Available online at www.sciencedirect.com
SCIENCE DIRECT®
Infection, Genetics and Evolution 3 (2003) 287–292
Infection, Genetics and Evolution
www.elsevier.com/locate/meegid

Relationship between the *Pfcrt* T76 and the *Pfmdr-1* Y86 mutations in *Plasmodium falciparum* and in vitro/in vivo chloroquine resistance in Burkina Faso, West Africa

Halidou Tinto [b,c], Jean Bosco Ouédraogo [b], Annette Erhart [d], Chantal Van Overmeir [d], Jean-Claude Dujardin [d], Eric Van Marck [c], Tinga Robert Guiguemdé [a], Umberto D'Alessandro [d,*]

[a] *Centre Muraz, Bobo Dioulasso, Burkina Faso*
[b] *Institut de Recherche en Sciences de la Santé, Bobo Dioulasso, Burkina Faso*
[c] *University of Antwerp, Antwerp, Belgium*
[d] *Prince Leopold Institute of Tropical Medicine, Nationalestraat 155, Antwerp B-2000, Belgium*

Received 28 May 2003; received in revised form 6 August 2003; accepted 6 August 2003

Abstract

The relationship between *Pfcrt* T76 and *Pfmdr-1* Y86 mutations in *Plasmodium falciparum* was explored in samples from patients with uncomplicated malaria and tested in vitro and in vivo with chloroquine (CQ) in Burkina Faso. The two mutations were strongly related. The *Pfcrt* T76 mutation was found in 82% of the samples having the *Pfmdr-1* Y86 mutation too (odds ratio (OR) = 4.8 [95% CI: 1.7–13.3]; $P = 0.002$). However, only half (16/34) of samples with *Pfcrt* T76 mutation had also the *Pfmdr-1* Y86 mutation. The latter was apparently associated with in vitro resistance (OR = 4.8 [95% CI: 1.4–16.5]; $P = 0.01$) but such association disappeared ($P = 0.77$) after adjusting for the presence of the *Pfcrt* T76 mutation. This suggests that the occurrence of the *Pfmdr-1* Y86 mutation is dependent on that of *Pfcrt* T76 mutation and could explain previous reports linking the *Pfmdr-1* Y86 mutation with CQ resistance (CQR). The isolates carrying both the *Pfcrt* K76 and *Pfmdr-1* N86 alleles (wild/wild (WW)) and the single mutant *Pfmdr-1* Y86 (WM) had the lowest IC50 geometric mean (GMIC50) values, while those carrying both *Pfcrt* T76/*Pfmdr-1* Y86 alleles (mutant/mutant (MM)), and the single mutant *Pfcrt* T76 (MW) had the highest. Among pre-treatment samples there was a strong linkage disequilibrium with an excess of MM and WW and a deficit of single mutants (MW and WM), suggesting that parasite fitness is higher for the former and lower for the latter.

Keywords: Malaria; *Plasmodium falciparum*; Chloroquine resistance; *Pfcrt*; *Pfmdr-1*

1. Introduction

Despite increasing resistance, chloroquine (CQ) is still widely used for the treatment of uncomplicated malaria in several African countries. In Burkina Faso, between 1998 and 2000, CQ clinical failure has been estimated at around 12% and parasitological failure at 18% (Tinto et al., 2002). Although the mode of action and mechanisms of CQ resistance (CQR) have not been fully understood (Ursos et al., 2000, Durand et al., 2001), several molecular markers have been identified. CQR was initially attributed to mutations in the *Plasmodium falciparum* multidrug resistance gene (*Pfmdr-1* Y86) on chromosome 5 (Foote et al., 1990; Duraisingh et al., 1997; Adagu and Warhurst, 1999) and to a second candidate gene on chromosome 7, *cg2* (Su et al., 1997). However, this was not confirmed by later studies (Basco and Ringwald, 1998; Grobusch et al., 1998; Mungthin et al., 1999; Durand et al., 1999; Pillai et al., 2001). Subsequently, point mutations in the *P. falciparum* CQ resistance transporter, encoded by the *Pfcrt* gene, have been linked to CQR in laboratory parasite strains (Fidock et al., 2000), and the association with in vivo CQR was first reported from Mali (Djimde et al., 2001), the mutation T76 being the most important. However, the role of the *Pfmdr-1* Y86 mutation in determining CQR is not clear yet. A recent study carried out on isolates from Sudan reported that the presence of both *Pfmdr-1* Y86 and *Pfcrt* T76 might determine high-level in vitro CQR (Babiker et al., 2001). Nevertheless, it remains unclear how these two mutations relate to each other and, when associated, their role in

* Corresponding author. Tel.: +32-3-247-63-54.
E-mail addresses: tintoh@hotmail.com (H. Tinto), udalessandro@itg.be (U. D'Alessandro).

1567-1348/$ – see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/j.meegid.2003.08.002

determining CQ resistance. Therefore, we studied the relationship between the *Pfcrt* T76 and *Pfmdr-1* Y86 mutations and the in vitro and in vivo CQ resistance in patients with uncomplicated malaria.

2. Materials and methods

2.1. Study population

This is part of a larger study on CQR in 6 months–15 years old malaria patients carried out between 1998 and 2000 in Bobo Dioulasso, Burkina Faso. Details of the methodology and in vivo results have been largely described elsewhere (Tinto et al., 2002). The present analysis focus only on patients from the urban site where, both in vivo and in vitro tests have been done. Patients with fever (axillary temperature $\geq$37.5 °C) and a *P. falciparum* infection with a parasite density between 2000 and 100,000 parasites/μl were recruited for the in vivo test. The in vitro test was performed only in patients who had a parasite density of at least 4000 parasites/μl.

2.2. Treatment and follow-up

The follow-up was carried out according to the WHO 14 days in vivo test (WHO, 1996). CQ (Nivaquine® 100 mg) was administered orally under supervision and according to body weight (25 mg/kg in 3 days). The clinical outcome was defined according to the WHO 14 days clinical classification system: early treatment failure (ETF), late treatment failure (LTF) and adequate clinical response (ACR) (WHO, 1996). The parasitological outcome was defined according to the WHO parasitological resistance classification system: sensitive (S), resistance levels RI, RII and RIII (Guiguemdé et al., 1996).

2.3. In vitro test

Venous blood samples for the in vitro tests were collected at day 0 before treatment. The isotopic microtest (Desjardins et al., 1979) was used to measure the proliferation of *P. falciparum* in the presence of CQ. Full details of the methodology have been described elsewhere (Tinto et al., 2000). Most of the tests were carried out the same day of collection and few (<10%) within 48 h after sampling. ^{3}[H]-hypoxanthine (Amersham, Little Chalfont, UK) was added to assess parasites growth. The results of cellular proliferation were expressed as the counts per minute (CPM) and analysed using linear regression to determine the 50% inhibitory concentration (IC50) values. The value of IC50 > 100 nM was used as the resistance threshold (Tinto et al., 2000).

2.4. Molecular analysis

Blood samples for the molecular analysis were collected on filter paper (Whatman 3, Maidstone, England) at day 0 before treatment. DNA was extracted using the Chelex-100 method (Plowe et al., 1995). The detection of the *Pfcrt* T76 and the *Pfmdr-1* Y86 mutations was done according to the protocol provided in Djimde et al. (2001). The allele-specific polymerase chain reaction (AS-PCR) was used for the detection of the *Pfcrt* T76 mutation: the first round PCR reaction was done by using 0.5 pmol of each primer (TCRP1 5′-CCGTTAATAATAAATACACGCAG-3′ and TCRP2 5′-CGGATGTTACAAAACTATAGTTACC-3′) in a 25 μl reaction mixture containing 0.2 mM of dNTPs, 1× PCR buffer (PROMEGA, Madison, WI, USA), 2.5 mM $MgCl_2$ (PROMEGA, Madison, WI, USA), 1 U of Taq polymerase (PROMEGA, Madison, WI, USA) and 1 μl of extracted parasite DNA. The denaturation was carried out at 94 °C (3 min initially and 30 s in each cycle). Primers were annealed at 56 °C for 30 s and extension at 60 °C for 1 min. After 40 cycles, the final extension was carried out at 60 °C for 3 min. For allele-specific PCR, 1 μl of the primary PCR product was used in a total reaction volume of 25 μl containing 0.2 mM of dNTPs, 1× PCR buffer (PROMEGA, Madison, WI, USA), 1.5 mM $MgCl_2$ (PROMEGA, Madison, WI, USA) and 1 U of Taq polymerase (PROMEGA, Madison, WI, USA). The common primer TCRP3 5′-TGACGAGCGTTATAGAG-3′ was used in combination with either a mutant-specific primer TCRP4m 5′-GTTCTTTTAGCAAAAATTG-3′ or a wild-type-specific primer TCRPw 5′-GTTCTTTTAGCAAAAATTT-3′. The PCR parameters were same as above for primary PCR, except that the number of cycles were 20 and the annealing and extension were carried out at 47 and 64 °C, respectively. The amplified DNA fragments were resolved by electrophoresis in a 2% agarose gel. Wild-type and mutant genotypes were designated on the basis of amplification of products of predicted sizes by the appropriate primers. A band of 366 bp was observed if successful. The *Pfmdr-1* Y86 mutation was detected by using PCR followed by sequence-specific restriction enzyme digestion (SS-RED): the first round was done by using primers MDR1 5′-ATGGGTAAAGAGCAGAAAGA-3′ and MDR2 5′-AACGCAAGTAATACATAAAGTCA-3′ and then, nested PCR was done by using primers MDR3 5′-TGGTAACCTCAGTATCAAAGAA-3′ and MDR4 5′-ATAAACCTAAAAAGGAACTGG-3′. The PCR amplification was performed in a 25 μl mixture containing 1× PCR buffer (PROMEGA, Madison, WI, USA), 2.5 mM $MgCl_2$ (PROMEGA, Madison, WI, USA), 0.2 mM of dNTPs, 0.5 pmol of each primers, 1 U of Taq polymerase (PROMEGA, Madison, WI, USA) and 1 μl of extracted parasite DNA. For the first round, the samples were incubated for 3 min at 94 °C for denaturation prior to cycles (94 °C for 30 s, 56 °C for 30 s and 65 °C for 1 min). After 35 cycles, primers extention was continued for 3 min at 65 °C. For the second round, the initial denaturation was carried out at 95 °C for 5 min, followed by 20 cycles (30 s at 92 °C, then 30 s at 48 °C and 1 min at 65 °C) and ended with 3 min at 65 °C. The nested PCR product was subjected to enzyme digestion with *AflII*

(New England Biolabs), which cuts only the mutant gene into 226 and 295 bp fragments. For each series of samples, water was used as a negative control, 3D7-clone DNA was used as the wild-type control and Dd2-DNA was used as the mutant control.

2.5. Statistical methods

Data were entered in Excel, Version 97 and analysed using STATA 7.0 software (Stata Corp. 2001). The frequencies of both *Pfcrt* 76 and *Pfmdr-1* 86 alleles before treatment were determined for the different outcomes of the in vivo and in vitro tests. The geometric mean of IC50 (GMIC50) determined for the presence of different alleles of the two loci was calculated. χ^2-test (or Fisher exact test when required) was used to test for significant differences between categorical variables. A value of $P < 0.05$ was considered statistically significant. Crude odds ratio (OR) were calculated to estimate the risk of in vitro resistance for each mutation and adjusted OR (for the combined effect of both mutations and/or the effect of age) were calculated using a logistic regression.

3. Results

For the molecular analysis, a total of 117 blood samples were collected from positive *P. falciparum* malaria patients. Among these, 93 patients were recruited for in vivo study and 85 had a complete follow-up (three were lost to follow-up, four had severe malaria and one developed another illness). Clinical failure (early + late) was 13% (11/85) and parasitological failure (RI + RII + RIII) 42% (36/85), most of them late RI. Children less than 5 years old were significantly more at risk of clinical (OR = 6.2 [95% CI: 1.1–45.2]; $P = 0.025$) and parasitological (OR = 3.3 [95% CI: 1.2–9.0]; $P = 0.008$) failure than older ones. in vitro CQ susceptibility could be determined in 71 out of 112 samples obtained from patients with a parasitaemia >4000 parasites/μl. Eighteen isolates (25.4%) were classified as CQ resistant (GMIC50 = 143.4 nM; 95% CI [119.2–187]) and 53 (74.6%) as CQ sensitive (GMIC50 = 9.9 nM; 95% CI [7.2–13.8]). Thirty-nine samples had both the in vitro and in vivo results that were interpretable.

3.1. Prevalence of pfcrt T76 and Pfmdr-1 Y86 mutations

The *Pfcrt* 76 and the *Pfmdr-1* 86 loci could be successfully genotyped in 114 and 94 samples, respectively. The *Pfcrt* T76 mutation was found in 61.4% (70/114) of the samples. About 35.7% (25/70) of them carried both the mutant (T76) and the wild (K76) alleles (mixed infection). The *Pfmdr-1* Y86 mutation could be found in 36% (34/94) of the samples and 32.3% (11/34) of them carried a mixture of the wild and mutant alleles for this locus.

3.2. Relation between Pfcrt T76 and Pfmdr-1 Y86 mutations and in vitro CQ susceptibility

The *Pfcrt* T76 mutation was found in all in vitro CQR isolates (18/18) and in about 36% of the sensitive ones (19/53) ($P < 0.0001$). The *Pfmdr-1* Y86 mutation was apparently associated with in vitro resistance (OR = 4.8 [95% CI: 1.4–16.5]; $P = 0.01$) but such association disappeared (OR = 1.3 [95% CI: 0.3–5.4]; $P = 0.77$) after adjusting for the presence of the *Pfcrt* T76 mutation. The effect of the association between the two loci alleles on the in vitro outcome and the IC50 geometric means could be explored in 59 samples (Table 1). Samples with only the single mutant *Pfcrt* T76 allele (MW), or both *Pfcrt* T76/*Pfmdr-1* Y86 alleles (mutant/mutant (MM)), had a significantly higher IC50 geometric mean, while those carrying both *Pfcrt* K76 and *Pfmdr-1* N86 alleles (wild/wild (WW)) or the single mutant *Pfmdr-1* Y86 (WM) had a lower mean. Most of the in vitro resistant isolates were MM while the majority of the sensitive ones were WW ($P < 0.0001$). This result did not changed after excluding the mixed infections with both the mutant and wild alleles for a given locus ($P < 0.0001$).

3.3. Relation between Pfcrt T76 and Pfmdr-1 Y86 and in vivo outcome

About two third (53/82) of the in vivo pre-treatment samples carried the *Pfcrt* T76 mutation and its prevalence did not vary with age (Table 2). The *Pfcrt* T76 mutation was detected in the majority of patients with clinical failure (10/11) (OR = 6.3 [95% CI: 0.7–143.4]; $P = 0.09$) but did not reach statistical significance. The mutation was associated with parasitological failure, more in younger (OR = 52.8 [95% CI: 4.8–1382]; $P < 0.0001$) than in older children

Table 1
Distribution of *Pcfrt* 76 and *Pmdr1* 86 alleles according to outcome and the IC50 geometric mean of 59 isolates tested in vitro

Pcfrt K76T/*Pmdr1* N86Y		CQR (%) ($n = 16$)	CQS (%) ($n = 43$)	GMIC50 (nM) ($n = 59$)	GMIC50 95% CI
K76/N86	WW	0 (0.0)	26 (60.4)	7.3	4.9–10.9
K76/Y86	WM	0 (0.0)	3 (7)	8.24	0.05–1350
T76/N86	MW	6 (37.5)	6 (14)	37.7	12.8–111.8
T76/Y86	MM	10 (62.5)	8 (18.6)	58.5	30.9–112

CQR: chloroquine resistant; CQS: chloroquine sensitive; W: wild; M: mutant or mixed (M/W); GMIC50: geometric mean of IC50.

Table 2
Pcfrt 76 and *Pmdr1* 86 genotypes and the parasitological outcome by age group

	Pcfrt K76T ($n = 82$)						*Pmdr1* N86Y ($n = 69$)					
	6 months–5 years			6–15 years			6 months–5 years			6 months–15 years		
	M* (*n* (%))	W (*n* (%))	Total (*n* (%))	M* (*n* (%))	W (*n* (%))	Total (*n* (%))	M* (*n* (%))	W (*n* (%))	Total (*n* (%))	M* (*n* (%))	W (*n* (%))	Total (*n* (%))
Parasitological resistance												
S	5 (29)	12 (71)	17 (100)	14 (48)	15 (52)	29 (100)	5 (36)	9 (64)	14 (100)	8 (31)	18 (69)	26 (100)
R**	22 (96)	1 (4)	23 (100)	12 (92)	1 (8)	13 (100)	6 (33)	12 (67)	18 (100)	6 (54)	5 (46)	11 (100)
Total	27	13	40	26	16	42	11	21	32	14	23	37

W: wild; M*: mutant + mixed (M/W); R**: RI (early + late) and RII + RIII.

(OR = 12.9 [95% CI: 1.4–300.1]; $P = 0.007$). After controlling for age, parasitological failure was still significantly associated to *Pfcrt* T76 mutation (OR = 29.6 [95% CI: 5.9–149.4]; $P < 0.001$). The *Pfmdr-1* Y86 mutation was found in 36.2% (25/69) of patients and was not found to be associated with clinical ($P = 1.0$) or parasitological failure ($P = 0.6$) (Table 2).

3.4. Association between Pfcrt 76 and Pfmdr-1 86 alleles and parasite population structure

The parasite population structure was studied by analysing the association between the *Pfcrt* and *Pfmdr-1* alleles in 94 pre-treatment samples (even those without an in vivo follow-up). When comparing the observed association between the *Pfcrt* and *Pfmdr-1* alleles with that expected under the hypothesis of free panmixy, there was a strong linkage disequilibrium (OR = 4.9 [95% CI: 1.6–15.7]; $P = 0.001$) with an excess of MM and WW (59/94), and a deficit of single mutants MW and WM (35/94). When considering all samples, the *Pfcrt* T76 mutation could be found in 82% (28/34) of the samples carrying the *Pfmdr-1* Y86 mutation too (OR = 4.8 [95% CI: 1.7–13.3]; $P = 0.002$). Such an association was even stronger when considering only the 71 samples tested in vitro (OR = 12.2 [95% CI: 3.0–50.0]; $P < 0.0001$). However, only half (28/57) of the samples with *Pfcrt* T76 mutation had also the *Pfmdr-1* Y86 mutation. Similar results are obtained also after excluding mixed infections with both alleles (mutant/wild) for a given locus (OR = 8.27 [95% CI: 2.1–38.6]; $P = 0.001$).

4. Discussion

We have analysed the relationship between the *Pfmdr-1* Y86 and *Pfcrt* T76 mutations previously identified as the main molecular markers for in vitro and in vivo CQ resistance. The *Pfcrt* T76 mutation was found in 82% of the samples having the *Pfmdr-1* Y86 mutation too. However, the opposite was not true as only half of the samples with the *Pfcrt* T76 mutation carried also the *Pfmdr-1* Y86 mutation. This suggests that the occurrence of the *Pfmdr-1* Y86 mutation is related with that of the *Pfcrt* T76 mutation. Although CQ treatment before inclusion into the study could have selected parasites with both mutations, it should be pointed out that the relation observed goes only in one direction (*Pfcrt* towards *Pfmdr*). Indeed, a study done in Sao Tome and Principe found the T76 mutation in all samples having the Y86 mutation too (Lopes et al., 2002) and supports the hypothesis that the occurrence of *Pfmdr-1* Y86 is dependent on that of *Pfcrt* T76. Several earlier studies have showed evidence that *Pfmdr-1* polymorphism is associated with CQ resistance (Basco et al., 1995; Foote et al., 1990; Duraisingh et al., 1997; Adagu and Warhurst, 1999). Such association is probably explained by assuming the contemporary presence of the *Pfcrt* T76 mutation. This is also confirmed by the disappearance of the association between the *Pfmdr-1* Y86 mutation and the in vitro CQR, after controlling for the presence of the *Pfcrt* T76 mutation.

The *Pfcrt* T76 was associated with clinical failure, but did not reach statistical significance probably because of the small sample size, and was significantly associated with parasitological and in vitro resistance, confirming its role in determining CQR. The stronger association observed in younger children confirms the role of host immunity in modifying the relation between molecular markers and resistance (Djimde et al., 2001; Dorsey et al., 2001).

The contribution of the *Pfmdr-1* Y86 mutation in determining CQR is still unclear. The mutation in itself might not be directly related to CQR but, due to its relation with *Pfcrt* T76, it probably plays an important role, possibly increasing the fitness of parasites carrying the *Pfcrt* T76 mutation. Indeed, the study of the association between the two loci alleles showed that the CQ resistance is mostly dependent on the presence of *Pfcrt* T76 but not on that of *Pfmdr-1* Y86. The association between the IC50 and the *Pfmdr-1* Y86 allele was found only in clones with the *Pfcrt* T76 and not in those with the *Pfcrt* K76 allele (Babiker et al., 2001). Similarly, a study from Senegal showed that the *Pfcrt* T76 and *Pfmdr-1* Y86 occurred together in 44% of CQR isolates but in only 10% of the sensitive ones (Thomas et al., 2002). We were unable to confirm such relationship in our study because of the small sample size (three samples) of isolates carrying both the *Pfcrt* K76/*Pfmdr-1* Y86 alleles (WM). Nevertheless, we observed that the IC50 geometric mean of the samples carrying both the T76/Y86 alleles (MM) was higher

compared with those carrying the single mutant T76/N86 (MW). Although the *Pfcrt* T76 could be used to estimate the prevalence of CQ resistance, the additional testing for the *Pfmdr-1* Y86 might increase the specificity of this approach (Jelinek et al., 2002).

In a population under CQ pressure, the parasite population structure should be strongly biased towards those having the *Pfcrt* T76 mutation. In our study, a strong linkage disequilibrium was indeed observed before treatment, as mutant (MM) and sensitive parasites (WW) for both genes were over-represented, while intermediate genotypes (MW and WM) were under-represented. A higher parasite fitness for the former and a lower for the latter could explain such finding. The in vitro fitness of parasites under CQ pressure should be distinguished from the global fitness of parasite populations in natural conditions (with selective factors such as drug, immunity and transmission level). The parasites with WW genotype, although susceptible to CQ, might have a high global fitness when the selective pressure exerted by the drug is low or decreases. In our study area, drug use is probably low during the dry season (January–June) because malaria transmission is low. In such conditions, parasite with the WW genotype might have an advantage. An important limitation to consider is that our samples were from patients attending a health facility and not from randomly selected individuals with asymptomatic infections. Therefore, the population structure of the parasites might not reflect that circulating in the study area.

In conclusion, the occurrence of *Pfmdr-1 Y86* mutation is mostly dependent on that of *Pfcrt* T76 mutation, which is the main determinant of *P. falciparum* CQR. Further investigations targeting other loci on the two genes (which have not been explored here) with more representative population of parasites are needed to confirm our findings and could help in explaining the relation between these two mutations (as they are on different chromosomes) and possibly the mechanisms involved.

Acknowledgements

We thank the parents of the children included in this study for their participation. We would also like to thank the health staff of the health centres where the study was conducted for their collaboration. Thanks also to Dr. Ambrose Talisuna for his contribution to the analysis of data. We thank the French Cooperation for its financial support for the field study and the Belgium cooperation for supporting the laboratory work through a training grant for Halidou Tinto.

References

Adagu, I.S., Warhurst, D.C., 1999. Association of *cg2* and *pfmdr1* genotype with chloroquine resistance in field samples of *Plasmodium falciparum* from Nigeria. Parasitology 119, 343–348.

Babiker, H.A., Pringle, S.J., Abdel-Muhsin, A., Mackinnon, M., Hunt, P., Walliker, D., 2001. High-level chloroquine resistance in Sudanese isolates of *Plasmodium falciparum* is associated with mutations in the chloroquine resistance transporter gene *pfcrt* and the multidrug resistance gene *pfmdr1*. J. Infect. Dis. 183, 1535–1538.

Basco, L.K., Ringwald, P., 1998. Molecular epidemiology of malaria in Yaounde, Cameroon. III. Analysis of chloroquine resistance and point mutations in the multidrug resistance 1 (*pfmdr1*) gene of *Plasmodium falciparum*. Am. J. Trop. Med. Hyg. 59, 577–581.

Basco, L.K., Le Bras, J., Rhoades, Z., Wilson, C.M., 1995. Analysis of *pfmdr1* and drug susceptibility in fresh isolates of *Plasmodium falciparum* from subsaharian Africa. Mol. Biochem. Parasitol. 74, 157–166.

Desjardins, R.E., Canfields, C.J., Haynes, J.P., Chnulay, J.D., 1979. Quantitative assessment of antimalarial activity in vitro by a semi-automated microdilution technique. Antimicrob. Agents Chemother. 16, 710–718.

Djimde, A., Doumbo, O.K., Cortese, J.F., Kayentao, K., Doumbo, S., Diourte, Y., Dicko, A., Su, X.Z., Nomura, T., Fidock, D.A., Wellems, T.E., Plowe, C.V., Coulibaly, D., 2001. A molecular marker for chloroquine-resistant Falciparum malaria. N. Engl. J. Med. 344, 257–263.

Dorsey, G., Kamya, M.R., Singh, A., Rosenthal, P.J., 2001. Polymorphisms in the *Plasmodium falciparum pfcrt* and *pfmdr-1* genes and clinical response to chloroquine in Kampala, Uganda. J. Infect. Dis. 183, 1417–1420.

Duraisingh, M.T., Drakeley, C.J., Muller, O., Bailey, R., Snounou, G., Targett, G.A., Greenwood, B.M., Warhurst, D.C., 1997. Evidence for selection for the tyrosine-86 allele of the *Pfmdr1* gene of *Plasmodium falciparum* by chloroquine and amodiaquine. Parasitology 114, 205–211.

Durand, R., Gabbett, E., Di Piazza, J.P., Delabre, J.F., Le Bras, J., 1999. Analysis of kappa and omega repeats of the *cg2* gene and chloroquine susceptibility in isolates of *Plasmodium falciparum* from subsaharian Africa. Mol. Biochem. Parasitol. 101, 185–197.

Durand, R., Jafari, S., Vauzelle, J., Dlabre, J.-F., Jesic, Z., Le Bras, J., 2001. Analysis of *Pfcrt* point mutations and chloroquine susceptibility in isolates of *Plasmodium falciparum*. Mol. Biochem. Parasitol. 114, 95–102.

Fidock, D.A., Nomura, T., Talley, A.K., Cooper, R.A., Dzekunov, S.M., Ferdig, M.T., Ursos, L.M., Sidhu, A.B., Naude, B., Deitsch, K.W., Su, X.Z., Wootton, J.C., Roepe, P.D., Wellems, T.E., 2000. Mutations in the *P. falciparum* digestive vacuole transmembrane protein Pfcrt and evidence for their role in chloroquine resistance. Mol. Cell 6, 861–871.

Foote, S.J., Kyle, D.E., Martin, R.K., Oduola, A.M., Forsyth, K., Kemp, D.J., Cowman, A.F., 1990. Several alleles of the multidrug-resistance gene are closely linked to chloroquine resistance in *Plasmodium falciparum*. Nature 345, 255–258.

Grobusch, M.P., Adagu, I.S., Kremsner, P.G., Warhurst, D.C., 1998. *Plasmodium falciparum*: in vitro chloroquine susceptibility and allele-specific PCR detection of *pfmdr1 Asn86Tyr* polymorphism in Lambarene. Gabon. Parasitol. 116, 211–217.

Guiguemdé, R.T., Gbary, R.A., Coulibaly, S.O., Ouédraogo, J.B., 1996. Comment réaliser et interpréter les résultats d'une épreuve de chimiorésistance de *Plasmodium falciparum* chez les sujets malades en zone tropicale. Cahiers Santé 6, 187–191.

Jelinek, T., Aida, A.O., Peyerl-Hoffmann, G., Jordan, S., Mayor, A., Heuschkel, C., El Valy, A.O., Von Sonnenburg, F., Christophel, E.M., 2002. Diagnostic value of molecular markers in chloroquine-resistant falciparum malaria in Southern Mauritania. Am. J. Trop. Med. Hyg. 67, 449–453.

Lopes, D., Nogueira, F., Gil, J.P., Ferreira, C., do Rosario, V.E., Cravo, P., 2002. *pfcrt* and *pfmdr1* mutations and chloroquine resistance in *Plasmodium falciparum* from Sao Tome and Principe, West Africa. Ann. Trop. Med. Parasitol. 96, 831–834.

Mungthin, M., Bray, P.G., Ward, S.A., 1999. Phenotypic and genotypic characteristics of recently adapted isolates of *Plasmodium falciparum* from Thailand. Am. J. Trop. Med. Hyg. 60, 469–474.

Pillai, D.R., Labbé, A.C., Vanisaveth, V., Hongvangthong, B., Pomphida, S., Inkathone, S., Kain, K.C., 2001. *Plasmodium falciparum* malaria in Laos: chloroquine treatment outcome and predictive value of molecular markers. J. Infect. Dis. 183, 789–795.

Plowe, C.V., Djimdé, A., Bouare, M., Doumbo, O., Wellems, T.E., 1995. Pyrimethamine and proguanil resistance-confering mutations in *Plasmodium falciparum* dihydrofolate reductase: polymerase chain reaction methods for surveillance in Africa. Am. J. Trop. Med. Hyg. 52, 565–568.

Su, X., Kirkman, L.A., Fujioka, H., Wellems, T.E., 1997. Complex polymorphisms in an approximately 330 kDa protein are linked to chloroquine-resistant *P. falciparum* in Southeast Asia and Africa. Cell 91, 593–603.

Thomas, S.M., Ndir, O., Dieng, T., Mboup, S., Wypij, D., Maguire, J.H., Wirth, D.F., 2002. In vitro chloroquine susceptibility and PCR analysis of *pfcrt* and *pfmdr1* polymorphisms in *Plasmodium falciparum* isolates from Senegal. Am. J. Trop. Med. Hyg. 66, 474–480.

Tinto, H., Ouédraogo, J.B., Coulibaly, S.O., Traoré, B., Guiguemdé, T.R., 2000. Le microtest isotopique simplifié: une méthode pour l'étude de la chimio-résistance in vitro de *Plasmodium falciparum* aux antipaludiques. Cahiers Santé 10, 353–356.

Tinto, H., Zoungrana, E.B., Coulibaly, S.O., Ouédraogo, J.B., Traoré, M., Guiguemdé, T.R., Van Marck, E., D'Alessandro, U., 2002. Chloroquine and sulfadoxine-pyrimethamine efficacy for uncomplicated malaria treatment and haematological recovery in children in Bobo Dioulasso, Burkina Faso, during a 3-year period (1998–2000). Trop. Med. Int. Health 7, 925–930.

Ursos, L.M., Dzekunov, S.M., Roepe, P.D., 2000. The effects of chloroquine and verapamil on digestive vacuolar pH of *P. falciparum* either sensitive or resistant to chloroquine. Mol. Biochem. Parasitol. 110, 125–134.

World Health Organization, 1996. Assessment of therapeutic Efficacy of Antimalarial Drugs for Uncomplicated Falciparum Malaria in Areas with Intense Transmission. WHO/MAL/96.1077.