Occurrence of multiple chloroquine-resistant Pfcrt haplotypes and emergence of the S_{aglt} VMNT type in Cameroonian Plasmodium falciparum

Huguette Gaelle Ngassa Mbenda and Aparup Das*

Evolutionary Genomics and Bioinformatics Laboratory, Division of Genomics and Bioinformatics, National Institute of Malaria Research, Sector 8, Dwarka, New Delhi 110077, India

*Corresponding author. Tel: +91–11-25307-322; Fax: +91–11-25307-377; E-mail: aparup@mrcindia.org

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Objectives: The main objective of this study was to unravel the distribution of different Pfcrt genotypes in the central, littoral, eastern and southern regions of Cameroon and also in locations bordering Gabon and Equatorial Guinea. This is because (i) the chloroquine-resistant malaria parasite Plasmodium falciparum shows a wide occurrence in Cameroon, (ii) mutations in the 72nd to 76th amino acid positions of the Pfcrt gene are known to confer resistance to chloroquine, and (iii) only a single chloroquine-resistant haplotype (C72V73I74E75T76) has so far been reported in Cameroon.

Methods: We followed a molecular approach with DNA sequencing of the second exon of the Pfcrt gene to identify single nucleotide polymorphisms in 180 P. falciparum field isolates sampled in five different locations in Cameroon.

Results: The chloroquine-resistant Pfcrt CVIET haplotype was most abundant, followed by the wild-type CVMNK haplotype. Five hitherto unreported chloroquine-resistant Pfcrt haplotypes were detected for the first time in Cameroonian P. falciparum, including the surprise appearance of the S_{aglt} VMNT haplotype.

Conclusions: The high observed haplotype diversity of the chloroquine-resistant Pfcrt gene and the appearance of the S_{aglt} VMNT haplotype are daunting and can be attributed to drug pressure and/or the misuse of chloroquine and/or amodiaquine in Cameroon.

Keywords: chloroquine resistance, P. falciparum, malaria, Cameroon

Introduction

The evolution and spread of the chloroquine-resistant malarial parasite Plasmodium falciparum have posed a great challenge to malaria-control efforts in all countries where malaria is endemic. Although chloroquine has been banned from national malaria-control programmes in many countries with endemic malaria, it still continues to be used unofficially in many countries, including Africa, because of its high efficacy, easy availability and affordability.1–4 Furthermore, in countries with endemic malaria such as India and south-east Asia where the malarial parasites Plasmodium vivax and P. falciparum co-occur, and due to the susceptibility of the former to chloroquine, this drug is still used to treat uncomplicated P. vivax malaria. Thus, continuous drug pressure exerted by chloroquine usage has possibly helped in the emergence of new varieties of chloroquine-resistant P. falciparum isolates in these epidemiological settings that have thereafter spread to other countries with endemic malaria that have previously been dominated by chloroquine-susceptible isolates.
different genetic types based on the nucleotide sequence arrangements—S(agt)VMNT and S(tct)VMNT—and these have different evolutionary histories. While the S(agt)VMNT type has been reported to have originated in Papua New Guinea, the S(tct)VMNT has a South American origin.6,7 Malaria due to *P. falciparum* is highly endemic in Cameroon, with several reports of chloroquine-resistant isolates in the field.2,8,9 Genotyping of the 72nd to 76th amino acid positions of the *Pfcrt* gene is the best way to determine the molecular and genetic epidemiology of chloroquine-resistant malaria, and previous studies have indicated that, in Cameroon, the chloroquine-resistant malaria parasites are categorized mostly into the CVIET type.6 Considering the high migration potentiality of malarial parasites, with the recent entry of chloroquine-resistant *P. falciparum* to many African countries,5 we hypothesize that Cameroon, which is considered to be ‘Africa in miniature’ for malaria (H. G. Ngassa Mbenda, G. Awasthi, P. K. Singh, I. Gouada and A. Das, unpublished results), could also have been populated with chloroquine-resistant parasites with different *Pfcrt* genetic backgrounds. We therefore conducted a large-scale molecular epidemiological study with highly sensitive DNA sequencing of the *Pfcrt* gene in 180 *P. falciparum* parasites collected in the southern, eastern, littoral and central regions of Cameroon and also in locations bordering Gabon and Equatorial Guinea in order to describe the distributional patterns of different chloroquine-resistant *Pfcrt* haplotypes in Cameroon.

**Methods**

We used a PCR-based diagnostic assay10,11 to identify *P. falciparum* mono-infections following DNA isolation from finger-prick blood samples.11 Altogether, 180 monoclonal isolates from six different district hospitals in five different locations (Ebolowa: south, 41 isolates; Douala: littoral, 62 isolates (37 from District Hospital Bomassa and 25 from District Hospital New-Bell); Yaoundé: central, 24 isolates; Bertoua: eastern, 21 isolates; and Kye-ossi: bordering Gabon and Equatorial Guinea, 32 isolates) (Figure 1) covering almost all of the southern area (the most malarious region) of Cameroon were used in the present study. To determine the amino acid sequences of the *Pfcrt* polypeptide chain, the entire second exon of the *Pfcrt* gene was PCR-amplified using published primers12 following standard protocols: initial denaturation at 95 °C for 5 min; 35 cycles of denaturation at 95 °C for 50 s, annealing at 58.9 °C for 1 min and elongation at 72 °C for 1 min; and final elongation at 72 °C for 5 min. The PCR products were then purified using the Exonuclease-I and Shrimp Alkaline Phosphatase (Fermentas, Life Sciences) and processed for DNA sequencing following standard protocols from Applied Biosystems.

Each DNA fragment was sequenced in both directions (2× coverage) and the products were run in an ABI 3730xl DNA Analyzer (in-house facilities at the National Institute of Malaria Research). The DNA sequences were assembled and edited using the SeqMan and EditSeq computer programs (DNAStar, Madison, USA), respectively. All 180 sequences were aligned using the MegAlign computer program (DNAStar, Madison) and scanned for single nucleotide polymorphisms (SNPs) present in the 72nd to 76th amino acid positions to define different *Pfcrt* haplotypes. The study was approved by the Ethics Committee of Cameroon (No003/CNE/SE/2012) and written informed consent was obtained from all adult patients and the guardians of the minor patients participating in the study.

**Results and discussion**

Scanning for SNPs in the 72nd to 76th amino acid positions of the *Pfcrt* gene in 180 *P. falciparum* isolates from five locations in...
Cameroon revealed the presence of seven different haplotypes including the wild-type C275Y73M2N47K76. It is known that the CVMNK haplotype represents chloroquine–susceptible parasites, and in the present study this haplotype was found to be distributed in all five locations of Cameroon in variable frequencies (Figure 1). In total, 65 isolates (36%) were of CVMNK type—meaning that about one-third of P. falciparum isolates are still susceptible to chloroquine in Cameroon. Out of the remaining 115 P. falciparum isolates bearing the chloroquine-resistant Pfcrt genotype, a major proportion (87%) was of CVIET type, corroborating previous observations in Cameroon.\(^4\) The remaining 13% of P. falciparum isolates bearing chloroquine-resistant Pfcrt genotypes were segregated into five different haplotypes (SVMNT, 5.2%; SVMET, 1.7%; CVMDT, 3.5%; CVMET, 0.9%; and CVMNT, 1.7%) (Figure 1). Notably, in previous studies,\(^2,4,13\) none of these five haplotypes was reported in Cameroon.

Out of the five locations in Cameroon, samples from Douala were found to bear all seven Pfcrt haplotypes including CVMNK (Figure 1), representing the highest haplotype diversity of the Pfcrt gene. However, in Douala, the CVMNK haplotype was present in a fairly low proportion (27.4%) in comparison with the four other locations. The SVMNT haplotype, which is considered to be the fittest one in a chloroquine environment in comparison with other haplotypes,\(^6,14\) was found only in Douala. Since our dataset is based on DNA sequencing, we were able to further determine the nucleotide composition of the SVMNT haplotype. Interestingly, all six parasite isolates bearing the SVMNT haplotypes were found to be of S\(\text{agt}^\text{VMNT}\) type.\(^15\) In addition, the other four chloroquine-resistant Pfcrt haplotypes found in Douala were found to be distributed in comparatively higher frequencies than in the four other Cameroonian locations.

The results indicating a high haplotype diversity of the chloroquine-resistant Pfcrt gene essentially mean that the Pfcrt gene governing chloroquine resistance in P. falciparum is under massive genetic reconstruction in Cameroon, as has been proposed for India.\(^5,16\) Such genetic reformation might have been propelled by (i) selection pressure exerted by a misuse of chloroquine in the field\(^16\) and/or (ii) pressure exerted by amodiaquine, an antimalarial that is part of WHO-recommended artemisinin combined therapy for the treatment of uncomplicated P. falciparum malaria in Cameroon. The second contention is justified by the fact that amodiaquine has a very similar genetic target (Pfcrt) to chloroquine.\(^6,14\) Although the S\(\text{agt}^\text{VMNT}\) haplotype was found only in Douala, and considering its higher fitness property and the fact that Douala is a cosmopolitan city in Cameroon, it could spread to other parts fairly quickly.

Whatever the case may be, observations on the high haplotype diversity of the chloroquine-resistant Pfcrt haplotypes coupled with the appearance of the S\(\text{agt}^\text{VMNT}\) type is daunting and could pose a greater challenge than before to Cameroon’s malaria control programme. This is true since it has been predicted from previous studies\(^5,16\) that the continued use of amodiaquine in combination therapies is hazardous in regions where P. falciparum isolates bearing the SVMNT haplotype occur. Similar large-scale DNA sequencing-based surveys of the Pfcrt gene in other parts of Cameroon and in other African countries would help not only in unravelling the extent of the diversity of different chloroquine-resistant Pfcrt haplotypes, but also in choosing appropriate chemotherapeutic combinations for the treatment of malaria in Africa.

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Transparency declarations
None to declare.

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