/440) and was similar among subjects ≤ 5 years (10.6%, 5/47) and subjects > 5 years (5.3%, 21/393) of age (χ^2 = 2.12; degree of freedom = 1; p = 0.146). Use of antimalarial drugs and insecticide-treated nets (ITNs) was low among the subjects (Table 4).

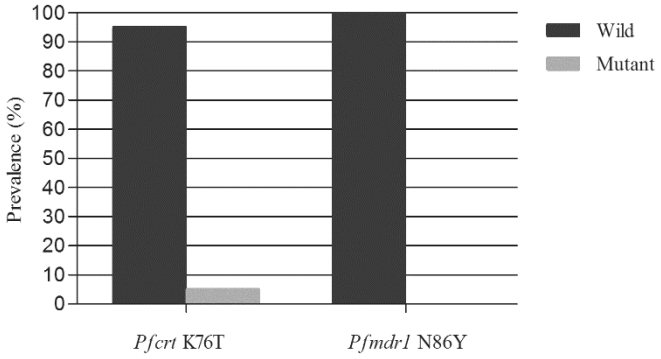
Asymptomatic falciparum malaria among the subjects

The prevalence of parasitemia (asexual) and gametocytemia (sexual) among the subjects were 12.7% (56/440) and 8.6% (38/440), respectively. The geometric mean parasite density (GMPD) was 240 (160–630) parasites/µL, while the geometric mean gametocyte density (GMGD) was 53 (24–96) gametocytes/µL. The GMPD was higher among subjects ≤ 5 years (p = 0.027) of age. There was no difference in prevalence of parasitemia (p = 0.637), gametocytemia (p = 0.561), and GMGD (p = 0.212) among the age groups studied (Table 5). The mean hematocrit was significantly lower among parasitic subjects (36.1% ± 5.6%) than non-parasitic subjects (38.5% ± 4.0%) (p < 0.0001).

Genetic polymorphisms of Pfcrt K76T and Pfmdr1 N86Y

All 56 (100%) and 52 (92.9%) samples were successfully amplified by nested PCR for *Pfcrt* K76T and *Pfmdr1* N86Y, respectively, and were used for the analysis. The prevalence of mutant *Pfcrt* 76T allele was 5.4% (3/56) compared with that of wild *Pfcrt* K76 allele of 94.6% (53/56); none of the samples harbored mixed alleles (*Pfcrt* 76T and K76). No mutant *Pfmdr1* 86Y allele was detected (0.0 %, 0/52) (Figure 1).

Figure1. Genetic polymorphism of *Pfcrt* K76T and *Pfmdrl* N86Y



Discussion

The present study assessed AFM among a cohort of Nigerian children (*almajirai*) who are usually neglected in most malaria control programs. Previously reported prevalence of asymptomatic malaria among African children varies greatly [9,35]. The prevalence of 12.7% reported in this study is higher than the findings of Strom *et al*. [35], who reported no asymptomatic malaria parasitemia among Tanzanian children, and those of Nkoghe *et al*. [9], who reported rates of 1%–8.7% among Gabonese children. This discordance could be attributed to two factors, namely age and use of ITNs. Subjects in the present study were relatively older, and a very small proportion (1%) used ITNs compared with the 97.2% found in a previous study [35]. Studies have shown that asymptomatic malaria increases with age as pre-immunity gradually develops [36], and that the use of ITNs is a reliable malaria preventive measure [37]. In contrast, the prevalence in the present study was lower than the 43.7% reported in western Kenya [38] and could be partly attributed to consumption of herbs, as 10.9% of the subjects who participated in the present study consumed herbs that may have antiplasmodial activities [39]. In addition, *almajirai's* lifestyle exposes them to many hardships,

Table 5. Asymptomatic falciparum malaria among the subjects.

Variables	Subject ≤ 5 years	Subject > 5 years	Total	p value
Number enrolled	47	393	440	-
Parasitemia (asexual)				
Prevalence (%)	14.9 (N = 7)	12.5 (N = 49)	12.7 (N = 56)	0.637
GMPD (/µl)	305 (160 – 630)	192 (160 – 430)	240 (160 – 630)	0.027
Gametocytemia (sexual)				
Prevalence (%)	6.4 (N = 3)	8.9 (N = 35)	8.6 (N = 38)	0.56
GMGD (/µl)	59 (24 – 80)	48 (24 – 96)	53 (24 – 96)	0.21

GMGD: Geometric mean gametocyte density; GMPD: Geometric mean parasite density.

including infections [25]; thus, they could have experienced sufficient malaria episodes resulting in pre-immunity even at an early age. The clinical implication of this finding is that clinical examination alone may not be adequate for malaria diagnosis in this cohort and could be a challenge to malaria diagnosis and control.

Malaria transmission depends largely on the presence of viable gametocytes in peripheral blood, which are picked up by anopheline mosquitoes during a blood meal [40]. The gametocyte carriage of 8.6% observed in the present study is similar to the rate of 10.8% reported in Kenya [38]. Gametocyte carriers are reservoirs of infection that play a key role in continuous malaria transmission [5,10,41]; thus, the *almajirai* could serve as a source of infection to larger populations, challenging control measures.

Point mutations at codons of *Pfcrt* and *Pfmdr1* genes are associated with CQ resistance in *P. falciparum* [20,21,42]. Low levels of mutant alleles (*Pfcrt* 76T and *Pfmdr1* 86Y) detected in the present study indicated high CQ sensitivity among the population. Therefore, it could be opined that CQ sensitivity may be returning to northeast Nigeria years after the withdrawal of CQ due to widespread resistance [14]. This is in accordance with previous studies that have shown a decline in prevalence of *Pfcrt* 76T allele in parts of Africa such as Mali [43], Malawi [22], Kenya [44], Tanzania [45], Senegal [46], and now Nigeria. However, this calls for a larger study to re-assess CQ sensitivity in Nigeria.

Conclusions

This study revealed the presence of AFM among *almajirai* in northeast Nigeria and a low prevalence of mutant *Pfcrt* 76T and *Pfmdr1* 86Y alleles. These findings have significance implications on malaria diagnosis and morbidity and could jeopardize malaria control measures. It is suggested that this cohort of Nigerian children should be incorporated into various malaria control programs to ensure the success of the fight against malaria in Nigeria at large.

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Authors' contributions

STB developed the research proposal under the tutelage of UKS and FAF, performed the molecular aspect of the laboratory work, analyzed the data, and drafted the manuscript. DNB, WAA, and KOO contributed to subject enrolment, laboratory analyses, and manuscript drafting. UKS and FAF were advisors at all stages of the study.

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