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Research Article

Re-Emergence of Chloroquine Sensitive *Plasmodium falciparum* after Several Years of Chloroquine Withdrawal in Bamenda, North West Cameroon

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Abstract

Background: Plasmodium falciparum resistance to chloroquine (CQ) denied health care providers access to cheap and effective antimalarial drug. Plasmodium falciparum resistance to CQ had been proven to be point mutations in the parasite Plasmodium falciparum chloroquine resistance transporter gene (Pfcrt) at codon 76. This study was to assess the current state of Plasmodium falciparum resistance to CQ in Bamenda, North West Region of Cameroon.

Methods: Blood was collected on Whatman filter paper from 198 malaria patients. Malaria parasite DNA was extracted from 191 out of the 198 samples using chelex method. The extracted DNA was used as a template in nested polymerase chain reaction amplification of Pfcrt at codon 76. Apo1 restriction enzyme was used to digest the amplified DNA to identify the samples as CQ sensitive (wild type) and CQ resistance (mutant) at codon 76.

Results: The prevalence of the resistance gene was 12.6% at Chi square ($\chi 2$) = 11.582; probability ($p = 0.003$) at the 5% level of significance. This result indicates that there is a reduction in the prevalence as compared to 67% that was reported in the same study area in the year 2000.

Conclusion: This could be as a result of the withdrawal of CQ as antimalarial drug in Cameroon. This study should be replicated in other areas in Cameroon so as to determine the re-emergence of CQ sensitive Plasmodium falciparum nationwide.

Keywords: *Plasmodium falciparum; Chloroquine; Anti-Malaria Resistance; Malaria; Pfcrt; Codon 76; Bamenda*

Background

Malaria is now second to HIV/AIDS as the global cause of death from infectious diseases but remains the main global cause of death from parasitic infectious diseases [1]. Malaria threatens approximately half the world's population and causes debilitating illness in more than half a billion people. The degree of infection is particularly high in Sub-Saharan Africa, where children below five years are at risk of infection, clinical disease and death [1]. *Plasmodium falciparum* remains the deadliest of the malaria parasite species in Africa [1] and also wreaks significant economic havoc in highly endemic areas, substantially decreased gross domestic product of affected countries relative to malaria free countries [2]. There were 216 million reported confirmed cases of malaria and 445000 reported deaths in the world in 2016 [3]. Although malaria endemicity remains substantial in Cameroon, the country successfully implemented health policies which improved and sustained nationwide coverage of malaria control measures and consequently documented a substantial decline in

morbidity and mortality amongst the population at risk [4]. In spite of these measures and gains, the appearance and possible spread of resistance to artemisinin derivatives threatens the world's malaria control and elimination efforts making the need for a fast development of effective and safe malaria vaccine, development of new antimalarial drugs or the bringing back of old drugs such as chloroquine.

For more than three decades, chloroquine had been a cheap and effective antimalarial drug particularly in Africa and raised hopes to eradicate malaria. However, these hopes were shattered by the emergence of chloroquine resistance in the late 1960s which spread on the African continent leading to high level of treatment failures and increased morbidity and mortality associated with malaria. Chloroquine and amodiaquine were the first-line drugs for the treatment of uncomplicated malaria until 2002 in Cameroon. Chloroquine resistance led to the gradual withdrawal of chloroquine and the use of amodiaquine as the first-line drug for the treatment of uncomplicated malaria during the transition period between 2002 and 2004 in Cameroon. This was followed by the adoption of artemisinin-based combination therapy (artesunate-amodiaquine, with artemether-lumefantrine as alternative) in 2006 as first-line treatment [5]. *Plasmodium falciparum* resistance to chloroquine has been proven to be point mutations in the *Plasmodium falciparum* resistance transporter (Pfcrt). A single nucleotide polymorphism encoding an amino acid change at codon 76 (K76T) is the hallmark of chloroquine resistance in *Plasmodium falciparum* worldwide.

There have been documented reports about the re-emergence of chloroquine sensitive *Plasmodium falciparum* in different areas in Africa [5-7]. However, in Bamenda, North West of Cameroon it remains unclear whether years of reliance on antimalarials other than chloroquine has led to the re-emergence of chloroquine sensitive *Plasmodium falciparum* and thus permits the re-introduction of this safe and affordable drug. In a bit to bridge this gap in knowledge, this cross sectional study was conducted with the aim to determine the prevalence of chloroquine resistance several years after chloroquine withdrawal in Bamenda Cameroon. This was done by using molecular techniques to assess point mutation of Pfcrt at codon 76. The participants for the study were residence in Bamenda. Bamenda is an urban area with genetically diverse population.

Methods

Study site

The study was carried out in Bamenda the head quarters of the North West Region of Cameroon and lies between latitude 6N and longitude 10.1E. The average altitude is 1218m above sea level. The climate is classified as a tropical monsoon with two seasons; dry season from November to March and rainy season from April to October. Annual precipitation is 2300mm. Bamenda has a population of about 500000 people and is located 366 kilo meters North West of the Cameroonian Capital, Yaounde. It is an urban area and has people from different ethnic groups, this renders its population genetically diverse. The transmission of malaria is permanent, occurring all year long. Malaria prevalence is higher during the rainy season, and drops during the dry season. Blood samples were collected from subject participants from June to August, 2015. Study sites were: Bamenda Regional Hospital, Nkwen Sub Divisional Hospital, Family Foundation clinic and Mankon Sub Divisional Hospital.

Study Population

Patients visiting the Regional Hospital Bamenda, Family Foundation Clinic Bamenda, Mankon Subdivisional Hospital Bamenda and Bamenda III Sub-divisional Hospital were enrolled in the study based on the following criteria: subjects age 1 to 80 years inclusively, subjects with acute manifestation of infection with *Plasmodium falciparum* determined by microscopy and rapid diagnostic test and subjects with temperature ≥ 37.5^{0}C. The following subjects were excluded: subjects with complicated malaria, pregnant women and subjects who were out of Bamenda for three weeks before the study.

Sample collection

Blood samples were collected from patients by finger prick. Blood samples that were positive for malaria parasite as determined by microscopy and rapid diagnostic test were placed on Whatman filter papers. The filter papers were packaged individually in a zip locked bags and labeled based on the participant identification number.

Plasmodium falciparum DNA extraction

The portion of the filter papers containing blood spots were cut and put into eppendorf tubes. The tubes were labeled based on the participant identification number. 400 µl of 5% chelex reagent was put into each of the eppendorf tube. The tubes were put into a shaking water bath and incubated at a temperature of 56^0C for one hour. The tubes were then incubated at a temperature of 95^0C for 30 minutes in the same shaking water bath. The tubes were removed and centrifuged at maximum speed (13500 Rpm) for 10 minutes. The supernatant was transferred into newly labeled eppendorf tubes and stored at -20^0C. The supernatant contained the extracted Plasmodium DNA.

Amplification of Pfcrt gene

PCR was done on a Gene Amp® PCR System 9700 machine. The PCR mixture contained 6.25 µl master mix (One Taq Hot Start DNA Polymerase form New England Biolabs), 0.5 µl primer (Forward: 5'GCGCGCGCATGGCTCACGTTTAGGTGGAG3' and Reverse: 5' GGGCCCGGC-GGATGTTACAAAACTATAGTTACC3'), 4.75 µl nuclease-free water and 1 µl DNA template (from the extracted DNA). The final volume of the PCR mixture was 12.5 µl. Nested PCR was conducted with the product of the first round providing the DNA template for the second round. The mixture was the same as that of the first round apart from the primers (Forward: 5'TGTGCTCATGTGTTTAAACTT3'and Reverse: 5'CAAAACTATAGTTACCAATTTTG3') That were different. The PCR profile was set at 95^0C for 30 seconds, 94^0C for 30 seconds, 57^0C for 30 seconds, 68^0C for 1 minute, 68^0C for 5 minutes and then halted at 5^0C. The PCR took 1 hour 30 minutes for each of the round. Pf3D7 DNA was used as positive control.

Restriction digests of Pfcrt gene

Allele specific restriction analysis was done using restriction endonuclease ApoI (from New England Biolabs). Briefly, the mixture consisted of 0.2 µl ApoI, 1 µl of 10×NE Buffer, 2.3 µl of nuclease-free PCR water and 6.5 µl of DNA template (from the second round of PCR products). The mixture had a final volume of 10 µl. The mixture was incubated at a temperature of 50^0C for 15 minutes. The digested samples were analysed on a 2% agarose gel in TBE buffer for 45 minutes at 120V. Molecular weight marker was run in adjacent wells on the gel.

Ethical consideration

Ethical clearances were obtained from the Institutional review board of the Regional Hospital Bamenda and the Institutional review board at the Faculty of Health Science University of Buea. Administrative authorization to conduct the study in the North West Region was obtained from the North West Regional Delegation of Public Health. Approval to use hospital laboratories was obtained from the Directors of the hospitals that were used in this study. Only individuals who fulfilled the specific inclusion criteria by signing a written informed consent and volunteered to participate after adequate sensitization on the research objectives, possible risks and benefits were enrolled into the study.

Results

A total of 198 blood samples were collected from 198 different subjects that were recruited in the study. *Plasmodium falciparum* DNA was extracted from 191 samples out of the 198 blood sample collected given a 96.5% yield. There were 129 females and 69 males that took part in the study. Parasite density ranged from 3700 to 12500 asexual parasite/µl of blood. All the samples including the control (Pf3D7) were amplified successfully and had the characteristic 145bp fragment. Digestions with ApoI restriction enzyme yielded: 2 bands with 99 bp and 46 bp for chloroquine sensitive, 1 band with 145bp for chloroquine resistance and 3 bands with 145 bp, 99 bp and 46 bp for mixed (Figure 1). Among the 191 samples collected; 149 (78%) were chloroquine sensitive, 24 (12.6%) chloroquine resistance and 18 (9.4%) mixed (Table 1).

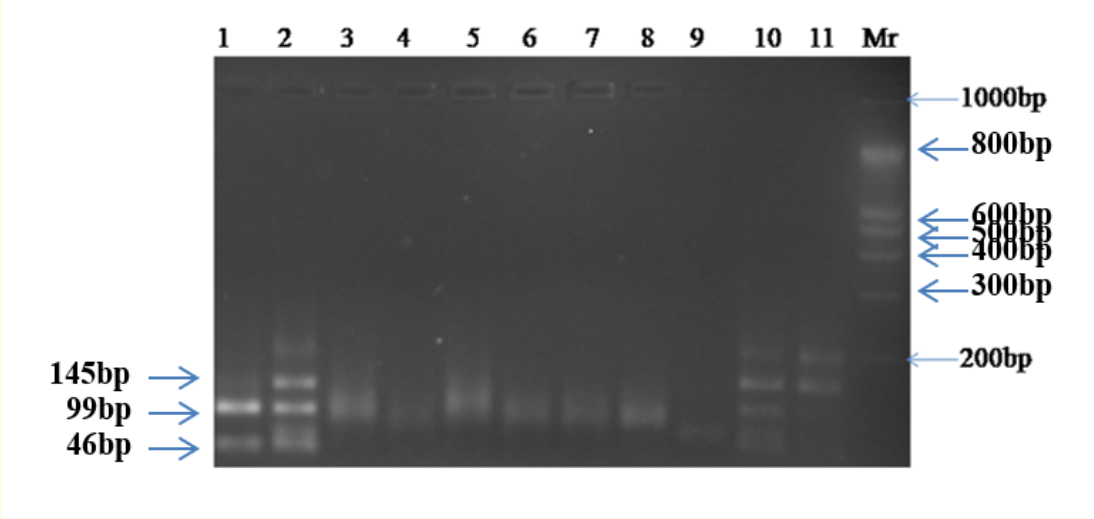

Figure 1: Restriction digests of Pfcrt gene with ApoI. Lane 1-11: Samples wells, Lane Mr: Molecular weight marker.

Haplotype	Numbers	%
CVMNK	149	78
CVIET	24	12.6
Mixed	18	9.4

Table 1: *Prevalence of Pfcrt haplotypes in Bamenda, North West Cameroon. CVMNK (wild type, chloroquine sensitive) and CVIET (mutant, chloroquine resistance).*

Discussion

The origin and spread of chloroquine resistance in *Plasmodium falciparum* have grossly hampered global malaria control programmes. The wide spread parasite resistance to chloroquine on the African continent in the 1990s led to its official ban as a first line treatment drug for uncomplicated malaria [8]. The loss of this effective and cheap antimalaria drug led policy makers to opt for alternative drugs to treat malaria. In the early 2000, the prevalence of chloroquine resistance in Cameroon ranged from 67% in the (Littoral, Central, South, West and North- West), 27% in the East and 13 - 25% in the North [9]. This indicated the failure of chloroquine as a first line antimalarial drug. A key reason behind this treatment failure was the sustained use of chloroquine as a monotherapy in the treatment of uncomplicated malaria for decades since its development [10]. Chloroquine resistance led to the gradual withdrawal of chloroquine and the use of amodiaquine as the first-line drug for the treatment of uncomplicated malaria during the transition period between 2002 and 2004 in Cameroon. This was followed by the adoption of artemisinin-based combination therapy (artesunate-amodiaquine, with artemether-lumefantrine as alternative) in 2006 as first-line treatment [5].

There are five chloroquine resistance haplotypes (CVIET, SVMNT, SVIET, CVMNT and CVTNT). Almost all African countries are populated predominately by the CVIET haplotype except Tanzania and Congo [11]. In the absence of chloroquine, the CVIET haplotype bearing *Plasmodium falciparum* are known to revert back to chloroquine sensitive *Plasmodium falciparum*. This view is supported by the non-usage of chloroquine in some African countries such as Malawi [8] and Kenya [7] which has resulted in reversion to ancestral state

of chloroquine sensitive (CVMNK haplotype) from chloroquine resistance CVIET haplotype. Pfcrt gene located on chromosome 7 has 13 exons and encodes an integral membrane protein located on the intra-erythrocytic parasite digestive vacuole. 20 point mutations in the Pfcrt gene have been associated with chloroquine resistance. These association studies have shown that substitution of threonine (T) for lysine (K) at position 76(K76T) is the hall mark of chloroquine resistance in parasite worldwide [12].

The aim of this study was to assess the current state of *Plasmodium falciparum* resistance to CQ in Bamenda, North West Region of Cameroon. Out of the 198 blood samples collected from participants, DNA was extracted from 191 samples, given a 96.5% yield. This high yield might be due to the use of chelex100 in the extraction process. Chelex100 limits destruction of DNA by inactivating nucleases and chelating heavy metals that may damage parasite DNA. This study indicates that the prevalence of chloroquine resistance in Bamenda is 12.6%. This is a great reduction as compared to 67% in the year 2000. This decrease is in concordance with the Malawi [8], Kenya [7], Senegal [13] and Mozambique [14] reductions after several years of chloroquine withdrawal in these countries. This reduction of chloroquine resistant allele and the re-emergence of chloroquine sensitive allele might be due to the withdrawal of chloroquine as a first line drug in the treatment of uncomplicated malaria in Cameroon. The absence of drug pressure is believed to be the key driver in the re-emergence of chloroquine sensitive parasite in the field [7]. The absence of drug pressure causes the *Plasmodium falciparum* bearing chloroquine resistance allele to lose fitness causing the *Plasmodium falciparum* bearing chloroquine sensitive allele to outgrow the resistance strains [7].

However, the presence of parasite bearing the resistance allele still in circulation in Bamenda might be due to drug pressure exerted by the use of amodiaquine (in the form of Artesunate amodiaquine) in the treatment of uncomplicated malaria. Chloroquine and amodiaquine are 4-aminoquinoline agents. These two drugs act in similar manner against *Plasmodium falciparum* by inhibiting the polymerization of heme. These drugs have similar genetic target (Pfcrt) so amodiaquine resistance by *Plasmodium falciparum* will also favour chloroquine resistance by the parasite [15]. The presence of mixed alleles indicates that an individual can habour both *Plasmodium falciparum* sensitive and resistance strains in a single malaria attack. However, due to loss of fitness as a result of absence of chloroquine, the sensitive strain might out grow the resistance strain. Resistance and sensitive strains will co-occur in the same host but in the absence of drugs, the sensitive clone competitively suppressed the resistant clone; this resulted in lower asexual parasite densities of the resistant clone and also reduced transmission of the resistant clone to the mosquito vector [16]. Nevertheless, in Lagos Nigeria there is a persistence high rate of chloroquine resistance allele (91.6%) four years after chloroquine withdrawal. The key reason is the clandestine use of chloroquine as a monotherapy against uncomplicated malaria in Nigeria [16]. This result from Nigeria suggests that the reduction observed in Bamenda may not be replicated in areas in Cameroon where Chloroquine is clandestinely used in the treatment of uncomplicated malaria.

Conclusion

The result obtained on the prevalence of chloroquine resistance (12.6%) in Bamenda, provides the first evidence of the decline in chloroquine resistance in this area. This decline could be attributed to the withdrawal of chloroquine as first line treatment of uncomplicated malaria in Cameroon. However, the 12.6% is still higher than world Health organisation maximum threshold of 10% drug resistance which ensures the removal of an antimalarial drug on the basis of failed efficacy. It is important to ensure the rational use of Artemisinin combination therapies (ACTs) for the treatment of uncomplicated malaria in this area. While doing so, it would be advisable to investigate if the results of this study can be replicated in other areas in Cameroon. If chloroquine sensitive *Plasmodium falciparum* is restored in Cameroon, chloroquine which is a cheap and effective antimalarial drug can be in future incorporated into malaria chemotherapy alongside ACTs for the treatment of uncomplicated malaria.

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