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Genetic Polymorphisms of Pfcrt K76T and Pfmdr1 N86Y among Asymptomatic School Children in Forest Communities of Ekondo Titi Subdivision along the Cameroon-Nigeria Border Area

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Authors' contributions

This work was carried out in collaboration among all authors. Author TKNA conceived the study, participated in the design, the write-up of the manuscript and revision of the manuscript. Author DNA conceived the study, performed the experiments, participated in the design, revision of the manuscript and purchased of reagents for molecular analysis. Author BT participated in the design of the study, collection of data, performed the experiments and the write-up of the manuscript. Author JFC designed the study, performed the experiments and revised the manuscript. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Aims: The study sought to quantify *Plasmodium* infection and molecular markers for chloroquine resistance among asymptomatic school children.
Study Design: The study was cross-sectional.
Place and Duration of Study: The study was carried out in Ekondo Titi Subdivision near

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Cameroon's south-western border with Nigeria from March to May and from September to October 2014.

Methodology: The prevalence of human *Plasmodium* species was determined by nested PCR (Polymerase Chain Reaction) using DNA from dried blood spot in six primary schools. A PCR/RFLP analysis (Restriction Fragment Length Polymorphism) was used to determine the prevalence of chloroquine resistance (CQR) associated *pfcrt* 76T and *pfmdr* 1 56Y point mutations in *Plasmodium falciparum* asymptomatic school children.

Results: A nested PCR amplifying the 18S small-subunit ribosomal RNA (SSU *rRNA*) gene of *Plasmodium* in 205 samples confirmed 76.1% of the isolates as asymptomatic *P. falciparum* infections, with a substantial proportion 22% of *P. malariae* infection. Among these, 3.6% were single *P. malariae* infections and 15.1% were *P. falciparum* and *P. malariae* mixed infections. Mixed *P. falciparum* and *P. ovale* infections were 2.0%. Of the 156 *Plasmodium falciparum*, positive samples by species-specific PCR, 107 samples with *P. falciparum* mono-infection were analyzed for the presence of drug resistant alleles *pfcrt* 76T and *pfmdr1*- Y 86. The prevalence of *pfcrt* 76T mutation (74.6%) was higher than that of the *pfmdr1*-Y86 mutation (25.4%). Logistic regression analysis of socio-demographic factors predicted no significant association between *pfcrt* 76T mutation with gender and communities.

Conclusions: The results indicated a high prevalence of *P. malariae* and mixed infection in the area under study. The high-level distribution of the *pfcrtT76* observed in the study could be possibly attributed to the fact that CQ remained widely used at the community level more than 14 years after withdrawal.

Keywords: P. Malariae; school children; asymptomatic; border; mutation; pfcrt; pfmdr1; surveillance.

1. INTRODUCTION

Malaria is a tropical, vector-borne infectious disease caused by protozoan parasites of the genus *Plasmodium* and transmitted by the bite of a female infected *Anopheles* mosquito. Malaria remains a public health problem for many tropical countries around the world despite all efforts. Some 2.97 billion people are at higher risk for malaria resulting in 216 million people infected with malaria parasites in sub-Saharan Africa leading to 445 000 deaths worldwide [1]. Malaria remains a major killer of children, taking the life of a child every 2 minutes [2]. However, the incidence rate of malaria is estimated to have decreased by 18% between 2010 and 2016. Reductions in this rate need to be accelerated in Africa countries if the GTS (Global Technical Strategy for Malaria 2016–2030) milestone of a 40 % reduction in incidence rates by 2020 is to be achieved [2]. However, many countries find difficulties in achieving the control and elimination targets because of the threat of malaria importation, re-introduction [3] and spread of drug resistance especially in border areas adjacent to high malaria-endemic countries. As the scientific community is pushing towards elimination, the failure to control the borders of a country is a concern for public health authorities. Malaria does not respect national borders and *Plasmodium* carries no traveling documents. Understanding malaria transmission dynamics at border areas will facilitate the identification of potential barriers to elimination. “Border malaria” is defined as malaria transmission or potential for transmission that takes place across adjacent administrative areas that share an international border (or lie at a specified distance from an international border) [4]. Border regions are not only difficult to access, but human migration across the porous borders, lower access to health services, health-seeking behaviour of forested and tribal populations, difficulties in deploying prevention programmes to remote and isolated communities and difficult terrain, also pose further obstacles to achieving total malaria control and elimination. On the border of Thailand, *Plasmodium falciparum* has become resistant to nearly all available antimalarial drugs [5]. The potential spill over of resistant strains of *Plasmodium falciparum* to border community's demands strengthened control efforts and close surveillance. To develop cross-border malaria surveillance and control programmes in conjunction with national strategic plans to control cross-border malaria transmission, active and passive case detections as well as entomological surveillance are recommended [6]. Surveillance of antimalarial drug resistance in south-western Cameroon border with Nigeria is vital as drug resistance has historically developed in forested border regions of south-east Asia.

Routine malaria surveillance in endemic regions largely relies on microscopy of Giemsa-stained blood smears, but nowadays, molecular methods of detection provide a more accurate estimate of malaria epidemiology [7]. Asymptomatic mixed-species infections harbouring *P. malariae* or *P. ovale* might represent a threat to meet the Target 3.3 of the Sustainable Development Goals (SDGs) meant to end the epidemics of AIDS, tuberculosis and malaria by 2030. Asymptomatic infected individuals do not seek treatment for their infection, they constitute a reservoir of the parasites. Asymptomatic infections may represent an important reservoir for resistance genes that confer a fitness disadvantage relative to wild-type alleles [8].

Recent data showed that the burden of malaria in Cameroon has declined in 2016 compared to 2000 [2]. However, the reduction in the number of clinical cases of the disease occurred mainly in the urban and peri-urban centres. In Cameroon, chloroquine (CQ) treatment of uncomplicated malaria was discontinued in 2002 due to an unacceptable treatment failure rate of 60% [9]. In spite of the ban on the importation of CQ by the Cameroonian government in the same year, the drugs are still available in the country through illicit trading. Studies have been carried out in Cameroon regarding the genetic polymorphisms associated with drug resistance among symptomatic malaria patients [10,11,12].

The municipality of Ekondo Titi was chosen because of the population movements across the international border with Nigeria, which is located 2.5 hours by waterways away. The municipality of Ekondo Titi is tribal and forested communities and no previous studies of this kind have ever been carried out in the area. The Sub-division is composed of the maritime and the mainland area. The mainland area is made up of 26 villages while the maritime constitutes the 19 kombos 'fishing ports' inhabited by Nigerians. The sub-division has two maritime entry points from Nigeria; the German Beach and the Ekondo Titi Beach. The German Beach has no State services and is the entry point for illegal immigrants and all sorts of smuggling products. Official statistics neither record the illegal immigrants nor the status of immigrants after crossing the border. Ekondo Titi Beach is government controlled and provides all State services. Ekondo Titi Beach also has a periodic market taking place every Saturday where several types of dried fish brought by Nigerian fishermen from 'fishing ports' are sold. Many families living in the municipality have close relatives living in Nigeria's border villages and there is frequent movement in both directions. School-age children living in the 'fishing ports' are also enrolled in a public school located on the mainland area of the municipality. Besides, Cameroon harbours a great number of gorillas and chimpanzees [13]. Lalremruata [14] has established that in South America *P. malariae* was circulating freely between monkeys and humans.

In Cameroon, since the withdrawal of CQ, and the subsequent introduction of ACTs, there was a paucity of information related to the distribution of the markers of CQ resistance. Studies on border malaria are currently lacking. A prevalence study was conducted of mutations in *Plasmodium falciparum* that are associated with chloroquine resistance among asymptomatic school-children in Ekondo Titi near Cameroon's south-western border with Nigeria. The study will provide important information regarding the epidemiology of asymptomatic *P. falciparum* malaria cases and the associated drug resistance capability. The implications of findings for the elimination strategy of malaria in the study area will be outlined.

2. MATERIALS AND METHODS

2.1 Study Area and Study Population

The study took place in Ekondo Titi Subdivision which has been described elsewhere [15]. The study population consisted of 213 children of both sexes randomly selected from six primary schools. The inclusion criteria included: i) obtaining consent from the parent or legal representative of the child, ii) attended school the day of collection, iii) axillary body temperature of <37.5ºC, iv) absence of symptoms suggestive of malaria or any other severe systemic illness.

2.2 Sample Collection and Storage

Blood was collected by finger prick blotted in triplicate onto Whatmann 3 MM (Whatman (Brentford, United Kingdom) paper and allowed to air dry at room temperature. Whatmann 3 MM paper was individually placed in sealed plastic bags containing silica gel, marked with patient's study numbers and date of collection before being transported to the Biotechnology Unit of the Clinical Diagnosis Laboratory of the Faculty of Science at the University of Buea for

molecular biology analysis. The blood-spotted filter papers were stored at room temperature until DNA extraction was done.

2.3 DNA Extraction

Parasite DNA was extracted from the dried blood spot using the QIAGEN DNA Mini kit (Ilden, Germany) according to the manufacturer's instructions. The prepared samples were kept at −20ºC until use.

2.4 *Plasmodium* Speciation by Species-specific nPCR

Genus and species-specific nested PCR assays based on the 18S SSU rRNA gene were used to detect and identify *Plasmodium species* as previously reported [16,17]. A total of 213 samples were randomly selected and confirmed for the presence of *Plasmodium falciparum* species by microscopy. The Nest 1 PCR was carried out using genus-specific primers (rPLU5 and rPLU6) and the Nest 2 PCR was carried out using species-specific primers including rFAL1/rFAL2, rVIV1/rVIV2, and rMAL1/ rMAL2 (Table 1).

Negative control with dH2O in place of the DNA template and a positive control with 3D7 template were always included. Ready-to-Go PCR beads (GE Healthcare, Illustratm puReTaq Ready-To-GoTM PCR Beads, UK, Limited, Amersham Place, Little Chalfort, Buckinghamshire, HP79NA UK) that contained DNA polymerase, dNTPs, and buffers were used. The 25 µl PCR reaction for Nest 1 amplification contained; the PCR bead, 21 µl PCR grade water, 1 µl of forward (rPLU5), 1 µl reverse primers (rPLU6) and 2 µl of DNA template. The cycling parameters for the Nest 1 reaction using the T100 programmable thermal cycler (BIO-RAD, T100, Thermal Cycler, USA) were as follows: 1) Initial denaturation 98ºC for 2 minutes; 2) Denaturation at 98ºC for 10 seconds; 3) Annealing at 70ºC for 15 seconds; 4) Extension at 72ºC for 15 seconds; 5) Repeat steps 2-4 45 times; 6) Final extension at 72°C for 2 minutes; 7) On hold at 40ºC.

For the Nest 2 reaction, two (02) µl of the nest1 amplification product served as the DNA template for the 25 µl Nest 2 amplification. The concentrations of the Nest 2 primers were identical to the nest 1 amplifications. The cycling parameters for the Nest 2 reaction using the P-100 were identical to those of Nest 1 except that the annealing temperature was 62.6ºC.

2.4.1 Analysis of the amplified gene products

The PCR products were separated on a 2% agarose gel alongside a 100 bp DNA ladder and the gel photographed under UV light [16].

2.5 SNP Determination at Codons 86 and 186 by Nested PCR-RFLP

Drug-resistant associated genes were detected by a nPCR followed by an RFLP analysis of the SNPs resulting in point mutation change in the codons 76(K76T) and 86 (N86Y) in *pfcrt* and *pfmdr1* genes respectively as reported by [18,19]. The *pfcrt* and *pfmdr1* mutation-specific primers are shown in Table 2.

2.5.1 Nested PCR and RFLP for Pfcrt mutation-specific detection

NEST 1

For a 25 µl reaction, 21 µl PCR grade water was added to the PCR bead, 1 µl forward primer (*crtp*1), 1 µl reverse primer (*crtp*2) and 2 µl of DNA sample. Primers used for *pfcrt* K76T nest 1 amplification included *crtp1* and *crtp2*. Prepared master PCR mix with dH2O in place of the DNA template was used for all samples to be amplified for a negative control (with dH2O in place of the DNA template). A programmable thermal cycler T100 was used with the following amplification protocol for the first reaction (NEST 1): Step 1 = Initial denaturation at 94ºC for 3 minutes, Step 2 = Denaturation at 94ºC for 30 seconds, Step 3 = Annealing at 56ºC for 30seconds, Step 4 = Extension at 60ºC for 1 seconds, Step 5 = Repeat steps 2-4 45 times, Step 6 = Final extension at 60ºC for 3 minutes, Step 7 = On hold at 40ºC.

NEST 2

The Nest 2 PCR was conducted by using the forward primer *crtp3* and the reverse primer *crtp4*. A programmable thermal cycler T100 was used with the following amplification protocol for the first reaction (NEST 2): Step 1 = Initial denaturation at 94 ºC for 3 minutes, Step 2 = Denaturation at 94ºC for 30 seconds, Step 3 = Annealing at 56ºC for 30seconds, Step 4 = Extension at 60ºC for 1seconds, Step 5 = Repeat steps 2-4 45 times, Step 6 = Final extension at 60ºC for 3 minutes, Step 7 = On hold at 40^0C.

Table 1. Primers for NEST 1 and 2 reactions

S./No.	Species' name	Primers' name	Oligonucleotide sequences
1	*Plamodium spp*.	rPLU5	5'-CCTGTTGTTGCCTTAAACTTC-3'
		rPLU6	5'-ACACAATGAACTCAATCATGACTACCCGTC-3'
2	*P. falciparum*	rFAL1	5'-TTAAACTGGTTTGGGAAAACCAAATATATT-3'
		rFAL2	5'-ACACAATGAACTCAATCATGACTACCCGTC-3'
3	*P. vivax*	rVIV1	5'-CGCTTCTAGCTTA ATCCACATAACTGATAC-3'
		rVIV2	5'-CTTCCAAGCCGAAGCAAAGAAAGTCCTTA-3'
4	*P. malariae*	rMAL1	5'-ATAACATAGTTGTACGTTAAGAATAACCGC-3'
		rMAL2	5'-AAAATTCCCATGCATAAAAAATTATACAAA-3'
5	*P. ovale*	rOVA1	5'-ATCTCTTTTGCTCATTTTTAGTATTGGAGA-3'
		rOVA2	5'-GGAAAAGGACACATTAATTGTATCCTAGTG-3'

Table 2. PCR primer sequences for amplification of codon 76 and 86 of *P. falciparum* chloroquine resistance transporter gene and *P. falciparum* multidrug 1 gene respectively

	Primer	Pairs sequence
pfcrt	Crtp1 (forward primer)	5'- CCGTTAATAATAAATACACGCAG -3'
	Crtp2 (reverse primer)	5'- CGGATGTTACAAAACTATAGTTACC -3'
	Crtd1 (forward primer)	5'- TGTGCTCATGTGTTTAAACTT-3'
	Crtd2 (reverse primer)	5'-CAAAACTATAGTTACCAATTTTG-3'
pfmdr1	Mdr1 (forward primer)	5'-GCGCGCGTTGAACAAAAGAGTACCGCGTG-3'
	Mdr2 (reverse primer)	5'- GGGCCCTCGTACCAATTCCTGAACTCAC-3'
	Mdr3 (forward primer)	5'-TTTACCGTTTAAATGTTTACCTGC-3'
	Mdr4 (reverse primer)	5'-CCATCTTGATAAAAAACACTTCTT-3'

2.6 Nested PCR and RFLP for *pfmdr1* Mutation-specific Detection

NEST 1

For a 25 µl reaction, 21 µl PCR grade water was added to the PCR bead, 1 µl foward primer (*mdr*1), 1 µl reverse primer (*mdr*2) and 2 µl of DNA sample. Primers used for *pfmdr* 86Y nest 1 amplification included *mdr*1 and *mdr*2. Prepared master PCR mix with dH2O in place of the DNA template was used for all samples to be amplified for negative control (with dH2O in place of the DNA template). A programmable thermal cycler T100 was used with the following amplification protocol for the first reaction (NEST 1): Step 1 = Initial denaturation at 95ºC for 5 minutes, Step 2 = Denaturation at 95ºC for 30 seconds, Step 3 = Annealing at 45ºC for 30seconds, Step 4 = Extension at 65ºC for 45 seconds, Step 5 = Repeat steps 2-4 45 times, Step 6 = Final extension at 72ºC for 5 minutes, Step 7 = On hold at 40^{0}C.

NEST 2

The nest 2 PCR was conducted by using the forward primer *mdr3* and the reverse primer *mdr4*. A programmable thermal cycler T100 was used with the following amplification protocol for the first reaction (NEST 2): Step 1 = Initial denaturation at 95ºC for 5 minutes, Step 2 = Denaturation at 95ºC for 30 seconds, Step 3 = Annealing at 45ºC for 30seconds, Step 4 = Extension at 65ºC for 45 seconds, Step 5 = Repeat steps 2-4 45 times, Step 6 = Final extension at 72ºC for 5 minutes, Step 7 = On hold at 40ºC.

2.6.1 Restriction digests of the pfcrt and pfmdr1 gene

Digestion assays of *pfcrt* and *pfmdr1* comprised 35 µl of PCR grade water, 5 µl of nest 2 amplicon, 5 µl of 10x NEB buffer and 5 µl of Apo 1(New England Biolabs Inc., Frankfurt Am Main, Germany) in 50 µl reactions. Digestion assays were incubated for 2 hours at 500ºC. In the PCR products, the DNA sequence was cleaved at the wild-type codon site (132 bp), while the mutant allele was not cut (265 bp).

2.6.2 Analysis of the amplified gene products

The digested products were separated by electrophoresis in a 3% agarose gel containing ethidium bromide and DNA was visualised by ultraviolet transillumination (Gel Doc XRt, Biorad, USA).

2.7 Statistical Analysis

Qualitative variables were summarized as proportions and percentages, while quantitative variables were presented as median (95% CI). Differences among qualitative variables were determined using Chi-square test. Differences among quantitative variables were determined using Kruskal Wallis test. Logistic regression was used to analyze the associations between baseline variables such as sex, age, and residence with the presence of *pfcrt* T76 and *pfmdr1 Y86* mutations in *Plasmodium falciparum* isolates. Results of logistic regression were provided as odds ratios (ORs; 95% confidence interval [CI], *P* values). Means ± standard deviations (SD) were compared using independent sample t-test, Mann-Whitney U test, and analysis of variance (ANOVA). The statistical significance level was set at α=0.05 for all tests (SPSS version 20; SPSS, Chicago, Illinois, USA).

3. RESULTS

3.1 Demographic Characteristics of the Study Population

A total of 213 selected positive samples by microscopy from previous studies [15], were subjected to PCR, of which 205 were positive. The characteristics of the subjects who provided samples for analysis are shown in Table 3. The age varied between 3 to 14 years (mean age 7.97 ± 2.20) out of which 45.5 % (121/213) were males with a mean age of 7.0 years (8.39 ± 2.30) and 54.5 % (92/213) female with a mean age (7.41 ±1 .93). The mean levels of haemoglobin (Hb) in the school children were 1138.69 ± 1103.74. No statistical difference was observed between males and female (*P*=11).

3.2 Detection of Malaria Parasites in Asymptomatic Populations

In the PCR analysis of 213 samples (all positive by microscopy), the infection was confirmed in 96.3% (n = 205) and 3.7% (n=8) children positive for microscopy were negative by PCR. The majority of the asymptomatic malarial infections among the school children were due to *P. falciparum* mono-infections with a prevalence of 76.1% (156/205), with a substantial proportion 45/205 (22%) of *P. malariae* infection among them (Table 4). Among these, 6.3 % were single for *P. malariae* infection and 15.1% had *P. falciparum* and *P. malariae* mixed infection (Fig. 1). Mixed *P. falciparum* and *P. ovale* infection was 2.0% (4/205). No single or mixed infection with *P. vivax* was observed (Fig. 2).

Haemoglobinaemia, age group and gender were not significantly associated with children with single and mixed infection. Mixed infections *P.f./P.m.* were not evenly distributed in the study area. Interestingly, school children of Bongongo Barombi and Kita Balue had no single *P.m.* mono-infection (Table 5).

Fig. 1. Electrophoresis after *Plasmodium*-specific PCR amplification. M = 100 bp Ladder molecular marker, PC= positive control, NC= negative control, F = *P. falciparum M* = *P. malariae*, O = *P. ovale,* V = *P. Vivax*

Table 3. Baseline characteristics of study population

Category	Number examined	Mean age (±SD)	Mean trophozoite (±SD)	Mean Haemoglobin(±SD)
Sex				
Male	121	8.39±2.30	1371.9±12223.83	10.50±1.94
Female	92	7.41±1.93	831.96±834.01	10.87±1.13
Total	213	7.97±2.20	1138.69±1103.74	10.66±1.64
Level of significance		**F=10.74;*P*=.001**	**F=13.23; *P* =.000**	**F=2.58; *P* =.11**
Age group (years)				
≤5	32	4.87±0.34	1010.62±806.91	10.16±1.34
6-9	126	7.5±1.12	1100.95±1000.31	10.69±1.72
10-14	55	10.84±1.23	1299.64±1432.92	10.88±1.58
Total	**213**	**7.97±2.20**	**1138.69±1103.74**	**10.66±1.64**
Level of significance		**F=343.23; *P* =.000**	**F=0.87; *P* =0.419**	**F=1.98; *P* =0.141**
Patient location				
Bongogo B	26	7.00±2.29	1058.46±806.39	9.35±1.81
Kita Balue	37	7.84±2.53	538.92±327.68	11.11±0.68
Lipenja	61	9.21±2.16	1526.56±1302.78	10.64±1.42
Lobe Estate	20	7.30±1.34	2214.00±1314.41	11.78±1.23
Ngolo Metoko	41	7.29±1.85	845.85±812.37	10.41±2.32
Sacred Heart	28	7.79±1.55	821.43±961.71	10.90±0.99
Total	**213**	**7.97±2.20**	**1138.69±1103.74**	**10.66±1.64**
Level of significance		**F=6.98; *P* =0.000**	**F=10.47; *P* =0.000**	**F=6.84; *P* =0.000**

Table 4. Comparison of PCR with microscopy for the identification of *Plasmodium species* in asymptomatic school children

***Plasmodium species* n(%)**	**Microscopy diagnosis n=485**	**Nested PCR n=213**
P. falciparum	360 (72.4)	156(73.2)
P. malariae	0(0.0)	14(6.4)
P. falciparum / P. malariae	0(0.0)	31(14.5)
P. falciparum /P. ovale	0(0.0)	4(1.8)
Negative	125(25.4)	8(3.7)

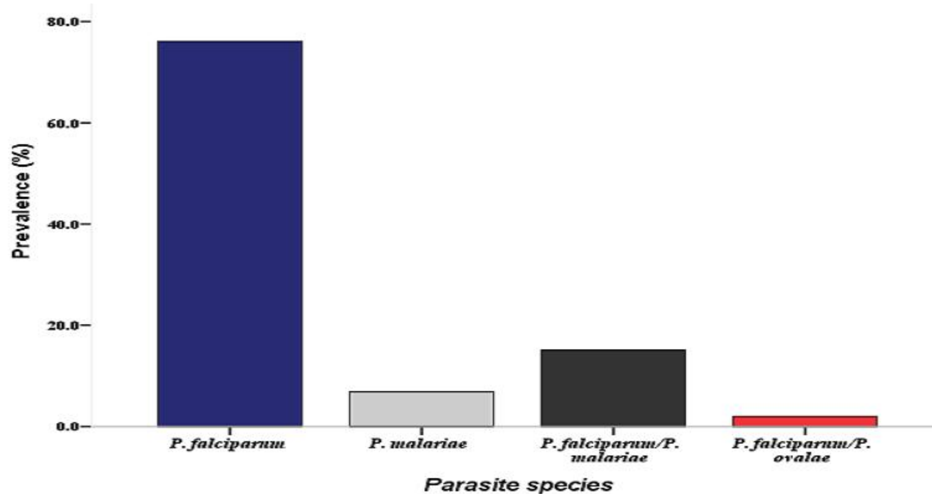

Fig. 2. Distribution of *Plasmodium species* detection by nested PCR in children with asymptomatic malaria

Table 5. Distribution of the different parasite species among the different categories of sex, age group, subject's location, parasite density and anaemic levels

Categories	Number examined positive by PCR	*Plasmodium species*				χ^2	P
		Pf	***Pm***	***Pf/Pm***	***Pf/Po***		
Sex							
Male	116	79.3(92)	6.9(8)	12.1(14)	1.7(2)		
Female	89	71.9(64)	6.7(6)	19.1(17)	2.2(2)	**2.082**	**0.556**
Total	**205**	**76.1(156)**	**6.8(14)**	**15.1(31)**	**2(4)**		
Age group							
≥5	31	74.1(23)	6.5(2)	12.9(4)	6.5(2)		
6-9	122	76.2(93)	8.2(10)	13.9(17)	1.6(2)	**6.126**	**0.409**
10-14	52	76.9(40)	3.8(2)	19.2(10)	0.0(0)		
Total	**205**	**76.1(156)**	**6.8(14)**	**15.1(31)**	**2(4)**		
Patient location							
Bongogo B	26	73.1(19)	0.0(0)	19.2(5)	7.7(2)		
Kita Balue	35	82.9(29)	0.0(0)	17.1(6)	0.0(0)		
Lipenja	58	75.9(44)	6.9(4)	17.2(10)	0.0(0)	**25.676**	**0.042**
Lobe Estate	20	70.0(14)	10.0(2)	10.0(2)	10.0(2)		
Ngolo Metoko	41	73.2(30)	9.8(4)	17.1(7)	0.0(0)		
Sacred Heart	25	80.0(20)	16.0(4)	4.0(1)	0.0(0)		
Total	**205**	**76.1(156)**	**6.8(14)**	**15.1(31)**	**2(4)**		
Anemic level							
Non anemic	94	78.7(74)	5.3(5)	13.8(13)	2.1(2)		
Moderate	104	74.0(77)	8.7(9)	15.4(16)	1.9(2)	**2.560**	**0.862**
Severe	7	71.4(5)	0.0(0)	28.6(2)	0.0(0)		
Total	**205**	**76.1(156)**	**6.8(14)**	**15.1(31)**	**2(4)**		
Parasite density							
Low	80	87.5(70)	0.0(0)	10.0(8)	2.5(2)		
Moderate	122	68.3(84)	11.4(14)	18.7(23)	1.6(2)	**14.994**	**0.022**
High	2	100(2)	0.0(0)	0.0(0)	0.0(0)		
Total	**205**	**76.1(156)**	**6.8(14)**	**15.1(31)**	**2(4)**		

Fig. 3. Electrophoresis after cut by the restriction enzyme. Lane1-100 pb ladder: Lane 2-10, 12, 14-18, K 76 polymorphism (uncut full length 132 bp). Lane 11 and 13 T76 polymorphism (cutted by Apo1 restriction enzyme to 98 and 265 bp). Lane 23,25, *Y86* polymorphism (uncut full length 265 bp). Lane 24 and 26 *Y86* polymorphism (cut by Apo1 restriction enzyme to 132bp)

3.3 Prevalence of Drug Resistant Molecular Markers

Of the 156 *Plasmodium falciparum* positive samples by species-specific PCR, 107 participants with *P. falciparum* mono-infection were analyzed for the presence of drug resistantgene *pfcrt* 76 *T* and *pfmdr1*-Y86 (Fig. 3). The prevalence of mutant *pfcrt* 76T (74.6 %) allele was higher than that of the mutant *pfmdr186 Y* (25.4 %) allele (Fig. 4). The proportion of the *pfmdr* 86 Y allele in Bongongo II was the highest while *pfcrt* 76T allele takes prominence in other localities (Fig. 5). The distribution of *pfcrt* 76 T and *pfmdr* 1 allele by age (Fig. 6) and school locations (Fig. 5) revealed no significant difference in study population.

3.4 Potential Risk Factors of Drug Resistance

The associations between the prevalence of *pfcrt* 76T and *pfmdr1Y86* mutations and some potential risk factors are presented in Table 6. Logistic regression analysis of socio-demographic factors predicted no significant association between *pfcrt* 76T mutation with gender and location of the schools respectively.

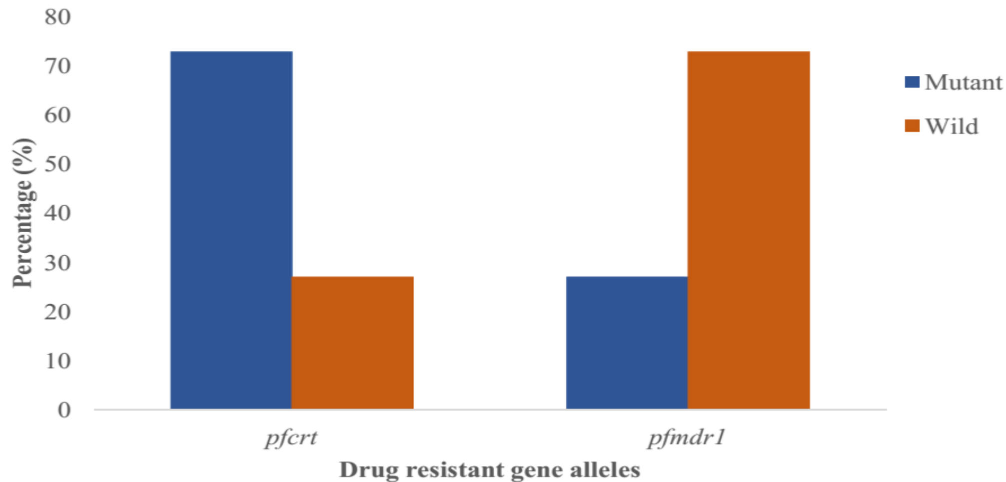

Fig. 4. Prevalence of *pfcrt* T76 and *pfmdr 1Y86* mutations in *Plasmodium falciparum* isolates

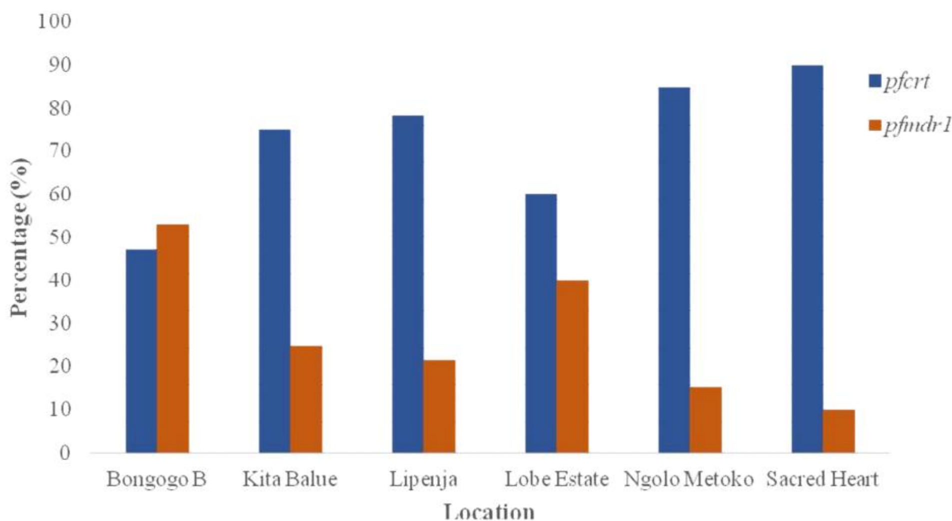

Fig. 5. Distribution of the *Pfcrt* T76 and *Pfmdr1Y86* mutations in *P. falciparum* isolates by school locations(*P*=.063)

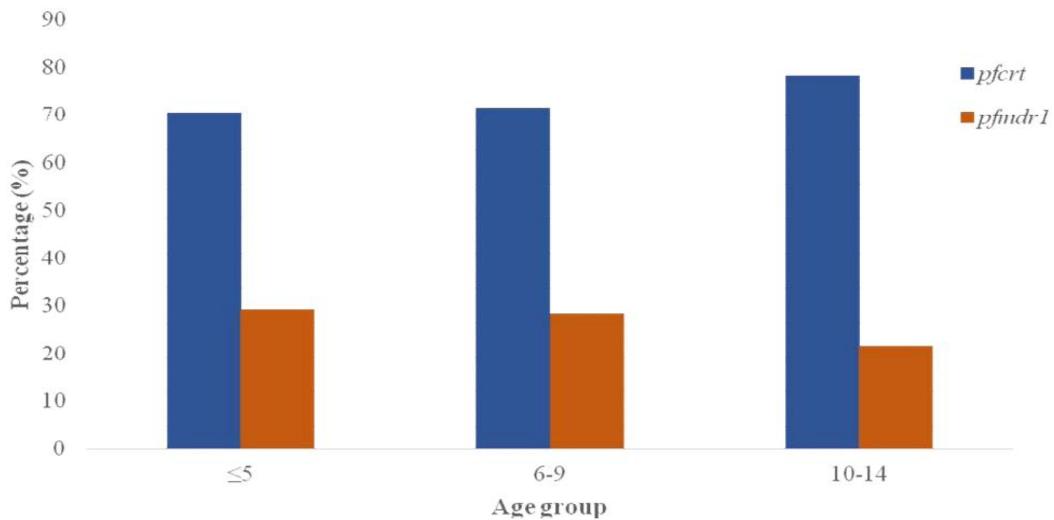

Fig. 6. Distribution of the *Pfcrt* T76 and *Pfmdr1Y86* mutations in *P. falciparum* isolates according to age groups (P=.806)

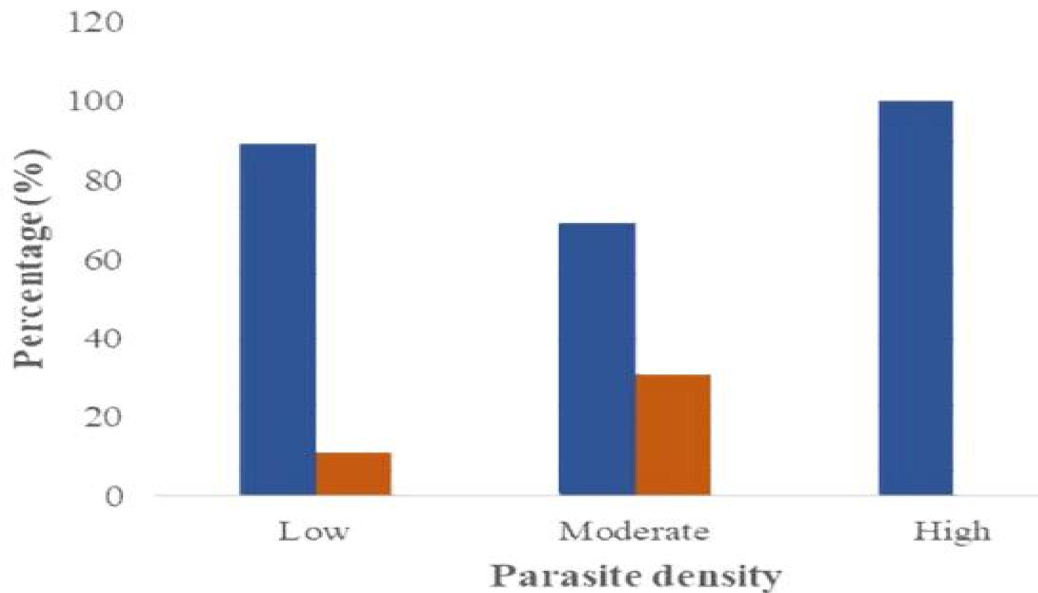

Fig. 7. Distribution of the *Pfcrt* T76 and *Pfmdr1Y86* mutations in *P. falciparum* isolates by Parasite density (*P*=.156)

4. DISCUSSION

The aims of the present study, were to determine the prevalence of the circulating *Plasmodium species* using molecular methods and secondly to assess the prevalence of mutations in *Plasmodium falciparum* that are associated with chloroquine resistance. The results showed that *P. falciparum* was the predominant parasite species in the population being analyzed, followed by *P. malariae.* No *P. malariae* was detected in microscopic diagnosis, thereby agreeing with previous reports in Cameroon [20,21]. Available data indicated an absence [22,21] or scanty presence of *P. malariae* in the country [23]. A recent study of asymptomatic individuals in Yaoundé found a much lower prevalence of *P. malariae* [20]. The prevalence of *P. malariae* found in the present site is higher than that reported in other African

Table 6. Factors associated with *pfcrt* T76 and *pfmdr1Y86* mutations (using the multinomial linear or binary logistic regression where appropriate)

	Number positive by PCR =205	Number examined for resistant gene allele	Number examined for resistant gene =107							
Variable	**Pf by PCR n**		***Pfcrt T76* % (n)**	**OR**	**95% CI**	**P value**	***Pfmdr1 Y86* % (n)**	**OR**	**95% CI**	**P value**
Sex										
Male	116	71	67.6(48)	0.558	0.308-1.011	0.054	32.4(23)	0.233	0.088-0.618	0.003
Female	89	36	83.3(30)				16.7(6)			
Age group										
≤5	31	17	70.6(12)				29.4(5)			
6-9	122	67	71.6(48)	0.811	0.504-1.305	0.388	28.4(19)	0.674	0.358-1.269	0.222
10-14	52	23	78.3(18)				21.7(5)			
Residence										
Bongogo B	26	17	47.1(8)				52.9(9)			
Kita Balue	35	8	75(6)				25(2)			
Lipenja	58	37	78.4(29)				21.6(8)			
Lobe Estate	20	15	60(9)	1.036	0.86-1.249	0.710	40(6)	0.747	0.566-0.986	0.040
Ngolo Metoko	41	20	85(17)				15(3)			
Sacred Heart	25	10	90(9)				10(1)			

countries for *P. malariae* [24,25]. An interesting finding was that no *P. vivax* was detected. Conversely, data from highland area specifically Dschang in the West Region of Cameroon confirmed the circulation of *P. vivax* [26]. Though, a cross sectional study, our data provide a different epidemiological panorama, where *P. malariae* was relevant regarding both single and mixed infections. Located in the African deep rain forest region with good proximity between humans and non-human primates, Ekondo Titi provides an ideal setting where the risk of parasite exchange between monkeys and humans cannot be overlooked. Lalremruata [14] has established that in South America, *P. malariae* is circulating freely between monkeys and humans. Geographically, Ekondo Titi shares border with Nigeria, cases of imported malaria infections should not be excluded since many school children move between Cameroon and Nigeria during school holidays. Besides, the presence of a weekly market at Ekondo Titi Beach provides a platform for exchange of *Plasmodium species* between fishermen and other traders from Nigeria and local communities. Routine malaria surveillance in endemic regions largely relies on microscopy of Giemsa-stained blood smears. However, *P. malariae* infections are often present with a low parasitaemia and occur as mixed infections with *P. falciparum.* Expertise in the malarial microscopy capable of distinguishing different species in clinical practice are scared and could subsequently, lead to false-positive reports. Malaria parasites were found in 213 samples (microscopy) while only 205 were confirmed by PCR. Since nested-PCR is a highly sensitive method for the identification of malaria parasites, this discrepancy may be due to a slight misinterpretation of microscopic diagnosis. Similar differences have been reported in Pakistan where nearly 15 % of samples positive by microscopy were parasite negative by PCR [27]. *P. falciparum* was mostly detected as mono-infection, whereas *P. malariae* and *P. ovale* were usually combined with *P. falciparum*. The most predominant mixed infection was observed to be *Pf/Pm* (77.86%) which is lower than the findings of Achonduh [28] but higher than that of [24]. This is in line with epidemiological findings in India [29] where the less prevalent malaria parasite (*P. malariae*, and *P. ovale*) mostly occurred as coinfection with *P. falciparum.* Identification of *P. malariae* is critical because *P. malariae* has been associated with chronic infections persisting for years in persons as reservoirs for ongoing transmission as it remains undetected in the host for longer periods. It has been reported that blood stages of *P. malariae* persist for extremely long periods, often for the life of the human host. Most published data showed that the prevalence of *P. malariae* has been underestimated apparently due to low parasitaemia, the morphological resemblance with *P. falciparum* and occurrence as mixed infections with the major parasite species [30]. The 6.8% of non- *P. falciparum* infections recorded in the current study revealed the dynamic and the changing pattern of transmission of malaria in the border communities. The rapid spread of antimalarial drug resistance over the last few decades has increased the need for monitoring the emergence of drug resistance to ensure proper management of clinical cases, to allow for early detection of changing patterns of resistance and to suggest where national malaria treatment policies should be revised. To appropriately address the challenges, raised by the failure rate of several antimalarial in most of the malaria-affected areas, a close monitoring of the epidemiology and dynamics of drug resistance is imperative.

Out of the 107 isolates, the *pfcrtT76* mutant allele was present in 74.6% of samples while *pfmdr1* 86Y was detected in 25.4%. The high-level distribution of the *pfcrtT76* observed in the study could be possibly attributed to the fact that CQ remained widely used at the community level more than 14 years after its withdrawal. The frequency of the *pfcrt* 76 T among parasite isolates from the school children was higher than data from other parts of Cameroon far from international borders which have shown a decreased prevalence of mutants *pfcrt* gene [31,32]. This might be attributed to ignorance of drug regimen change-over, self-medication and defective communication strategies for rural and forested areas among others. Furthermore, the potential spill over of resistant strains of *Plasmodium falciparum* to chloroquine from Nigeria to Cameroon border communities should not exclude. Nigeria contrary to Cameroon government has not implemented the ban of chloroquine in spite of adopting ACTs as the first-line treatment of uncomplicated malaria. In 2012, a report by in Nigeria found from a household survey that 54% of children who received an antimalarial drug were given CQ even though the national malaria drug policy had changed to ACTs seven years previously [33].

Amodiaquine (AQ), a compound belonging to the group of 4-aminoquinolines, structurally similar to

CQ, acts as a partner molecule in ABC therapy. AQ may as well sustain some selection pressure for CQ-resistant parasites [34]. In an association study showing a cross-resistance between CQ and AQ may suggest that the use of artesunate plus AQ contributes to the continued persistence of the mutant *pfcrt* genotype. Reduced artemether sensitivity has been observed in parasites containing 86N, 1034S or 1042N alleles or more than equal 4 copies of the *pfmdr1* gene [35]. The selection of the mutant *pfcrtt76* and *pfmdr1y86* alleles indicates the primary involvement of the two genes in the mediation of AQ resistance. Thus, similar to CQ, AQ resistance in *P. falciparum* may depend primarily on mutations in *pfcrt T76* and additional mutations in *pfmdr1.* Compared with the high prevalence of the *pfcrt T76* mutation, fewer samples had mutations in codon 86 of *pfmdr*1. The low prevalence of the *pfmdr1* 86Y mutant allele among *P. falciparum* isolates from Ekondo Titi, is in discordant with the findings of [36] in Yemen and [10] in Cameron. Moreover, [35] has shown that the parasites with the *pfmdr1* 86Y allele produce a higher number of gametocytes, which would gain the advantage in terms of transmission.

Age is one of the most important factors that correlate with protective immunity in areas in which malaria is endemic. Individuals acquire immunity from several episodes of malaria during the early part of their lives [37]. The *pfcrt* 76T mutation was neither significantly associated with any age groups (OR = 1.038, 95% CI = 0.308-1.011 P = .05), nor location of the schools (OR = 1.036, 95% CI = 0.86-1.249, P = 0.710). This is in line with studies by [38]. In contrast, [39] found above 10 years of age children to be significantly associated with *Pfcrt* 76T mutations.

In a region of very high malaria transmission intensity, children harbouring *P. falciparum* containing mutations associated with resistance to CQ and AQ were less likely to be febrile suggest that wild-type parasites are more capable of causing clinical illness than those with resistance-mediating mutations in *pfcrt* and *pfmdr1* [40]. Our results indicated that, the prevalence of *pfcrt* was similar among the residence areas, except for *pfmdr1*whose prevalence was the lowest in Sacred Heart primary school.

Interestingly, the present data reported a trend between age, residence and *pfmdr1Y86* mutations. Our results also indicated no significant association of *pfcrt* 76T mutation with moderate/low parasitaemia and this is consistent with findings from Sudan and Congo [41,42]. The relationship between *pfcrt* 76T mutation and malaria severity is a controversial issue. The finding is however contradictory to the studies that reported a significant association of *pfcrt* 76T mutation with the severity of malaria in India [43, 44]. The reasons for these observations are yet be understood [44]. The study had a few limitations. The findings obtained cannot be generalized because of the small number of samples analysed, the limited geographical area covered and the inability to collect samples in both side of the border. The lack of molecular data on the prevalence of artemisinin and antifolate resistance markers to complement the present survey.

5. CONCLUSION

Epidemiology of malaria in Cameroon is shifting from the sole predominance of *P. falciparum* to multi-species infection with two minor parasite species of *P. malariae* and *P. ovale spp*. occurring. Our results provide evidence that *P. malariae* is still being transmitted in the study area. The current work highlighted the need for an active case surveillance to determine the prevalence of malaria in border areas of the country, aiming at the identification and management of asymptomatic carriers. Finally, the present study emphasized the need to re-strategize drug policy by the government of Cameroon. Close monitoring with molecular markers to detect the emergence of antimalarial drug resistance is imperative, particularly at the border areas.

CONSENT AND ETHICAL APPROVAL

The study was conducted following the Helsinki Declaration and the national laws and regulations in force. The study obtained ethical clearance from the Regional Delegation of Public Health, Buea. A supportive letter was obtained from the District Health Services at Ekondo Titi. Before collection of data, written informed consent was obtained from voluntary participants. The subjects were assured of their right to withdraw from the interview at any time during the exercise without any explanation.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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