MOLECULAR EPIDEMIOLOGY OF MALARIA IN YAOUNDE, CAMEROON V.
ANALYSIS OF THE OMEGA REPETITIVE REGION OF THE
PLASMODIUM FALCIPARUM CG2 GENE AND CHLOROQUINE RESISTANCE

LEONARDO K. BASCO and PASCAL RINGWALD
Institut de Recherche pour le Développement (ORSTOM) and Laboratoire de Recherche sur le Paludisme, Laboratoire Associé
Francophone 302, Organisation de Coordination pour la lutte contre les Endémies en Afrique Centrale (OCEAC),
Yaoundé, Cameroon

Abstract. A novel Plasmodium falciparum gene, denoted cg2 gene, has been recently discovered, and a distinct
genotype, characterized by 12 point mutations and 3 size polymorphisms, has been shown to be associated with
chloroquine resistance in laboratory-adapted parasite strains. One of the polymorphic regions, denoted the omega
region, consists of 16 tandem repeat units in chloroquine-resistant strains, while the chloroquine-sensitive strains
have either ≤ 15 or ≥ 17 repeat units. In this study, the in vivo and in vitro responses were compared with the
number of repeat units in the omega region of the cg2 gene for 75 Cameroonian isolates determined either by
DNA sequencing or agarose gel electrophoresis. The 16-repeat units that characterize the resistant strains were
found in 10 chloroquine-sensitive isolates (50% inhibitory concentration [IC50] < 100 nM) and 30 chloroquine-
resistant isolates (IC50 ≥ 100 nM). Thirty-five isolates (28 chloroquine-sensitive isolates and 7 chloroquine-resistant
isolates) displayed ≤ 15 or ≥ 17 repeat units. Of the 18 patients responding with treatment failure, 15 were infected
with parasites carrying 16 repeat units. Twenty-eight patients (11 with isolates carrying 16 repeat units and 17 with
isolates carrying ≤ 15 or ≥ 17 repeat units) showed an adequate clinical response. The sensitivity, specificity, and
predictive value were 81% (83%), 74% (61%), and 75% (58%), respectively compared with in vitro (or in vivo)
responses. Neither the level of IC50 nor the key P. falciparum multidrug resistance gene 1 (pfmdr 1) allele at
position 86 was associated with the number of omega repeat units. Although in vitro and in vivo resistance to
chloroquine was statistically associated with the presence of 16 repeat units in the omega region (P < 0.05), the
number of omega repeat units did not adequately discriminate patients infected with chloroquine-resistant parasites
from those infected with chloroquine-sensitive parasites. Further studies on the cg2 gene are needed to determine
whether cg2 gene is a reliable genetic marker for chloroquine resistance.

Chloroquine is still the first-line drug for the treatment of acute uncomplicated Plasmodium falciparum malaria in
many African countries. Chloroquine is safe, well-tolerated, and highly effective against P. vivax, P. ovale, P. malariae
and drug-sensitive P. falciparum. The drug is also cheap and widely available in sub-Saharan Africa. Chloroquine is
generally perceived to be a standard drug by local populations who are directly exposed to malaria. It probably plays an
important role in self-medication, especially for the initial treatment of sick children at home, which may prevent or
delay the development of cerebral malaria. In addition, chloroquine, with or without proguanil, is currently one of the
major chemoprophylactic regimens for pregnant women in endemic countries. These reasons reinforce the place that
chloroquine occupies among the essential drugs in tropical and subtropical countries and emphasize the need to under-
stand the mechanism of chloroquine resistance for the implementation of national drug policies and rational use of
antimalarial drugs.

Neither the mechanism of action of chloroquine nor the mechanism of resistance to chloroquine has been completely
elucidated. Its mechanism of action may be based on pH-dependent and/or transporter-mediated accumulation of the
drug in the digestive vacuole, followed by an inhibition of malarial enzymes and/or non-enzymatic heme polymeriza-
tion. The mechanism of resistance to chloroquine is probably based on the decreased accumulation of the drug in the
digestive vacuole, which may be due to either a rapid efflux of the drug, a slower influx process, or a decreased pH gra-
dient in the resistant strains. More experimental data are needed to determine which of these biochemical processes
predominate in resistant strains.

Studies on the parasite genome have identified 2 candidate genes that may explain the underlying cause of the chloro-
quine-resistant phenotype. The P. falciparum multidrug-resistant genes 1 and 2 (pfmdr 1 and pfmdr 2) are homologous
to the members of the gene family that are implicated in transport processes of various substrates, including drugs. Although the pfmdr 1 gene, but not pfmdr 2, codes for a transmembrane protein capable of transporting drugs across the membrane and point mutations may alter its function, its role in resistance to 4-aminoquinolines and aminoalcohols is
still unclear. A second candidate, cg2 gene, has been recently isolated and characterized. Its functional role is cur-
rently unknown.

Su and others have observed that in laboratory-adapted, chloroquine-resistant strains the genotype at 12 widely sep-
arated amino acid residues and 3 polymorphic regions is identical. In contrast, the genotype defined by these posi-
tions varies considerably in chloroquine-sensitive strains, with no single genotype associated with chloroquine sensi-
tivity. If the chloroquine-resistant genotype is indeed confirmed to be identical in all resistant strains from various
graphic regions, it may serve as a molecular basis to differentiate between a chloroquine-sensitive isolate from a
chloroquine-resistant isolate and may be a helpful clue for the development of a diagnostic tool. For diagnostic purpos-
es, however, it is impractical to determine the full sequence of the 3 polymorphic regions and 12 amino acid residues,
most of which do not correspond to a restriction site of commercially available restriction endonucleases. To simplify
the diagnostic test, which may be readily adopted in African research centers, we evaluated the reliability of a polymerase
Materials and Methods

Patients. The study was part of recent randomized clinical trials conducted at the Nongkak Catholic missionary dispensary in Yaoundé. Clinical isolates of *P. falciparum* were obtained by venipuncture before treatment from symptomatic Cameroonian patients residing in Yaoundé. The following inclusion criteria were used for enrollment: age ≥ 5 years old, fever at consultation (or history of fever within the past 24 hr), monoinfection with *P. falciparum* (parasite density > 5,000 asexual parasites/µl of blood), easy access to health services in Yaoundé for daily monitoring, and no recent history of self-medication with antimalarial drugs, as confirmed by a negative Saker-Solomons urine test result. Patients with signs and symptoms of severe and complicated malaria, as defined by the World Health Organization (WHO), severe anemia (hemoglobin < 5.0 g/dL), or moderate and severe malnutrition were excluded. Our previous studies have shown that clinical parameters and hematologic and blood biochemistry do not differ significantly between the adult population (> 15 years old) and the pediatric population (between 5 and 15 years old) in Yaoundé. Informed consent was obtained from either the patients or a guardian accompanying the sick children. The study was approved by the Cameroonian National Ethics Committee and the Cameroonian Ministry of Public Health.

Parasite DNA. Three reference clones (W2/Indochina, 7G8/Brazil, and 3D7/unknown origin) were maintained in a continuous *in vitro* culture, according to the standard technique described by Trager and Jensen. These clones were kindly provided by Professor David Walliker (Institute of Cell, Animal and Population Biology, University of Edinburgh, Edinburgh, United Kingdom).

Seventy-five isolates were selected from our collection of parasites obtained between 1994 and 1997 to analyze an equal number of chloroquine-sensitive and chloroquine-resistant isolates. An aliquot of 1.5–2 ml of red blood cell pellet was used to extract parasite DNA. Infected erythrocytes were suspended in 15 ml of ice-cold NET buffer (150 mM NaCl, 10 mM Tris, pH 7.5) and lysed with 0.015% saponin. The lysate was centrifuged at 2,000 × *g* for 10 min and the pellet was transferred to a 1.5-ml microfuge tube and suspended in 500 µL of NET buffer. The mixture was treated with 1% N-lauroylsarcosine and RNase A (100 µg/ml) at 37°C for 1 hr and proteinase K (200 µg/ml) at 50°C for 1 hr. Parasite DNA was extracted 3 times in equilibrated phenol (pH 8), phenol-chloroform-isomyl alcohol (v/v/v 25:24:1), and chloroform-isomyl alcohol (v/v 24:1) and precipitated by the addition of 0.3 M sodium acetate and cold absolute ethanol. The extracted DNA was dried and resuspended in TE buffer (10 mM Tris, 1 mM EDTA). Parasite DNA was stored at −20°C until use.

Polymerase chain reaction. Two pairs of specific oligonucleotide primers were designed based on the complete nucleotide sequence of the cg2 gene published by Su and others. The nested polymerase chain reaction was performed to amplify the omega repeat domain of the cg2 gene. A 4.7-kilobasepair DNA fragment was initially amplified by the polymerase chain reaction using the PTC-100 thermal cycler (MJ Research, Watertown, MA) under the following conditions: approximately 200 ng of genomic DNA, 20 pmol of primers 5′-GGAGACACACACACTAAAAGGGAATGAA-AAG-3′ (sense) and 5′-ATTCAATGAGCAATTATTTACCGA-TITTTCT-3′ (anti-sense), buffer (50 mM KCl, 10 mM Tris, pH 8.3), 2.25 mM MgCl2, 500 µM dNTP, and 2.6 units of a mixture containing Taq and Pwo DNA polymerases (Expand Long Template PCR System; Roche Diagnostics, Meylan, France) in a 50-µl reaction at 94°C for 2 min in the first cycle and 1 min in subsequent cycles, 60°C for 5 min in the first cycle and 2 min in subsequent cycles, and 68°C for 10 min in all cycles, with an additional extension step of 68°C for 15 min after the completion of 30 cycles. A second round of polymerase chain reaction was performed under the following conditions: amplification product diluted to 1/1,000 (2–4 µl), 15 pmol of internal primers 5′-GCCGAAAGGTAGATAAAGATGG-3′ (sense) and 5′-TGATATACTCCTCCTCCCACACTACATCG-3′ (anti-sense), buffer (50 mM KCl, 10 mM Tris, pH 8.3, and 1.5 mM MgCl2) for Tag DNA polymerase; 25 mM KCl, 5 mM (NH4)2SO4, 10 mM Tris, pH 8.8, and 2 mM MgSO4, for Pwo DNA polymerase; 200 µM dNTP and 1 unit of Tag or Pwo DNA polymerase in a 50-µl reaction at 94°C for 2 min for the first cycle and 30 sec in subsequent cycles, 60°C for 1 min for the first cycle and 30 sec in subsequent cycles, and 72°C for 1 min for all cycles, for 30 cycles, followed by a 10-min extension step at 72°C. The Pwo DNA polymerase was used to obtain amplification products that were sequenced. The amplified DNA fragments were resolved by electrophoresis in a 1.8% agarose gel, stained with ethidium bromide, and visualized under ultraviolet transillumination.

Sequencing of DNA. The sequences of 21 amplified products, including those of the reference clones W2/Indochina, 7G8/Brazil, and 3D7/unknown origin, were determined by a single-stranded sequencing protocol. The amplified products that yielded 2 or more distinct bands were not sequenced. Briefly, the nested polymerase chain reaction was performed as described above, using an oligonucleotide primer that was phosphorylated at the 5′-end by T4 polynucleotide kinase. The amplified products were purified with glass beads (Jetset; Genomed Inc., Research Triangle Park, NC) and treated with lambda exonuclease (Gibco-BRL Life Technologies, Cergy Pontoise, France) to generate single-stranded DNA. The single-stranded template was used to sequence the amplified product by the dyeoxy chain termination reaction.

According to the criteria set by Su and others, *in vitro* chloroquine resistance is associated with the presence of 16 repeat units in the omega polymorphic region. *In vitro* chloroquine sensitivity is associated with either 17 or more repeat units.
peat units or 15 or less repeat units. There are at least 6 variants of the omega repeat units composed of 6–8 amino acids. The size of the entire 16-unit fragment generally corresponds to 309 basepairs. To identify clinical isolates with size variations in the omega region, the size of the amplified bands was compared with that of W2/Indochina clone by agarose gel electrophoresis. Our reference band derived from the W2/Indochina clone is 405 basepairs, including the conserved sequences spanning the omega repeat region. The results of the size variations in the omega region were compared with the allele at position 86 of the pfmdr 1 gene, which was determined in our previous study. 

**In vitro drug sensitivity assay.** The in vitro assay was performed for all isolates without prior adaptation to in vitro culture conditions. Infected erythrocytes were washed 3 times in RPMI 1640 medium and suspended in RPMI 1640 plus 10% human serum (obtained from European blood donors without a history of malaria), 25 mM HEPES, and 25 mM NaHCO3 at a hematocrit of 1.5% and an initial parasitemia ranging between 0.2% and 1.0%. If the blood sample had a parasitemia > 1.0%, fresh, uninfected type A+ erythrocytes were added to adjust the parasitemia to 0.6%.

The isotopic semi-microtest and microtest used in this study were described in previous studies. Statistical analysis. The chloroquine-sensitive and the chloroquine-resistant isolates analyzed in this study were selected from our list of available samples (with in vitro results) arranged in the chronological order of patient inclusion in our randomized clinical studies. Since our in vitro studies conducted during the same period from 1994 to 1997 showed in a larger sample size (> 400 isolates) that about 50% of Cameroonian isolates obtained in Yaounde are chloroquine-resistant, any random selection of isolates from our sample is expected to yield an unbiased sample of approximately equal number of sensitive and resistant isolates. Based on the chloroquine-resistant genetic profile established by Su and others, data of clinical isolates presenting ≤ 15 or ≥ 17 omega repeat units were pooled together and compared with those of the isolates presenting 16 repeat units. Qualitative data were arranged in 2 × 2 contingency tables. Fisher’s exact test was used to test whether there is an association between the number of omega repeat units and chloroquine sensitivity or the pfmdr 1 genotype. The results of the diagnostic test based on the number of omega repeat units were gauged against the in vitro and in vivo responses to determine the validity of the test. The unpaired t-test was used to compare the mean chloroquine IC50 of the isolates with 16 repeat units and those with either ≤ 15 or ≥ 17 repeat units.

**RESULTS**

All 75 clinical isolates and 3 reference clones yielded an amplification product. The size of the amplified fragment varied from approximately 280 basepairs (corresponding to 9–10 repeat units) to 750 basepairs (corresponding to 32–33 repeat units). Sixty-eight of 75 samples yielded a single band. There were 2 or more visible bands in the other samples. DNA sequencing showed that 7G8/Brazil, W2/Indochina, and 3D7 reference clones have 14, 16, and 19 repeat units, respectively. The number of repeat units was determined by DNA sequencing for 18 clinical isolates. The number of repeat units in the omega region for 57 clinical isolates was determined by comparing their size with that of 18 isolates and 3 reference clones that were sequenced.
Since the exact number of repeat units cannot be determined with precision for all isolates, the results were expressed as 16 repeat units or either ≤15 or ≥17 repeat units. A typical result on an agarose gel is presented in Figure 1.

The in vitro sensitivity to chloroquine was determined in all 75 clinical isolates. Thirty-seven isolates were chloroquine-resistant in vitro, while 38 were chloroquine-sensitive (Table 1). Forty isolates (10 chloroquine-sensitive isolates and 30 chloroquine-resistant isolates) had 16 repeat units in the omega region. Five of these isolates had additional bands, suggesting polyclonality. Thirty-five isolates (28 chloroquine-sensitive isolates and 7 chloroquine-resistant isolates) displayed ≤15 repeat units (n = 18), ≥17 repeat units (n = 16), or 2 bands (n = 1). In vitro chloroquine resistance was associated with the presence of 16 repeat units (P < 0.001). Of the 75 patients enrolled in this study, 46 were treated with chloroquine and completed the 14-day follow-up. Of the 28 patients with an adequate clinical response, 11 were infected with isolates displaying 16 repeat units, and 17 had isolates with ≤15 and/or ≥17 repeat units. Of the 18 patients with treatment failure, 15 were found to be infected with parasites carrying 16 repeat units. The presence of 16 repeat units was statistically associated with therapeutic failure (P = 0.006). The sensitivity, specificity, and predictive value were 81%, 74%, and 75%, respectively, when compared with in vitro responses, and 83%, 61%, and 58%, respectively, in relation to in vivo therapeutic responses.

The presence of 16 omega repeat units in the cg2 gene was compared with the key allele (amino acid residue 86) of the pfmdr1 gene in 55 clinical isolates. A large majority of the isolates (42 of 55, 76%) had the mutant Tyr-86 allele in the pfmdr1 gene. There was no association between the pfmdr1 genotype and the number of repeat units in the omega region (P = 0.52, not significant).

The chloroquine-sensitive isolates and the chloroquine-resistant isolates presenting ≤15, 16 (with a single band or with additional bands indicating polyclonality), or ≥17 omega repeat units were grouped together and their mean levels of chloroquine sensitivity and chloroquine resistance were calculated for comparison (Table 2). The mean IC50 did not differ significantly for chloroquine-sensitive isolates presenting either ≤15 (n = 14 isolates) or ≥17 (n = 13 isolates) omega repeat units (P > 0.05), chloroquine-sensitive isolates with either 16 (n = 10) or ≤15 or ≥17 (n = 27) omega units (P > 0.05), and chloroquine-resistant isolates displaying either 16 (n = 30) or ≤15 or ≥17 (n = 7) repeat units (P > 0.05).

### Table 1

<table>
<thead>
<tr>
<th>No. of repeat units</th>
<th>In vitro test of therapeutic efficacy</th>
<th>pfmdr1</th>
</tr>
</thead>
<tbody>
<tr>
<td>In vitro assay</td>
<td>Adequate clinical response</td>
<td>Treatment failure</td>
</tr>
<tr>
<td>16</td>
<td>10</td>
<td>30</td>
</tr>
<tr>
<td>≤15</td>
<td>14</td>
<td>4</td>
</tr>
<tr>
<td>≥17</td>
<td>14</td>
<td>3</td>
</tr>
</tbody>
</table>

*Data are the number of clinical isolates with 16 (with additional bands in 5 polyclonal isolates), ≤15, or ≥17 repeat units in the omega polymorphic region of the cg2 gene. One chloroquine-sensitive isolate obtained from a patient with an adequate clinical response presented two bands (≤15 and ≥17 repeat units) and was classified as ≤17 repeat units. The presence of 16 units in the omega region was associated with in vitro chloroquine resistance by 2e and others.18*
**Table 2**

<table>
<thead>
<tr>
<th>No. of repeat units</th>
<th>Chloroquine-sensitive</th>
<th>Chloroquine-resistant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of isolates</td>
<td>Geometric mean</td>
</tr>
<tr>
<td>≤15</td>
<td>14</td>
<td>31.4</td>
</tr>
<tr>
<td>≥17</td>
<td>13</td>
<td>26.3</td>
</tr>
<tr>
<td>16</td>
<td>7</td>
<td>28.9</td>
</tr>
<tr>
<td>16 +</td>
<td>3</td>
<td>37.6</td>
</tr>
</tbody>
</table>

*Number of repeat units in the omega polymorphic region of the cg2 gene. IC50 refers to the presence of a band corresponding to 16 repeat units and additional band(s) after agarose gel electrophoresis. According to Su and others, the presence of 16 repeat units is associated with in vitro chloroquine resistance; chloroquine-sensitive parasites have ≥15 or ≤17 repeat units.

**DISCUSSION**

The regular surveillance of the *in vitro* chloroquine resistance conducted at our laboratory by isotopic microtest or semi-microtest has shown that 50–60% of *P. falciparum* isolates in Yaoundé have become resistant to the drug.25,29–31

We have also demonstrated that a similar proportion of Cameroonians, including adults and children ≥ 5 years old, failed to clear asexual parasitemia and symptoms associated with malaria infection within 14 days after the standard chloroquine therapy.18,19,27 Thus, our *in vitro* and *in vivo* data are concordant and support the observation that a relatively high proportion of *P. falciparum* isolates in Yaoundé are chloroquine-resistant. The epidemiology of drug-resistant malaria has not been studied regularly in other localities in Cameroon. Despite the spread of chloroquine resistance, Cameroonians health authorities still recommend the use of chloroquine for the first-line treatment of acute uncomplicated *falciparum* malaria, as in most of central and west African countries. However, unlike in the other African countries, amodiaquine is also recommended as an alternative first-line drug in Cameroon. The second-line and third-line drugs in Cameroon and many other central and west African countries are sulfadoxine-pyrimethamine and quinine, respectively.

Although chloroquine is still the first-line antimalarial drug in most of the African continent, its decreasing efficacy has been reported from all over the continent.28 Two methods, *in vitro* drug sensitivity assays and *in vivo* tests for drug resistance, have been used to define the spatial distribution of chloroquine resistance in Africa. The results of isotopic *in vitro* tests are moderately concordant with the standard, simplified *in vivo* test developed by the WHO, and *in vitro* assays may be a useful adjunct for the epidemiologic survey of drug resistance.27 However, isotopic *in vitro* assays require sophisticated equipment and technical skills. Thus, *in vitro* assays should probably be reserved for advanced African research centers. The new WHO *in vivo* test of therapeutic efficacy is adapted for field research in Africa,28 but its wide adoption and application to guide the national drug policy await further evaluation. In addition, because of various limitations in the interpretation of *in vitro* and *in vivo* responses to chloroquine in different epidemiologic situations,27 it is important to determine which measure should be the standard marker for drug resistance to which other markers for chloroquine resistance can be compared. The development of a standard method will also allow a more rational approach for the surveillance of drug resistance.

Another method that may be applicable in the field for mass surveys to define areas of chloroquine resistance is molecular approaches, as illustrated in the present study. Such studies are now feasible to map pyrimethamine and sulfadoxine resistance since the genes associated with drug resistance, dihydrofolate reductase-thymidylate synthase and dihydropterotate synthase genes, have been identified and characterized.25–37 We have applied both relatively simple molecular techniques based on the polymerase chain reaction and more sophisticated techniques involving vector cloning and DNA sequencing in an effort to define the epidemiology of drug resistance in Yaoundé.36–40

The present study is an extension of our previous work, which sought to apply relatively simple, rapid, and reproducible techniques based on molecular biology and develop novel approaches for the epidemiologic description of drug resistance in malaria parasites. Initial studies have suggested that the key point mutation in the amino acid residue 86 of the first gene candidate for chloroquine resistance, *pfmdr 1*, may be associated with the resistant phenotype.51,52 However, subsequent studies conducted at our study site did not confirm the association between the *pfmdr 1* allele and *in vitro* and *in vivo* chloroquine resistance in Cameroon.17,43

In the present study, we assessed the validity of the test based on the determination of the number of omega repeat units in the *cg2* gene to detect chloroquine resistance. Although the presence of 16 omega repeat units was statistically associated with chloroquine resistance and the sensitivity and specificity of the polymerase chain reaction–based diagnostic test were 81% and 74%, respectively, compared to the *in vitro* assay, 30 of 40 isolates (predictive value = 75%) carrying 16 omega repeat units were actually chloroquine-resistant in *in vitro*. The predictive value of the diagnostic test, when gauged against the *in vivo* therapeutic test, was even lower. In addition, the number of omega repeat units was not associated with the level of *in vitro* chloroquine resistance expressed as IC50 values. These results seem to suggest that, at least in individual patients, the diagnostic test based on the number of omega repeat units is probably not sufficiently accurate to discriminate patients infected with chloroquine-resistant parasites from those infected with chloroquine-sensitive parasites. Further correlational studies between the drug-resistant phenotype and *cg2* genotype are needed to determine whether *cg2* gene is a reliable genetic marker for chloroquine resistance.

Acknowledgments: We thank Sisters Solange Menard and Marie-Solange Oko and their nursing and laboratory staff at the Nlongkak Catholic mission for precious aid.

Financial support: The study was financed in part by Agence Universitaire de la Francophonie and the French Ministry of Cooperation and Development.

Authors' address: Leonardo K. Basco and Pascal Ringwald, OCEAC/IRD, BP 288, Yaoundé, Cameroon.
REFERENCES


