GENOME METHODS

Detection of RAPD Markers Correlated with Chloroquine Resistance in *Plasmodium falciparum*

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We described the use of the random amplified polymorphic DNA (RAPD) technique on *Plasmodium falciparum* DNA to detect genetic markers for chloroquine-resistant strains. Fourteen RAPD primers were tested, three of which generated banding patterns correlated with chloroquine resistance. To measure this correlation, the RAPD profiles were analyzed using the Nei and Li similarity coefficient. Detection of distinctive RAPD bands allowed us to synthesize specific PCR primers to be used on whole-blood samples. Two primer sets were synthesized and tested on sensitive and resistant strains for their ability to amplify the DNA fragment corresponding to the RAPD marker. These results suggest that RAPD and PCR techniques can be used as powerful tools for the detection of genetic markers associated with drug resistance.

[The nucleotide sequence data described in this paper have been submitted to the EMBL, GenBank, and DDBJ Nucleotide Sequence databases under accession nos. A863W25C and U854T57L]

Malaria, with an estimated 300 to 500 million cases and 1.5–2.7 million deaths per year (World Health Organization 1996), is a major health problem. Today, one of the main obstacles encountered in the control of this disease is the rapid spread of drug resistant *Plasmodium falciparum* strains (Slater 1993). Chloroquine, the drug most widely used in the world, is the main issue involved with this problem. Extensive literature deals with biochemical and genetic mechanisms of chloroquine resistance but there is still no substantial data available to assess the precise mechanisms underlying this phenomenon. Chloroquine-resistant parasites are known to accumulate less of the drug in their digestive vacuole than sensitive parasites, however the biochemical basis is yet to be determined as to whether it is linked with increased efflux of pre-accumulated drug or reduced chloroquine uptake (Slater 1993). The hypothesis of a rapid efflux system linked to the *Pfmdr 1* gene (Krogstad et al. 1987; Wilson et al. 1989; Foote et al. 1990) has now been questioned by a number of authors (Slater 1993; Cremer et al. 1995). The genetic basis is also controversial. Data has been published suggesting the linkage of chloroquine resistance with either a unique locus (Wel-

lems et al. 1991) or a multigenic mechanism (Karcz and Cowman 1991; Ward et al. 1995).

In this context, it would be of great interest to determine genomic profiles of malaria parasites and to compare those obtained with chloroquine-sensitive and chloroquine-resistant *P. falciparum* strains. As no similar data is available, we performed the random amplified polymorphic DNA (RAPD) analysis, which represents a powerful tool for genome characterization (Welsh and McClelland 1990; Williams et al. 1990; Lymbery 1996), on 13 different strains (5 sensitive and 8 resistant to chloroquine). The Nei and Li (NL) coefficient (Nei and Li 1979) was used to score the DNA polymorphisms. The purpose of this study was to find a correlation between RAPD banding patterns and the behavior of the parasite in relation to chloroquine sensitivity and therefore obtain genetic markers associated with the drug susceptibility phenotype of the *P. falciparum* strains.

RESULTS

RAPD Analysis

The parasite strains were screened using 14 10-mer primers: OPR1–OPR14. Three had generated fingerprints of interest—OPR1, OPR8, and OPR9. Figure 1 shows profiles obtained with the OPR9 primer. Values of the NL coefficient for OPR1, OPR8, and OPR9

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Figure 1 RAPD analysis with the OPR9 primer of the 13 *P. falciparum* strains used in the study. (H₂O) Negative control; (MW) molecular weight scale (1-kb DNA ladder—GIBCO BRL).

primers are listed in Tables 1, 2, and 3. OPR1, OPR8, and OPR9 were selected because they highlight a correlation between the RAPD banding patterns and the IC₅₀ values of the strains as shown by NL coefficient analysis. With the OPR1 primer, the NL coefficient allowed set up of two groups of strains—one joining the four most sensitive strains together and the other joining six resistant strains and one sensitive strain (M25). The NL coefficient values obtained with the OPR8 primer display a group of the three most sensitive strains (3D7, M164, and HB3) and a group with all the strongly resistant strains. The M25 strain has an intermediate position as it presents similarities between the two groups. With the OPR9 primer, six strongly resistant strains have the same profile. Furthermore, similarities exist between HB3, M25, and INDOCHINE strains and M25 and NF7 profiles share some bands.

The statistical analysis demonstrates that the NL values between M13 and the other strongly resistant strains (IC₅₀ > 300 nM) were higher than 0.66 with a frequency significantly above those expected by chance ($P < 0.001$). Also, this analysis

shows that the NL values between M13 and the six other strains (IC₅₀ < 300 nM) were more often inferior to 0.33 than expected by chance ($P < 0.001$). Therefore, the RAPD results permit to associate the seven strongly resistant strains together and to distinguish them from those with lower IC₅₀ values.

PCR Primer Synthesis

Two pairs of primers were synthesized after cloning and sequencing two RAPD bands that were amplified by the OPR1 primer correlated to a certain level of sensitivity to chloroquine. The first band was present in the profiles of seven of the eight resistant strains (INDOCHINE, L1, JEAN, FCR3, SGE1, M13, and LILI). The corresponding primer set was called R1f/R1r. The second DNA fragment was amplified in the banding patterns of the three most sensitive strains. This primer set was called S1f/S1r.

PCR Reactions

The Pff/Pfr primer set was used as the PCR reaction positive control. The *P. falciparum*-specific band was amplified for all the strains.

The R1f/R1r primers reacted with seven of the eight resistant strains (INDOCHINE, L1, JEAN, FCR3, SGE1, M13, and LILI) but with none of the sensitive strains (Fig. 2), like the corresponding RAPD band.

The reaction performed with the S1f/S1r prim-

Table 1. Nei and Li Coefficient Values Computed from RAPD Profiles Obtained with the OPR1 Primer

	3D7	M164	HB3	NF7	M25	INDO	L1	JEAN	FCR3	SGE1	M13	LILI	W2
3D7	1												
M164	0.86	1											
HB3	0.86		1										
NF7	0.67	0.5	0.8	1									
M25	0.29	0.4	0.4	0.4	1								
INDO	0.29	0.4	0.4	0.4	1	1							
L1	0.25	0.29	0.29	0.33	0.86	0.86	1						
JEAN	0.25	0.29	0.29	0.33	0.86	0.86	1	1					
FCR3	0.25	0.29	0.29	0.33	0.86	0.86	1	1	1				
SGE1	0.25	0.29	0.29	0.33	0.86	0.86	1	1	1	1			
M13	0.22	0.25	0.25	0.29	0.75	0.75	0.89	0.89	0.89	0.89	1		
LILI	0.22	0.25	0.25	0.29	0.25	0.25	0.44	0.44	0.44	0.44	0.4	1	
W2	0	0	0	0	0.25	0.25	0.22	0.22	0.22	0.22	0.2	0.2	1

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Table 2. Nei and Li Coefficient Values Computed from RAPD Profiles Obtained with the OPR8 Primer

	3D7	M164	HB3	NF7	M25	INDO	L1	JEAN	FCR3	SGE1	M13	LILI	W2
3D7	1												
M164	0.86	1											
HB3	0.86	0.75	1										
NF7	0.5	0.4	0.4	1									
M25	0.5	0.44	0.44	0.33	1								
INDO	0	0	0	0	0	1							
L1	0.36	0.33	0.33	0.22	0.46	0	1						
JEAN	0.22	0.2	0.2	0.29	0.54	0	0.86	1					
FCR3	0.22	0.2	0.2	0.29	0.54	0	0.86	1	1				
SGE1	0.22	0.2	0.2	0.29	0.54	0	0.86	1	1	1			
M13	0.2	0.18	0.18	0.25	0.5	0	0.93	0.92	0.92	0.92	1		
LILI	0.22	0.2	0.18	0.29	0.54	0	0.71	0.83	0.83	0.83	0.77	1	
W2	0.25	0.22	0.18	0.33	0.6	0	0.62	0.73	0.73	0.73	0.66	0.73	1

ers was positive with the three most sensitive strains (3D7, M164, and HB3) and was negative with all the resistant strains (Fig. 3), like the corresponding RAPD band. It must be noted that with this primer set, some nonspecific products were generated when the specific band was not amplified. This can be explained by the low annealing temperature used (T_m S1r = 52°C).

Therefore, S1f/S1r and R1f/R1r primer sets work and would be able to be tested in a wide scale study for their ability to detect sensitive or resistant *P. falciparum* strains, respectively.

DISCUSSION

Chloroquine resistance in *P. falciparum* has both important medical and economical consequences, notably in poorer endemic areas. This phenomenon is complex and its biochemical and genetic mechanisms are still poorly understood (Slater 1993). The discovery of genetic markers correlated with sensitivity or resistance to chloroquine would certainly be helpful in understanding the exact mechanisms of chloroquine resistance in *P. falciparum* (Carlton et al. 1995; Lym-

bery 1996). In this study, we used the RAPD technique, which has proven to be a powerful and rapid method for detecting such polymorphic markers (Welsh and McClelland 1990; Williams et al. 1990). This technique, based on PCR, involved the amplification of random segments of genomic DNA using a single, short primer, arbitrarily chosen, at a low stringency. This method does not require prior sequence information. The RAPD technique generates complex banding patterns and it is therefore necessary to use a similarity coefficient to analyze this data. We used the NL coefficient (Nei and Li 1979), which is recommended for routine computation of genetic similarities (Lambory 1994).

The analysis using the NL coefficient shows a correlation between the IC_{50} values of the *P. falciparum* strains and the profiles obtained with the three selected primers. Taken together, these results permit the classification of the strains into three groups. The sensitive group comprises the four most sensitive strains (3D7, M164, HB3, and NF7) and the resistant group consists of the seven strongly resistant strains (L1, JEAN, FCR3, SGE1, M13, LILI, and W2). All of the strains from each group present similarities between themselves, however both groups

Table 3. Nei and Li Coefficient Values Computed from RAPD Profiles Obtained with the OPR9 Primer

	3D7	M164	HB3	NF7	M25	INDO	L1	JEAN	FCR3	SGE1	M13	LILI	W2
3D7	1												
M164	0	1											
HB3	0	0	1										
NF7	0.2	0.22	0	1									
M25	0.2	0	0.44	0.33	1								
INDO	0	0	1	0	0.44	1							
L1	0.29	0	0	0.22	0	0	1						
JEAN	0.29	0	0	0.22	0	0	1	1					
FCR3	0.29	0	0	0.22	0	0	1	1	1				
SGE1	0.29	0	0	0.22	0	0	1	1	1	1			
M13	0.29	0	0	0.22	0	0	1	1	1	1	1		
LILI	0.29	0	0	0.22	0	0	1	1	1	1	1	1	
W2	0	0	0.22	0.22	0.22	0.22	0	0	0	0	0	0	1

Figure 2 PCR analysis with the R1f/R1r primer set of the 13 *P. falciparum* strains used in the study. Positive PCR reactions generate a product of 630 bp. (H₂O) Negative control; (MW) molecular weight scale (1-kb DNA ladder—GIBCO BRL).

are very distinct from each other. The third intermediate group includes the M25 and INDOCHINE strains, which present similarities between themselves as well as with strains from the two other groups. It must be noted that M25 and INDOCHINE both have IC₅₀ values close to the threshold of 100 nM. These results are consistent with the existence of a multigenic chloroquine resistance mechanism, as suggested by a number of authors (Slater 1993; Cremer et al. 1995). Such a system would determine several levels of chloroquine resistance, each associated with a particular modification of the genotype.

RAPD analysis of the *P. falciparum* strains has therefore provided genetic markers of potential interest for the study of chloroquine resistance mechanisms. These results, however, must be considered carefully for two reasons. First, RAPD, as its name indicates, generates random markers. Therefore, despite the strong correlation with the level of chloroquine resistance, the interference of other factors can not be ruled out. The statistical analysis, however, has shown that the role of chance is highly improbable. The geographical origin of the *P. falciparum* strains must also be considered. Most of the parasites are from Africa, which lie mainly in the resistant group. It is, however, difficult to explain

the strong similarities that exist between strains isolated from various parts of the continent that represent different biotopes and epidemiological features. Furthermore, important similarities present between strains coming from different continents such as HB3 and M164 or M25 and INDOCHINE (OPR1) do not correlate with the geographical origin of the parasites. However, the association of the RAPD markers with chloroquine resistance must be confirmed by using many strains. The second reason is that cultured lines of *P. falciparum* are known to present less genetic diversity than strains isolated from the field (Robson et al. 1992). It is therefore necessary to control the value of the genetic markers using strains obtained from patients. For such a large scale study, usage of the RAPD technique would be difficult to carry out. To resolve this problem, we propose the use of the PCR technique that is able to detect specific sequences of the *P. falciparum* genome in whole-blood sample extracts contaminated by human DNA (Tirasophon et al. 1991; Hang et al. 1995). Furthermore, PCR is more reproducible than RAPD (Ellsworth et al. 1993). Specific

Figure 3 PCR analysis with the S1f/S1r primer set of the 13 *P. falciparum* strains used in the study. Positive PCR reactions generate a product of 355 bp. (H₂O) Negative control; (MW) molecular weight scale (1-kb DNA ladder—GIBCO BRL).

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primer sets for PCR reactions were synthesized from RAPD fragments of interest after cloning and sequencing. Two pairs of primers obtained from RAPD bands generated with OPR1 were tested on the strains of the study. The results of the PCR reactions performed confirm their ability to amplify a DNA fragment corresponding to the RAPD band from which they were synthesized. In addition these results, as with those obtained with RAPD, allow the classification of the strains of the study into several groups that correlate with their chloroquine resistance level.

RAPD on cultured *P. falciparum* strains is therefore used to screen genetic markers associated with the drug susceptibility phenotype of the parasite. Furthermore, PCR permits confirmation of the value of the RAPD markers on whole-blood samples collected from patients infected by *P. falciparum*. The genetic markers obtained in this manner should be useful to map and identify genes (Dweikat et al. 1994; Demeke et al. 1996; Greif et al. 1996) involved in chloroquine resistance and should therefore lead to a better understanding of the precise mechanisms involved in this phenomenon.

METHODS

Parasite Strains

Thirteen strains of *P. falciparum* were used in this study. Table 4 specifies the area where strains have been isolated and their level of susceptibility to chloroquine.

P. falciparum Cultures In Vitro

P. falciparum was maintained in culture using the method described by Trager and Jensen (1976). In short, strains were cultivated using A⁺ human erythrocytes at 8% hematocrit in RPMI 1640 medium (GIBCO, Grand Island, NY) that was supplemented with 24 mM sodium bicarbonate, 35 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer, 10 µg/ml gentamycin and 10% A⁺ human serum. The medium was renewed daily. Parasitized red blood cells were maintained as shallow layers in 75-cm² tissue culture flasks at 37°C in an atmosphere of 90% N₂, 5% CO₂, and 5% O₂.

Sensitivity to Chloroquine In Vitro

Sensitivity of *P. falciparum* strains to chloroquine was determined by measuring the uptake of radio-labeled hypoxanthin by parasites while being cultivated in the presence of increasing concentrations of the drug (Desjardins et al. 1979). The level of resistance was evaluated using the IC₅₀ value, which represents the concentration of chloroquine that is able to inhibit 50% of parasite growth. IC₅₀ is expressed in nanomoles. The resistance threshold adopted was 100 nM (Carme et al. 1995).

DNA Extraction

Genomic DNA was extracted from parasitized red blood cells lysed with saponin. After parasite lysis (0.1 M Tris-HCl, at pH 8, 0.4 M NaCl, 1 mM EDTA, 1% SDS, 100 µg/ml of proteinase K, and 1 hr at 55°C), proteins and others impurities were removed by three steps of extraction (phenol, phenol-chloroform, chloroform-isoamyl alcohol). An RNase (100 µg/ml, 1 hr at 37°C) treatment was then performed and after this incubation, the RNase was removed by a phenol-chloroform extraction. The DNA was then precipitated and the pellet was

Table 4. The Strains of *P. falciparum*, Their Geographic Origin, Their Chloroquine Susceptibility Phenotype (as Defined by the *in Vitro* Test), and Their IC₅₀ Value

Strains	Origin	Phenotype	IC ₅₀ (nM)
3D7	Amsterdam (cloned from NF54)	sensitive	40
M164	Malawi	sensitive	70
HB3	Honduras (cloned from H 1)	sensitive	80
NF7	Amsterdam Airport	sensitive	80
M25	Zaire	sensitive	90
INDOCHINE	Southeast Asia	resistant	110
L1	Niger	resistant	360
JEAN	R.C.A.	resistant	600
FCR 3	Gambia	resistant	600
SGE 1	Gambia	resistant	>1000
M 13	Gambia	resistant	>1000
LILI	Cameroon	resistant	>1000
W 2	Southeast Asia	resistant	>1000

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resuspended in sterile, distilled water. Purity control of extracted DNA was assumed by measuring absorption spectra from 210–310 nm. DNA concentration was evaluated using absorption values at 260 nm.

RAPD

Decamer oligonucleotide primers were obtained from Operon Technologies, Inc. (Alameda, CA). Three had generated fingerprints of interest: OPR1 (5'-TGCGGGTCCT-3'), OPR8 (5'-CCCGTTGCCT-3'), and OPR9 (5'-TGAGCACGAG-3').

Amplification reactions were carried out in a 50- μ l volume containing 20 mM Tris-HCl, at pH 8.3, 100 mM KCl, 0.01% Tween 20, 0.01% NP-40, 1.5 mM MgCl₂, 200 μ M each of dATP, dCTP, dGTP, and dTTP, 0.5 μ M primer, 100 ng of *P. falciparum* genomic DNA, and 2 units of Tth DNA polymerase (Replitherm Tebu). Samples were overlaid with mineral oil and amplification was performed in a DNA Thermal Cycler (Perkin-Elmer Cetus). After an initial denaturation at 95°C for 3 min, 35 cycles of 30 sec at 95°C, 1 min at 35°C, and 2 min at 72°C were performed, followed by 5 min at 72°C. The amplification products were analyzed by electrophoresis on 1.5% agarose gels and were detected by ethidium bromide staining.

RAPD Data Analysis

All the strain profiles were compared by calculation of the NL coefficient (Nei and Li 1979): $NL = 2a/(b + c)$, where *a* is the number of similar bands from two strains, and *b* and *c* are the total number of bands from each strain. The NL coefficient allows a value of the similarity between two strains. This analysis provided interesting data about the genomic distance between different strains. The NL coefficient was used because it is recommended for routine computation of genetic similarities using RAPD data (Lamboy 1994).

The statistical analysis was performed using the X² test. It was carried out to establish whether the NL values obtained with the three selected primers allowed the seven strongly resistant strains (IC₅₀ > 300 nM) to associate and to differentiate this group from strains with lower IC₅₀ values. For the calculation, we used the NL values of M13, which was taken as reference for the group of strongly resistant strains. We considered that a NL value superior to 0.66 indicates a similarity between two strains and a NL value below 0.33 reflects a difference between two strains. If obtaining identical bands between two RAPD profiles was attributable to chance, the probability of obtaining a NL value >0.66 or <0.33 would be one-third.

DNA Cloning and Sequencing

DNA fragments amplified by RAPD can be used to synthesize specific primer sets. Bands that were of interest were collected from the agarose gel and the amplification products were cloned into the TA cloning vector (Invitrogen). The inserted DNA was sequenced using the Sequenase 2.0 kit (U.S. Biochemical) and [α -³⁵S]dATP (Amersham). Based on this sequence, the oligonucleotide primer sequences were selected, including the sequence of the RAPD primer (10-mers) plus 8–10 bases characteristic of the excised fragment, and were synthesized by Eurogentec (Belgium).

PCR

Three sets of primers were used. The first primer set (Pff: 5'-CGCTACATATGCTAGTTGCCAGAC-3' and Pfr: 5'-CGTG-TACCATACATCCTACCAAC-3') was specific to *P. falciparum* (Tirasophon et al. 1991) and was used as a positive control for the PCR reactions. The second primer set (R1f: 5'-TGCGG-GTCCTAAATTTAAAA-3' and R1r: 5'-TGCGGGTCCTC-CAAAAAATG-3') was obtained from a DNA fragment generated by RAPD using the OPR1 primer. The third primer set (S1f: 5'-TGCGGGTCCTAGATGAAA-3' and S1r: 5'-TGCGGG-TCCTTATAATCA-3') was also synthesized from a DNA fragment amplified using the OPR1 primer.

Amplification reactions were carried out in a total volume of 50 μ l containing 20 mM Tris-HCl, at pH 8.3, 100 mM KCl, 0.01% Tween 20, 0.01% NP-40, 1.5 mM MgCl₂, 100 μ M each of dATP, dCTP, dGTP, and dTTP, 0.5 μ M of each primer, 100 ng of *P. falciparum* genomic DNA and 1.25 units of Tth DNA polymerase (Replitherm Tebu). Samples were overlaid with mineral oil and amplification was then performed in a DNA Thermal Cycler (Perkin-Elmer Cetus). After an initial denaturation at 95°C for 3 min, 30 cycles of 10 sec at 95°C, 30 sec at 60°C for Pff/Pfr and R1f/R1r or at 50°C for S1f/S1r and 1 min at 72°C were performed. Amplification products were analyzed by electrophoresis on 1.2% agarose gels and were detected by ethidium bromide staining.

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